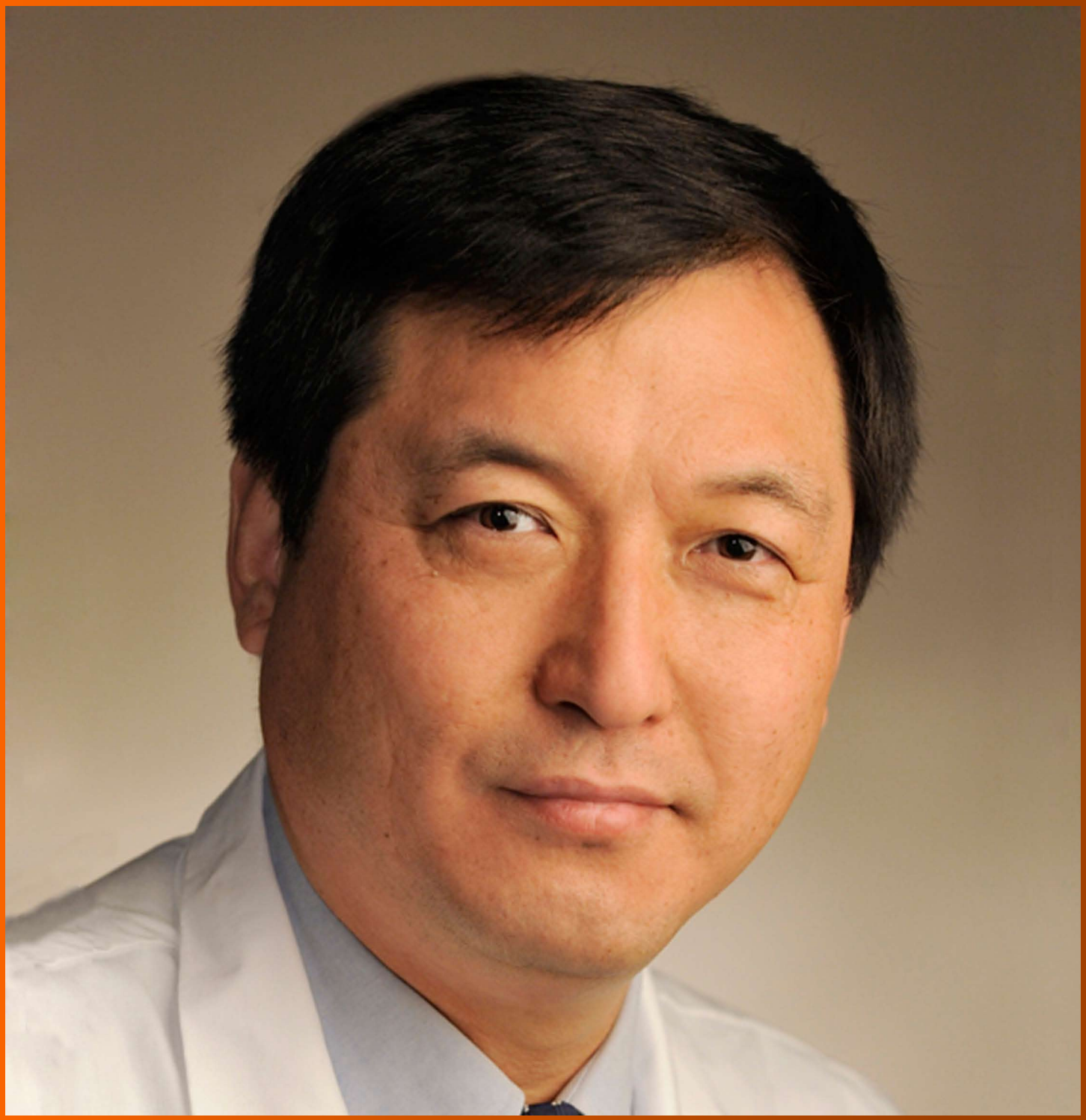
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REVIEW

Yersinia type Ⅲ effectors perturb host innate immune responses

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Abstract

The innate immune system is the first line of defense against invading pathogens. Innate immune cells recognize molecular patterns from the pathogen and mount a response to resolve the infection. The production of proinflammatory cytokines and reactive oxygen species, phagocytosis, and induced programmed cell death are processes initiated by innate immune cells in order to combat invading pathogens. However, pathogens have evolved various virulence mechanisms to subvert these responses. One strategy utilized by Gram-negative bacterial pathogens is the deployment of a complex machine termed the type Ⅲ secretion system (T3SS). The T3SS is composed of a syringe-like needle structure and the effector proteins that are injected directly into a target host cell to disrupt a cellular response. The three human pathogenic *Yersinia* spp. (*Y. pestis*, *Y. enterocolitica*, and *Y. pseudotuberculosis*) are Gram-negative bacteria that share in common a 70 kb virulence plasmid which encodes the T3SS. Translocation of the *Yersinia* effector proteins (YopE, YopH, YopT, YopM, YpkA/YopO, and YopP/J) into the target host cell results in disruption of the actin cytoskeleton to inhibit phagocytosis, downregulation of proinflammatory cytokine/chemokine production, and induction of cellular apoptosis of the target cell. Over the past 25 years, studies on the *Yersinia* effector proteins have unveiled tremendous knowledge of how the effectors enhance *Yersinia* virulence. Recently, the long awaited crystal structure of YpkA has been solved providing further insights into the activation of the YpkA kinase domain. Multisite autophosphorylation by YpkA to activate its kinase domain was also shown and postulated to serve as a mechanism to bypass regulation by host phosphatases. In addition, novel *Yersinia* effector protein targets, such as caspase-1, and signaling pathways including activation of the inflammasome were identified. In this review, we summarize the recent discoveries made on *Yersinia* effector proteins and their contribution to *Yersinia* pathogenesis.

Key words: Type Ⅲ secretion; *Yersinia*; Effectors; Innate; Virulence

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Core tip: The study of *Yersinia* type Ⅲ secretion system effector proteins has provided critical insights into bacterial pathogenic strategies and host innate immune responses. Identification of the crystal structure of YpkA revealed how a bacterial effector can counteract phagocytosis at multiple levels including inhibition of actin polymerization by sequestering actin, inhibition of actin signaling molecules *via* both its kinase and dissociation-like inhibitor domains, and inhibition of actin-cytoskeletal components *via* phosphorylation. YpkA/YopO multisite autophosphorylation may allow YpkA/YopO to bypass regulation by host phosphatases and thus prolong its ability to interfere with phagocytosis. Additionally, an emerging theme is the role of caspases in anti-*Yersinia* host defenses.

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INTRODUCTION

The *Yersinia* genus consists of Gram-negative coccobacilli or rod-shaped bacteria in which three are pathogenic to humans: *Y. pestis*, the causative agent of the plague, and the two enteric pathogens, *Y. enterocolitica*, and *Y. pseudotuberculosis*[1]. Infected fleas serve as a vector for the transmission of *Y. pestis* to humans. Alternatively, the pneumonic form of the plague can be transmitted from an infected individual to another person *via* aerosolized droplets. *Y. enterocolitica* and *Y. pseudotuberculosis* are transmitted through ingestion of contaminated food or water. Upon transmission, *Y. pestis* migrates to regional lymph nodes where it utilizes the type Ⅲ secretion system (T3SS; see below) to evade host immune cells. In doing so, *Y. pestis* is capable of replicating extracellularly and causes bubonic plague. If the infection becomes systemic it can result in the septicemic and pneumonic forms of plague. *Y. enterocolitica* and *Y. pseudotuberculosis* also have a trophism for lymphoid tissue whereupon ingestion the pathogens cross the specialized epithelial M cells found in the ileal tract of the small intestine. Once across the epithelial tissue of the small intestine *Y. enterocolitica* and *Y. pseudotuberculosis* infect the underlying lymphoid tissue such as the Peyer's patches and mesenteric lymph nodes resulting in gastrointestinal diseases[2]. Systemic infections by the two enteric *Yersinia* pathogens are rare in humans, but mouse infection models show colonization of other tissues such as the spleen and liver.

The T3SS is a virulence mechanism found in a wide array of Gram-negative bacteria that are pathogenic to mammals or plants, as well as in symbiotic bacteria of plants and insects[3]. The T3SS is composed of a needle-like syringe termed the injectisome and the effector proteins that are injected directly into a target host cell from the bacterium's cytosol to disrupt, hijack, or mimic host signaling proteins. Although the T3SS may be used for different functions depending on the life cycle and infection process of the pathogenic bacteria, it is primarily used to subvert the host response to favor survival of the pathogen. Bacterial pathogens lacking the T3SS, expressing a translocation-defective T3SS, or expressing an effectorless T3SS are attenuated *in vivo*, and thus underscore the essential role of the T3SS to bacterial virulence.

All three pathogenic *Yersinia* species share in common a 70 kb virulence plasmid that encodes proteins of the T3SS[4]. Expression of the proteins is observed at 37 ℃ under low Ca^{2+} concentration *in vitro* whereas *in vivo* it is also dependent on cellular contact of the *Yersinia* bacterium with the target host cell. Adhesion of the *Yersinia* bacterium to a target host cell is mediated through the binding of adhesion proteins such as invasin (Inv) and YadA to host cell surface proteins[5]. Once attached, the injectisome forms a pore at the plasma membrane of the host cell and subsequently translocates the effector proteins termed *Yersinia* outer proteins (Yops). There are 6 known Yop effector proteins that are translocated through the injectisome by pathogenic *Yersinia* species to disrupt the host response: YopE, YopH, YopT, YpkA (YopO in *Y. enterocolitica*), YopJ (YopP in *Y. enterocolitica*), and YopM (Table 1). Once in the target host cell these effector proteins function in concert to thwart the host response by altering the actin cytoskeletal structures to inhibit phagocytosis, as well as induce cell death and downregulate proinflammatory responses (Figure 1)[6].

Both the innate and adaptive immune systems mount a response to combat against invading pathogens with the former being the first line of defense[7]. Macrophages, neutrophils, dendritic cells, and natural killer (NK) cells are innate immune cells that respond to resolving the infection. Upon ligand binding by receptors such as pattern recognition receptors innate immune cells engage in phagocytosis, production of reactive oxygen species (ROS), and induction of inflammation and cell death to combat the pathogen. All three *Yersinia* species are capable of downregulating innate immune responses to promote survival of the *Yersinia* pathogens. Cells of the innate immune system are also involved in activating the adaptive immune response, and thus highlight the significance of why pathogens evolved effective mechanisms to disarm the innate immune system.

Recent studies are unveiling new target substrates and signaling pathways that are inhibited by the *Yersinia* effector proteins. The discovery of the crystal structure of YpkA, the identification of multisite YpkA autophosphorylation to activate its kinase domain, and the establishment that YpkA phosphorylates actin-binding proteins are shedding light on YpkA and its

Table 1 The *Yersinia* effector proteins, their host substrates, and cellular effects

Yop effectors	Enzymatic function	Target substrates	Cellular effects
YpkA/YopO	Serine/threonine kinase	Gαq, EVL, VASP, WASP, WIP, gelsolin, mDia1, INF2, and cofilin	Disruption of the actin cytoskeleton to contribute to inhibition of phagocytosis; additional effects are unknown
	Guanine nucleotide dissociation-like inhibitor	RhoA, Rac1, and Rac2	Disruption of the actin cytoskeleton to contribute to inhibition of phagocytosis
YopE	GTPase activating protein	RhoA, Cdc42, Rac2, and RhoG	Disruption of the actin cytoskeleton to contribute to inhibition of phagocytosis; inhibition of caspase-1 activation and maturation of IL-18 and IL-1β
YopH	Protein tyrosine phosphatase	FAK, $p130^{Cas}$, paxillin, Fyb, SKAP-HOM, PRAM-1, SLP-76, Vav, PLCγ2, p85, Gab1, Gab2, Lck, and LAT	Disruption of focal adhesion complexes and actin stress fibers to inhibit phagocytosis; inhibition of proinflammatory cytokine and MCP-1 production; inhibition of early calcium response and ROS production; inhibition of the PI3K/Akt pathway
YopP/J	Acetyltransferase/cysteine protease/deubiquintase	TRAF2, TRAF6, IκBα, MAPKKKs, MAPKKs, IKKβ, RICK, and eIF2α	Inhibition of adhesion molecules, proinflammatory cytokine and chemokine production; activation of caspase-1 and maturation of IL-18 and IL-1β; induction of apoptosis
YopT	Cysteine protease	RhoA, Rac1, Cdc42, and RhoG	Disruption of the actin cytoskeleton to contribute to inhibition of phagocytosis; inhibition of NFκB
YopM	Leucine-rich repeat protein	RSK and PRK isoforms, caspase-1, and IQGAP1	Inhibition of proinflammatory cytokine production; inhibition of caspase-1 activation; induction of apoptosis; induction of anti-inflammatory cytokine production

IL: Interleukin; FAK: Focal adhesion kinase; PLC-γ2: Phospholipase C-γ2; MAPK: Mitogen-activated protein kinase; MAPKK: MAPK kinase; MAPKKK: MAPKK kinase; Akt: Protein kinase B; NFκB: Nuclear factor kappa b; IKKβ: Inhibitor of kappa B kinase beta; IκBα: Inhibitor of kappa B alpha; IQGAP1: IQ motif containing GTPase activating protein 1; Lck: Lymphocyte-specific protein tyrosine kinase; LAT: Linker for activation of T cell; eIF2α: Eukaryotic initiation factor 2; PRK: Protein kinase C-related kinase; RSK: Ribosomal S6 protein kinase; VASP: Vasodilator-stimulated phosphoprotein; WASP: Wiskott-Aldrich syndrome protein; WIP: WASP-interacting protein; $p130_{Cas}$: Crk associated tyrosine kinase substrate; Fyb: Fyn-binding protein; SKAP-HOM: Src kinase-associated phosphoprotein 55 homologue; PRAM-1: PML-retinoic acid receptor alpha regulated adaptor molecule 1; SLP-76: SH2 domain containing leukocyte protein of 76 kDa; EVL: Ena/VASP-like; INF2: Inverted formin 2; TRAF: TNF-receptor associated factor; RICK: Rip-like interacting caspase-like apoptosis-regulatory protein kinase; MCP-1: Monocyte chemotactic protein 1; ROS: Reactive oxygen species; PI3K: Phosphatidylinositol 3-kinase; mDia1: Mammalian diaphanous 1; Gab: Grb-associated-binder.

molecular function within the host cell. Additionally recent findings emphasize the central role of caspases in anti-*Yersinia* host defenses. *Yersinia* effector proteins and their effects on the host innate immune response are the focus of this review. Although we will be focusing our discussions on findings within the past 5 years, we will also draw upon relevant studies performed in the prior years.

YERSINIA OUTER PROTEINS

YopH inhibits early response of innate immune cells to enhance Yersinia virulence

YopH is a 50 kDa protein tyrosine phosphatase (PTPase) containing an N-terminal substrate binding domain and a C-terminal PTPase domain[8,9]. Mouse infection studies showed that YopH is essential to enhance *Yersinia* virulence, especially at the early stages of an infection to evade innate immune cells[10-13]. Studies on YopH and its PTPase activity demonstrate that it inhibits phagocytosis by epithelial cells, macrophages, neutrophils, and dendritic cells when working in concert with other Yops[10,14-19]. Translocation of YopH into these immune cells results in inhibition of the early calcium signaling and ROS production, as well as the production of some proinflammatory cytokines [tumor necrosis factor-α, interleukin (IL)-10, and IL-1β] and the chemokine, monocyte chemotactic protein 1[18,20-22]. YopH was recently implicated to function in concert with YopE to inhibit integrin β1-mediated inflammasome activation in epithelial cells; however whether YopH also inhibits inflammasome activation in immune cells still remains to be determined[23].

YopH targets kinases and/or adapter proteins of innate immune cells

To date, it has been reported that YopH binds to and dephosphorylates the focal adhesion kinase (FAK), Crk associated tyrosine kinase substrate ($p130^{Cas}$), and paxillin in epithelial cells whereas $p130^{Cas}$, Fyn-binding protein, and SKAP-HOM are targeted in macrophages[17,24-26]. Although not discussed here, YopH also affects the adaptive immune system where it targets lymphocyte-specific protein tyrosine kinase, linker for activation of T cell, as well as SLP-76 in T-cells[27,28]. Interestingly, YopH only targets a subset of these proteins in a cell specific manner. Recently, the translocation of YopH was shown to directly or indirectly affect phosphorylation of the PRAM-1/SKAP-HOM and the SLP-76/Vav/phospholipase C-γ2 (PLC-γ2) signaling cascades in polymorphonuclear neutrophils (PMNs)[21]. Moreover, the Grb-associated binder 1 and 2 (Gab1 and Gab2) adapter proteins, the phosphatidylinositol 3-kinase (PI3K) subunit, p85, and the Vav adapter protein were shown to associate with recombinant YopH in a pulldown experiment. The dephosphorylation of the p85 subunit of PI3K by YopH accounts for the previously reported YopH-mediated dephosphorylation of PI3K to perturb PI3K-

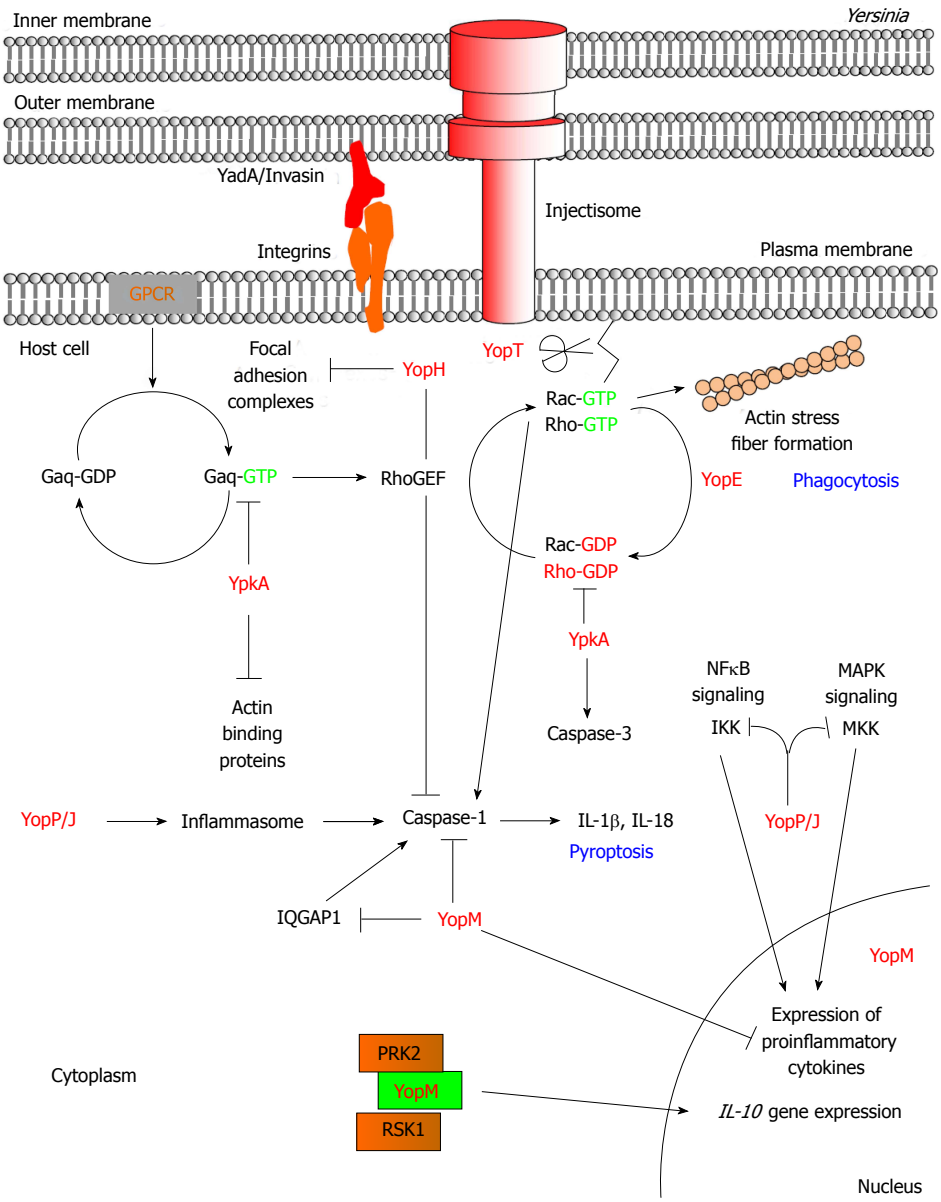


Figure 1 Modulation of host signaling pathways by the *Yersinia* effector proteins. Pathogenic *Yersinia* utilizes the T3SS to subvert the host innate immune response. During an infection, the *Yersinia* bacterium adheres onto the target host cell by using adhesion proteins such as YadA and invasin to bind onto host β1 integrin. This enables the injectisome of the T3SS to form a pore at the host plasma membrane and the subsequent translocation of the *Yersinia* effector proteins directly into the cytoplasm. The *Yersinia* effector proteins localize to distinct subcellular locations where they mimic host proteins/functions to disrupt host signaling pathways. The Ser/Thr kinase YpkA binds actin monomers resulting in YpkA autophosphorylation and kinase activation. Upon GPCR activation of the host G protein Gαq, YpkA binds to and phosphorylates Gαq to inhibit Gαq-mediated activation of the Rho GTPases and subsequent actin stress fiber formation. YpkA also uses actin as bait for actin binding proteins (*i.e.,* VASP, cofilin, and WASP) whereby YpkA then phosphorylates these proteins. In addition, YpkA binds onto the Rho GTPases, RhoA and Rac1, to inhibit the exchange of GDP for GTP. YopE is a GAP protein that facilitates the intrinsic GTPase activity of the Rho proteins resulting in their inactivation. The cysteine protease, YopT, disrupts the actin cytoskeleton by cleaving post-translationally modified Rho GTPases. Cleavage of the Rho proteins results in the mislocalization and inactivation of the Rho proteins. YopH, a PTPase, dephosphorylates focal adhesion components such as FAK, $p130^{Cas}$, paxillin, Fyb, SKAP-HOM, PRAM-1, and SLP-76 to disrupt focal adhesion complexes, β1 integrin signaling, and activation of the Rho protein, Rac1. Together, the enzymatic activity of YpkA, YopE, YopT, and YopH affects organization of the actin cytoskeleton to inhibit phagocytosis. YopP/J is an acetyltransferase with deubiquitinase activity and a putative cysteine protease function. YopP/J targets signaling components of the MAPK and NFκB signaling pathways following activation of Toll-like receptor 4 to inhibit production of proinflammatory cytokines. In addition to inhibiting the MAPK and NFκB signaling pathways, YopP/J activates the inflammasome complex resulting in the maturation of caspase-1, the production of IL-1β and IL-18, and the cell death process termed pyroptosis. YopE and YopT were implicated in inhibiting inflammasome and caspase-1 activation by targeting Rac1. YopM is a protein with leucine-rich repeats that localizes to the cytoplasm and the nucleus of the cell. The translocation of YopM results in the inhibition of proinflammatory cytokine production. Cytoplasmically-localized YopM inhibits caspase-1 activity by binding to the upstream component, IQGAP1, to pro-caspase-1, or to mature caspase-1. YopM also binds onto the PRK and RSK isoforms and inhibits phosphatase-mediated deposphorylation of these two host kinases. The underlying function of targeting these two kinases by YopM is still unclear, but is linked to the production of the anti-inflammatory cytokine, IL-10. T3SS: Type III secretion system; GPCR: G protein-coupled receptor; IL: Interleukin; FAK: Focal adhesion kinase; MAPK: Mitogen-activated protein kinase; NFκB: Nuclear factor kappa b; IQGAP1: IQ motif containing GTPase activating protein 1; PRK: Protein kinase C-related kinase; RSK: Ribosomal S6 protein kinase; GAP: GTPase activating protein; VASP: Vasodilator-stimulated phosphoprotein; WASP: Wiskott-Aldrich syndrome protein; $p130^{Cas}$: Crk associated tyrosine kinase substrate; Fyb: Fyn-binding protein; SKAP-HOM: Src kinase-associated phosphoprotein 55 homologue; PRAM-1: PML-retinoic acid receptor alpha regulated adaptor molecule 1; SLP-76: SH2 domain containing leukocyte protein of 76 kDa.

dependent chemokine production[22,29]. Interestingly, the p85 subunit of the PI3K protein complex (p85/p110) binds onto the Gab proteins enabling signal transduction through the PI3K signaling pathway following activation of the cell surface receptor[30]. Thus, YopH may exploit the Gab1, Gab2, and Vav adapter proteins in order to gain access to signaling molecules.

YopE facilitates GTPase activity of the Rho family of GTPases

YopE is a 23 kDa GTPase activating protein (GAP) that is translocated by *Yersinia* into the target host cell where it exerts its GAP activity on the Rho family of GTPases (for a review refer to[31]). YopE is essential to *Yersinia* virulence *in vivo* due to its effective ability to inhibit phagocytosis by innate immune cells[32]. Earlier findings showed that YopE stimulates the GTPase activity of RhoA, Rac1, and Cdc42 *in vitro*; however the selective targeting of the Rho proteins *in cellulo* or *in vivo* depends on the cellular localization of YopE and the Rho proteins[31,33-36]. In addition to the well studied Rho GTPases, YopE also targets RhoG and Rac2[33,35,37]. Intriguingly, activation of a Rho GTPase can mediate the activation of another Rho protein, and thus YopE may be targeting multiple Rho GTPases at once to enhance *Yersinia* virulence.

YopE-mediated inhibition of the Rho GTPases also perturbs inflammasome activation and ROS production

The GAP activity of YopE on Rho proteins causes disruption of the actin cytoskeleton and inhibits phagocytosis; however, inactivating the Rho proteins also has other effects[38]. Thinwa *et al*[23] demonstrated that infection of epithelial cells with the YopE-deficient *Y. enterocolitica* mutant in part induces the maturation of caspase-1 and IL-18; a hallmark of inflammasome activation[39]. The discovery that YopE inhibits inflammasome activation is not new, but does corroborate the previously reported finding that YopE inhibits maturation of caspase-1 and caspase-1-mediated responses in macrophages[40].

Once in the lymphoid tissue *Yersinia* utilizes the T3SS to evade the host immune response. Infection of mice with a *Y. pseudotuberculosis* mutant strain deficient in expressing YopE showed higher association of *Yersinia* with PMNs one day post infection. Furthermore, the mutant can colonize and disseminate better in PMN-depleted mice[13]. The translocation of YopE into neutrophils enables *Yersinia* to thwart the neutrophil response. The inhibition of ROS production along with phagocytosis by YopE is crucial for *Yersinia* colonization of the spleen of infected mice[37]. Since Rac2 is primarily expressed in hematopoetic cells it is likely that YopE targets Rac2 in immune cells such as neutrophils to perturb the killing of *Yersinia* during an infection. Additionally, the YopE-mediated inhibition of RhoG was speculated to affect proper neutrophil functions since $RhoG^{-/-}$ murine neutrophils are deficient in proper ROS production when stimulated with different agonists[35,41]. Although the reported Rac2-dependent ROS production is independent of RhoG, the activation of Rac2 at the early time points upon agonist stimulation was affected in RhoG knockout neutrophils[37,41]. Thus, it appears that YopE may be targeting multiple signaling pathways in immune cells that mediate the production of ROS.

YopT targets the Rho family of GTPases

The cysteine protease, YopT, is a 35.5 kDa protein that is translocated into the target host cell by pathogenic *Yersinia* to target the Rho family of GTPases[42-44]. YopT is a member of the "CA" clan of cysteine proteases containing the conserved Cys, His, and Asp amino acids required for the catalytic activity of cysteine proteases[43]. The catalytic action of YopT was observed to act upon the post-translationally modified Rho GTPases where YopT cleaves upstream of the isoprenylated cysteine residue resulting in the mislocalization of the membrane-bound Rho protein, and the disruption of the actin cytoskeleton[45]. Unlike the other Yops, YopT is not found in all pathogenic *Yersinia* species namely in some serotypes of *Y. pseudotuberculosis* due to an internal deletion of the *yopT* gene. Thus, the molecular role of YopT is complemented by the other translocated Yops such as YopE in the *yopT*-deficient *Yersinia* strain as demonstrated in mouse infection studies[42,46]. Moreover, the inhibition of Rac1 by YopT and YopE inactivates caspase-1 suggesting an overlap of function between YopT and some of the other Yop effector(s)[40].

YopT has been demonstrated to play a role in inhibiting phagocytosis by macrophages and neutrophils, but not by dendritic cells[47]. The Rho GTPases are key signaling molecules involved in remodeling the actin cytoskeleton to mediate phagocytosis[48,49]. YopT has been shown to cleave the post-translationally modified RhoA, Rac1, and Cdc42 *in vitro*[50]. However, the enzymatic activity of YopT appears to be more selective in targeting different Rho protein(s) depending on the cell type and subcellular localization of the Rho proteins[35,51]. In addition to targeting the well studied Rho proteins, YopT also cleaves membrane bound RhoG to inhibit RhoG-mediated uptake of *Yersinia*; however, since RhoG is also required for proper neutrophil functions, it remains to be tested whether YopT also affects other response mediated by RhoG in immune cells[35,41].

YopT induces expression of host's KLF2 and GILZ to inhibit NFκB signaling

The translocation of YopT results in decreased IL-8 production suggesting that the catalytic activity of YopT on Rho proteins not only results in the disruption of the actin cytoskeleton, but also modifies the host transcriptional profile[22,46,52,53]. YopT was shown to mediate expression of the zinc-finger transcription factor, Kruppel-like factor 2 (KLF2), and the leucine zipper, glucocorticoid induced-leucine zipper (GILZ), in macrophages and/or epithelial cells upon infection with *Yersinia*[54,55]. In host cells both KLF2 and GILZ inhibit the NFκB signaling pathway to downregulate

NFκB-mediated proinflammatory responses[56,57]. Thus, YopT usurps a normal host regulatory mechanism to counteract anti-*Yersinia* immune responses. However, it is still unclear at what stage(s) of *Yersinia* infection KLF2 and GILZ expression are required to promote *Yersinia* pathogenesis.

The multi-domains of YpkA/YopO

Yersinia protein kinase A (YpkA; YopO in *Y. enterocolitica*) is an 80 kDa serine/threonine (Ser/Thr) kinase that when translocated into the target cell causes disruption of the actin cytoskeleton[58]. Interestingly, YpkA is a multifaceted effector protein with regards to the functional eukaryotic-like enzymatic domains that include a Ser/Thr kinase domain and a guanine nucleotide dissociation-like inhibitor (GDI) domain[59,60]. YpkA is the only *Yersinia* effector protein with two enzymatic domains. Activities of both domains affect the actin cytoskeleton by targeting the activation state of the Rho GTPases, RhoA and Rac1. Rho GTPases cycle between an active GTP-bound and an inactive GDP-bound state where the former is catalyzed by guanine nucleotide exchange factors (GEFs) to exchange GDP for GTP. Active Rho proteins activate downstream effectors to remodel the actin cytoskeleton to regulate cellular processes such as phagocytosis[49]. The intrinsic Rho GTPase activity is facilitated by GAP that hydrolyze GTP resulting in the inactivation of the Rho proteins. Inactive Rho proteins are bound to GDI and are kept in an inactivate state. Similar to host GDI, the GDI domain of YpkA binds directly to the Rho GTPases to inhibit GTP loading whereas the kinase domain targets the alpha subunit of the heterotrimeric G protein complex, Gq, to inhibit activation of downstream Rho proteins by the LARG RhoGEF[60,61]. However, the YpkA GDI domain alone can mediate the disrupted actin cytoskeleton phenotype, and thus underscores the prediction that the kinase activity of YpkA also targets additional host signaling pathways (see below). Although mouse infection studies with *Yersinia* mutant strains expressing either a kinase inactive YpkA mutant or only the GDI domain controversially argue one domain being more essential than the other, these studies taken together suggest that both domains of YpkA contribute to *Yersinia* virulence[30,59,62,63].

The secretion/translocation domain of YpkA (Sec/Trans; amino acids 1-77) located at its N-terminus mediates translocation of YpkA into the host cell[64]. Intriguingly though, multiple regulatory domains also overlap with the Sec/Trans domain of YpkA. To date, the chaperone binding domain (amino acids 20-77), the membrane localization domain (MLD; amino acids 20-90), and the substrate-binding domain (SBD; amino acids 40-49) all overlap with the Sec/Trans domain[65,66]. Salomon *et al*[67] also showed that residues located within amino acids 1-125 of YpkA mediate binding to phosphoinositides to perhaps localize YpkA to the plasma membrane after being translocated. Altogether, the MLD, SBD, and phosphoinositide-binding residues may regulate an aspect of YpkA activity such as the phosphorylation of Gαq and/or the selective inhibition of Rho proteins. Downstream of these domains is the kinase domain (amino acids 150-400), the GDI domain (amino acids 431-612), and the actin binding domain (ABD; amino acids 709-729).

Kinase activation of YpkA and the targeting of actin regulating proteins

YpkA is expressed as an inactive kinase in the *Yersinia* bacterium[59]. The kinase activity of YpkA is activated by binding onto globular actin *via* the ABD upon translocation into the target cell[68]. The crystal structure of the *Y. enterocolitica* YopO-actin complex showed that actin binding allosterically positions the catalytic and activation loops of YpkA in an active conformation[69]. Actin binding induces YpkA autophosphorylation on Ser90 and Ser95 *in vitro*[59,66,68]. Interestingly, mutation of Ser90 and Ser95 to alanine on YpkA does not affect its kinase activity *in cellulo* whereas mutation of all serine residues on YpkA resulted in a catalytically inactive kinase[66]. Further analysis showed that mutation of all serine residues to alanine within amino acids 436-710 to alanine does not affect YpkA kinase activity *in vitro* or *in cellulo*. Moreover, mutation of serine residues to alanine within amino acids 1-150 or 150-400 results in decreased YpkA autophosphorylation *in vitro*, but has no effect on its kinase activity *in cellulo*; however, mutation of all serine residues to alanine within amino acids 1-400 results in an inactive YpkA kinase mutant[66]. Together, the results suggest that once translocated YpkA auto-phosphorylates on multiple serine residues within its N-terminus to activate its kinase domain. Additionally, due to the fact that YpkA is translocated at a lower level relative to other *Yersinia* effectors, it appears that the YpkA kinase activity has been fine tuned to where it can function efficiently within the target host cell by autophosphorylation on multiple serine residues[58]. It is predicted that this multisite autophosphorylation mechanism by YpkA enables it to bypass the regulatory control imposed by host proteins such as phosphatases.

YpkA-mediated phosphorylation of Gαq results in the inhibition of the Gαq signaling cascade[61]. Activation of Gαq and its effector, phospholipase C, regulates an array of cellular responses such as neuronal signaling, platelet aggregation, cell growth and proliferation, and development[70]. However, further study is still needed to establish the molecular contribution of YpkA-mediated inhibition of Gαq to *Yersinia* pathogenesis. Although it remains to be explored, YpkA may be targeting Gαq-mediated NFκB activation through the scaffolding protein, CARMA3. CARMA family members form a complex with Bcl10, MALT1, and TRAF6 to activate NFκB resulting in the induction of a proinflammatory response[71]. Alternatively, YpkA may be targeting Gαq to inhibit activation of the dual oxidase-dependent production of ROS[71,72]. The production of ROS is involved in mediating killing of pathogens by directly damaging the pathogen cellular components (*i.e.*,

DNA damage and amino acid modification), as well as indirectly by regulating responses such as phagosomal protease activity and immune signaling[73]. In addition to binding directly to Gαq to mediate phosphorylation, YpkA utilizes actin as bait to recruit the actin filament elongators (EVL and VASP), the formin proteins (mDia1 and INF2), the nucleation-promoting factors (WASP and WIP), the actin filament severing protein (gelsolin), and the actin depoylmerizing protein (cofilin) for phosphorylation *in vitro*[69]. Since all of these proteins are involved in regulating actin assembly and disassembly, these proteins are likely targeted by YpkA to inhibit some aspect of phagocytosis as reported for VASP[74]. An alternative mechanism of phagocytosis (also known as bacterial uptake) is activated by binding of the *Yersinia* adhesion proteins, Inv and YadA, to host β1 integrin. Subsequently, focal adhesion proteins and Rac1 signal downstream molecules to remodel the actin cytoskeleton resulting in uptake of *Yersinia*[1]. Uptake of *E. coli* expressing the YadA protein by human umbilical vein endothelial cells was inhibited by the kinase activity of YpkA[68]. Phosphorylation of proteins regulating actin polymerization by YpkA may be responsible for the inhibition of bacterial uptake *via* the YadA-β1 integrin signaling pathway.

YpkA inhibits the Rac GTPases

Early studies on YpkA identified the Rho GTPases as target substrates of the YpkA GDI domain where binding of YpkA to RhoA and Rac1 inhibits their activation[60,75,76]. Moreover, phagocytosis of opsonized sheep red blood cells (IgG-sRBC) by COS-7 cells expressing the Fcγ receptor showed that YpkA localizes to phagocytic cups and inhibits phagocytosis[77]. This is achieved through the selective inhibition of Rac1 by the GDI domain of YpkA suggesting that once translocated into innate immune cells YpkA inhibits Fcγ receptor-mediated phagocytosis. Interestingly, the Rac2 isoform was also identified as an interacting partner of YpkA[69,77]. Rac2 is primarily expressed in hematopoietic cells at varying levels depending on the immune cell type and is involved in activation of the NADPH oxidase to produce ROS, as well as phagocytosis[78]. Thus, it is tempting to suggest that aside from targeting Rac1 for inhibition YpkA also inhibits Rac2 activity in immune cells. Additionally, the Rac1- and Rac2-regulated protein, PLC-γ2 isoform, was identified as an interacting partner with the YopO-actin complex[69,79]. This further supports the speculation that YpkA also targets Rac2 signaling in immune cells.

The many target substrates of YopP/J

YopJ (YopP in *Y. enterocolitica*) is a 34 kDa acetyltransferase with a deubiquitinating and putative cysteine protease function which was previously shown to inhibit production of proinflammatory molecules. YopP/J suppresses a proinflammatory response by interfering with the mitogen-activated protein kinase (MAPK) and NFκB signaling pathways[80-84]. More recently, YopJ was reported to acetylate the MAPKK kinase family member, TAK1, and the Ser/Thr kinase, RICK, to inhibit their activity[85,86]. Production of chemoattractants (KC, MIP-2, and G-CSF) and adhesion molecules are also effected by YopP/J presumably to inhibit the early recruitment of neutrophils to the site of infection; however, inhibition of chemoattractant expression levels *in vivo* may involve the function of other Yop effectors[87-89]. Moreover, the YopJ homolog, YopP, of *Y. enterocolitica* inhibits phosphorylation of the Tyk2 kinase and STAT4 of the Jak-STAT signaling pathway through a yet-to-be identified mechanism[90]. Of all the effectors that *Yersinia* translocates, it should be noted that YopP/J translocates at various amounts depending on the *Yersinia* strain which reflects its associated cytotoxicity[87,91,92]. This was further seen in studies conducted with different concentrations of recombinant YopJ[93-95].

YopP/J induces apoptosis through caspase-8

Similar to what was observed with extracellular *Yersinia*, translocation of YopJ by intracellular *Yersinia* induces apoptosis of macrophages[96]. Apoptosis is a process initiated by an extracellular "death" signal (extrinsic pathway) or an intracellular signal (intrinsic pathway) that converges on the mitochondria and the release of cytochrome C. The extrinsic and intrinsic pathway involve the activation of caspase-8 or caspase-9, respectively, and the subsequent processing of the effector caspases (*e.g.* caspase-3) to induce apoptosis[97]. YopP/J-mediated inhibition of the MAPK and NFκB signaling pathways, along with Toll-like receptor 4 signaling, induces apoptosis of macrophages and dendritic cells[98-102]. This reported YopJ-induced cellular apoptosis was shown to be a result of signal transduction *via* the receptor-interacting Ser/Thr kinase 1 (RIPK1), Fas-associated death domain and caspase-8 signaling cascade to induce apoptosis[103,104]. Additionally, activated caspase-8 mediates the maturation of the inflammasome-associated caspase-1[103,104]. Although inflammasome activation triggers the cell death process termed pyroptosis, YopJ-induced cell death is primarily through caspase-8-induced apoptosis[105,106]. However, in contrast to the experimental findings reported, YopJ-mediated activation of caspase-1 was observed in cells undergoing necrosis[107]. Since activated RIPK3 is downstream of RIPK1, and is the signaling molecule that triggers programmed necrosis termed necroptosis, it is likely the candidate for the observed necrosis[107,108]. In support of this is the evidence that YopJ-mediated activation of caspase-8 suppresses RIPK3 induced necrosis[103,104]. Thus, there appears to be an intimate crosstalk between caspase-8 and RIPK3 in determining the fate of a *Yersinia* infected cell containing YopP/J.

YopP/J utilizes host signaling pathways to promote Yersinia virulence

YopP/J activity affects macrophages, dendritic cells, NK cells, and to varying degrees neutrophils[90,98,109-111]. However, activity of YopP/J *in vivo* varies depending on the *Yersinia* strain, YopP/J variant, experimental para-

meters, and infection model[62,92,112-115]. Nevertheless, translocation of YopP/J results in the inhibition of key signaling pathways that mediate a proinflammatory response, and also induces production of specific cytokines[83,89,116-118]. In particular is the well established observation that YopJ activity on the MAPK and NFκB signaling pathways mediates the maturation of caspase-1[98,103-105]. Activated caspase-1 cleaves pro-IL-18 and pro-IL-1β to produce active IL-18 and IL-1β[39]. Studies using RIPK3/caspase-8 knockout mice showed reduced cytokine production in response to *Yersinia* infection, and thus, underscores the crucial role of caspase-8 and caspase-1 in mediating a host response to *Yersinia* when the MAPK and NFκB signaling pathways are inhibited[103,104]. Why would *Yersinia* utilize YopJ to induce the activation of caspase-8 and caspase-1? Is it a mechanism evolved by the host to counteract a *Yersinia* infection when the MAPK and NFκB signaling pathways are inhibited, or is it a virulence strategy employed by *Yersinia*? Recent studies are alluding to both situations being the case and are dependent on the association of caspase-1 with inflammasome components, NLRP12, NLRP3/ASC or NOD2[85,106,111]. Thus, although the functions of IL-18 and IL-1β induce an anti-*Yersinia* response, *Yersinia* may also exploit the normal functions of these cytokines at certain stages of infection to promote *Yersinia* virulence. YopJ was also linked to the inhibition of the host alpha subunit of the eukaryotic initiation factor 2 (eIF2α). Although the underlying mechanism and biological relevance is still unclear, modulating eIF2α activity by YopJ results in an increased cellular invasion of MEF cells by *Yersinia* and decreased cytokine production[119].

YopM is indispensable to Yersinia virulence

YopM is a leucine-rich repeat (LRR) protein that localizes to both the cytoplasm and the nucleus of the target host cell upon translocation[120,121]. The molecular contribution of YopM to *Yersinia* pathogenesis is still unclear; however, it has been shown to target immune cells to effect different immune cell populations of the spleen and liver of infected mice, downregulate proinflammatory responses, and upregulate the anti-inflammatory cytokine, IL-10[122-125]. IL-10 downregulates the production of proinflammatory cytokines by multiple innate immune cells, as well as regulates T cell function and proliferation[126]. The production of IL-10 was proposed to counteract the PMN response against *Yersinia*; however whether YopM is solely responsible for inducing production of IL-10 remains to be further explored[124]. YopM was also shown to inhibit platelet aggregation[127]. Although YopM is required to enhance *Yersinia* virulence in mouse infection models, varying results suggest that the route of infection and mouse strain used affects the reported contribution of YopM to *Yersinia* pathogenesis[122]. Moreover, this could also be attributed to the LRR motifs of the YopM variants, as well as the natural route of infection of the pathogenic *Yersinia* species.

YopM targets extra- and intracellular host proteins

Of the six effectors that are translocated by the T3SS of the pathogenic *Yersinia* species into the target host cell, YopM has been shown to also be secreted into the extracellular matrix where it binds α-thrombin and α1-antitrypsin[128-130]. Extracellular YopM can also penetrate culture cells[131]. Whether YopM enters the target host cells by crossing the lipid bilayer or *via* the T3SS it targets the ribosomal S6 protein kinase (RSK) and the protein kinase C-related kinase (PRK) isoforms[132,133]. Dephosphorylation of the RSK isoforms by phosphatase was inhibited by YopM *in cellulo* and *in vitro*[134]. The YopM mutant variant of *Y. pseudotuberculosis* 32777 that is defective in binding RSK1 and PRK2 was unable to induce IL-10 production[133]. More recently, YopM has been shown to inhibit the activity of mature caspase-1. This is achieved by the binding of YopM to the substrate-binding site of caspase-1, inhibition of recruitment of pro-caspase-1 to the inflammasome complex, and/or by targeting the scaffold protein, IQ motif containing GAP 1 (IQGAP1), which is known to activate caspase-1[135,136]. Moreover, YopM has also been shown to activate caspase-3 to presumably induce apoptosis of PMNs and/or macrophages in the liver of infected mice, and thus promote *Yersinia* virulence[137].

CONCLUSION

A major theme emerging from studies of the *Yersinia* effector proteins is the important role caspases play in host anti-*Yersinia* defenses. In particular are the effects of the *Yersinia* effector proteins on caspase-1 activity. Upon the detection of a pathogen by innate immune cells, the inflammasome complex is activated by the oligomerization of nucleotide-binding domain and LRR- containing (NLR) family of proteins. The caspase activation and recruitment domain found on NLRs or an associated adaptor protein such as ASC then recruits pro-caspase-1. Subsequently, autoproteolytic cleavage of pro-caspase-1 is induced to produce the mature caspase-1 form. Mature caspase-1 then mediates the maturation of IL-1β and IL-18, as well as induces pyroptosis. The targeting of Rac1 by YopE and YopT inhibits maturation of caspase-1 whereas the binding of IQGAP1, pro-caspase-1, and caspase-1 by YopM inhibits inflammasome activation, or the enzymatic function of caspase-1. Moreover, the dephosphorylation of FAK by YopH inhibits caspase-1 activation in epithelial cells which suggests that YopH also inhibits caspase-1 activity when translocated into innate immune cells. Alternatively, YopP/J was shown to activate caspase-1 through inflammasome activation, as well as the extrinsic cell death pathway. Whether this is beneficial or detrimental to *Yersinia* pathogenesis depends on the stage of infection; however, the kinetics of caspase-1 activation in the presence of multiple *Yersinia* effector proteins requires further exploration. An additional translocated Yop protein, YopK, which is involved in regulating translocation of the *Yersinia* effector proteins

also inhibits inflammasome activation (for a review on YopK refer to[138]. Lastly, although YpkA has not been shown to affect caspase-1 activation or its activity, a study has shown that YpkA induces cellular apoptosis of murine macrophages through the intrinsic pathway which activates caspase-3[139]. Altogether, the *Yersinia* effector proteins effectively enable pathogenic *Yersinia* spp. to thwart the host innate immune response by regulating some aspect of programmed cell death, as well as inhibit the induction of proinflammatory cytokine production and phagocytosis. Future studies may lead to the identification of novel targets for the *Yersinia* effector proteins and thus additional targets for therapeutic interventions.

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REVIEW

Complexity of vitamin E metabolism

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Abstract

Bioavailability of vitamin E is influenced by several factors, most are highlighted in this review. While gender, age and genetic constitution influence vitamin E bioavailability but cannot be modified, life-style and intake of vitamin E can be. Numerous factors must be taken into account however, *i.e.*, when vitamin E is orally administrated, the food matrix may contain competing nutrients. The complex metabolic processes comprise intestinal absorption, vascular transport, hepatic sorting by intracellular binding proteins, such as the significant α-tocopherol-transfer protein, and hepatic metabolism. The coordinated changes involved in the hepatic metabolism of vitamin E provide an effective physiological pathway to protect tissues against the excessive accumulation of, in particular, non-α-tocopherol forms. Metabolism of vitamin E begins with one cycle of CYP4F2/CYP3A4-dependent ω-hydroxylation followed by five cycles of subsequent β-oxidation, and forms the water-soluble end-product carboxyethylhydroxychroman. All known hepatic metabolites can be conjugated and are excreted, depending on the length of their side-chain, either *via* urine or feces. The physiological handling of vitamin E underlies kinetics which vary between the different vitamin E forms. Here, saturation of the side-chain and also substitution of the chromanol ring system are important. Most of the metabolic reactions and processes that are involved with vitamin E are also shared by other fat soluble vitamins. Influencing interactions with other nutrients such as vitamin K or pharmaceuticals are also covered by this review. All these processes modulate the formation of vitamin E metabolites and their concentrations in tissues and body fluids. Differences in metabolism might be responsible for the discrepancies that have been observed in studies performed *in vivo* and *in vitro* using vitamin E as a

supplement or nutrient. To evaluate individual vitamin E status, the analytical procedures used for detecting and quantifying vitamin E and its metabolites are crucial. The latest methods in analytics are presented.

Key words: Vitamin E metabolism; Long-chain metabolites of vitamin E; Vitamin E bioavailability; Vitamin E transport

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Core tip: Several factors influence vitamin E bioavailability. Gender, age and genetic constitution cannot be modified but life-style and vitamin E intake can be. Physiological handling of vitamin E involves intestinal absorption, vascular transport, hepatic sorting by intracellular binding proteins, and hepatic metabolism. These processes involve kinetics which vary between the different vitamin E forms. The coordinated metabolism of vitamin E is an effective physiological pathway to prevent excessive accumulation of non-α-tocopherol forms. Interactions with other nutrients or pharmaceutics occur. To evaluate vitamin E status, analytical procedures to detect and quantify vitamin E and metabolites are crucial. Current state-of-the-art analytics are presented.

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INTRODUCTION

Vitamin E is recognized as being essential for human health, yet fundamental questions remain. In the words of Eduardo Cardenas[1]: "This is a very exciting time in vitamin E research, yet it is evident that we are far away from making the final decision on the benefit *vs* risk for the potential use of vitamin E in human health". Underlying this lack of understanding is the immense complexity involved in the metabolism of vitamin E. Despite many decades of research on vitamin E, many relevant processes remain puzzling. In this review we will outline what is known today including more uncertain claims about vitamin E, to deepen insight into the physiological mechanisms and its metabolism.

Evans and Bishop were the first in 1922 to describe the relevance of vitamin E in the reproduction of rats and to characterize tocopherols (TOH) and tocotrienols (T3) including their α-, β-, γ- and δ-forms as vitamins[2]. Vitamin E belongs to the group of fat-soluble vitamins and occurs dominantly in oily plants; therefore, nuts, seeds and oils are good sources for vitamin E. Almonds, hazelnuts, germ oil and sunflower oil contain high amounts of α-TOH while walnuts, palm oil and soybeans predominantly contain γ-TOH[1]. T3 are widely found in some cereals, palm oil and rice bran oil[3]. Coconut oil, cocoa butter, soybeans, barley and wheat germ are also naturally occurring sources of T3s[4], whereas vegetables and fruits - with the exception of dried apricots, some legumes, avocado and green olives - contain lower levels of vitamin E forms[5]. The concentration of vitamin E forms contained in food depends on several factors, such as growing, harvesting and any further processing (refining or cooking)[5,6].

BIOAVAILABILITY

Bioavailability of vitamin E is influenced by numerous factors including: (1) the amount of vitamin E and intake of interfering nutrients; (2) proteins involved in vitamin E absorption and individual differences in the efficiency of vitamin E absorption, influenced by for example diseases; (3) vitamin E metabolism; (4) life-style factors; (5) gender; and (6) genetic polymorphisms. For an overview, see Figure 1.

Vitamin E intake: Recommendations

When considering dietary vitamin E, official intake recommendations are provided by various boards and institutes, and are theoretically defined amounts of a nutrient providing an adequate intake for the major part of a healthy population[7]. Here there exists a subtle difference in definitions describing levels of vitamin intake; whereas vitamin deficiency is caused by diseases or metabolic disorders, vitamin undersupply is characterized as an intake issue which does not achieve reference values[7].

As of today there is no generally accepted recommendation defining the value for an adequate intake of vitamin E. This is due to different references used to validate the recommended dietary allowance (RDA) for vitamin E, or α-TOH. Whereas in the United States the correlation of hydrogen peroxide-induced erythrocyte lysis and plasma α-TOH concentrations is used[8], in Germany, Austria and Switzerland the RDA for vitamin E is based on the effects of vitamin E on the prevention of lipid peroxidation[9]. At present, the German Society of Nutrition (Deutsche Gesellschaft für Ernährung) recommends a daily vitamin E intake of 12 mg/d for women and 13-15 mg/d for men, for both adolescents and adults; intake should be higher during pregnancy (13 mg/d) and breast-feeding (17 mg/d). The required amount of vitamin E increases with age for infants and children and decreases in the elderly independent of gender[10]. Generally, the recommended intake of vitamin E should correlate with the amount of polyunsaturated fatty acids in food: 1 g of diene fatty acid or rather diene equivalent requires an intake of 0.5 mg *RRR*-α-TOH.

Although several foods contain naturally occurring sources of vitamin E, it is frequently the case that the intake recommendations are not achieved. In Germany infants and children up to age twelve commonly do not

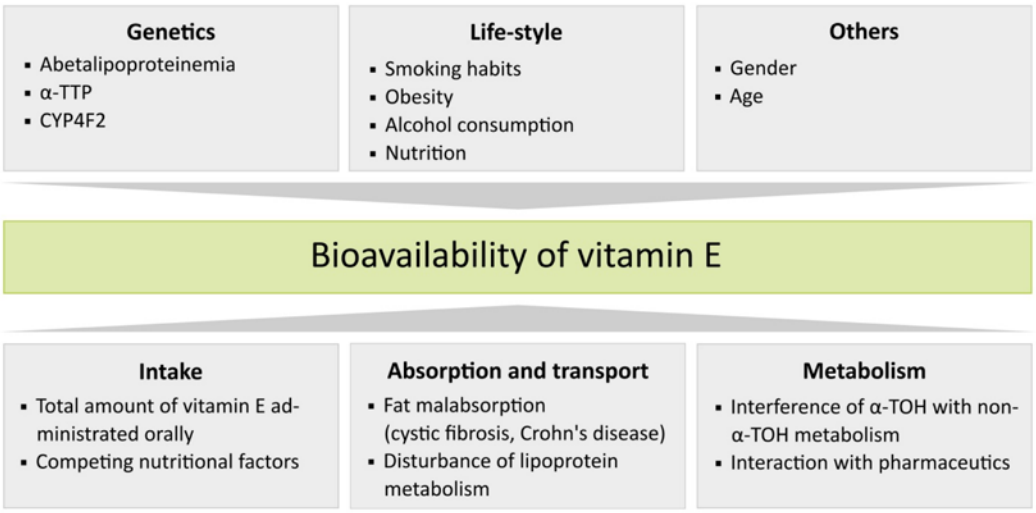


Figure 1 Factors influencing the bioavailability of vitamin E. Some factors affecting bioavailablity cannot be influenced, such as gender, age or genetic disposition, whereas others depend on individual habits and can be summarized as life-style factors. Variations in the physiological handling of vitamin E can also change its distribution status in the body. While uptake of vitamin E can be actively modulated, for example by the total amount of vitamin E intake and competing nutritional factors, absorption and transport principally depend on the state of health of the individual. In contrast, regulation of vitamin E metabolism is just partly influenced by exogenous factors, such as interfering pharmaceuticals.

reach the recommended levels of vitamin E intake[7], as shown in a number of studies including the VELS investigation (Verzehrsstudie zur Ermittlung der Lebensmittelaufnahme von Säuglingen und Kleinkindern; Food Consumption Survey of Babies and Infants) and the EsKiMo study (Ernährungsstudie als KiGGS-Modul, Nutritional Study as KiGGS modul), a follow-up of the KiGGS study (Kinder- und Jugendgesundheitssurvey; Children and Adolescence Health Survey). In addition some studies show that the elderly are often undersupplied with vitamin E[7,11-13]. Numerous research groups analyzing compliance to the vitamin E intake recommendations in Americans have found that a significant number of individuals consume insufficient amounts[14-16]. Dietary intake surveys by the United Kingdom and the United States National Health and Nutrition Examination Survey have revealed that at least 75% of the population consumes vitamin E far below recommended values[17]. Although the recommended amount of vitamin E is higher for men than for women, Dutch women consume less vitamin E more often compared to Dutch men[17]. Data by Traber[18] published last year suggest that more than 90% of United States Americans consume insufficient amounts of vitamin E.

As vitamin E is primarily stored in adipose tissue (about 90% of the body's total vitamin E content[19]), vitamin E deficiency is almost unknown under normal physiological conditions[19]. Symptoms of slight vitamin E undersupply become apparent after many years, usually after decades[19], since vitamin E can be mobilized from adipose tissue for years[20]. In contrast, serious vitamin E deficiency manifests in acute symptoms, such as neuropathy and myopathy, as vitamin E is essential for the development and maintenance of the central nervous system[21]. Ulatowski and Manor[16] categorized metabolic vitamin E deficiencies as: (1) a primary deficiency "(...) arising from specific alteration in vitamin E status"; and (2) a secondary deficiency, "(...) in which low levels of vitamin E are secondary to other global perturbations such as disorders in lipid malabsorption or lipoprotein metabolism and transport". Next to dietary habits, hereditary disorders are known to cause primary and secondary vitamin E deficiencies or inadequate vitamin E bioavailability[19].

Because the official intake recommedations of vitamen E are so seldom met, along with the rare occurance of deficiency symptoms (apart from being caused by disease, addressed later), Traber[18] recently questioned whether the recommended α-TOH intake is set too high and whether a diet low in dietary α-TOH intake has any biological significance. However as of now, circulating α-TOH concentrations below 9 mmol/L for men or below 12 mmol/L for women are considered as deficient and only marginal for healthy adults, respectively[18].

When supplements are used to maintain vitamin E balance, either self-medicated or by medical prescription, questions of toxicity and other safety concerns must be considered. Recent animal studies on reproduction or investigations on the developmental toxic effects of natural or non-natural vitamin E components have been negative but anti-mutagenic activity has been shown[22]. Physiological vitamin E intake of 100 mg/d (150 IU/d) can be increased up to 300 mg/d (450 IU/d) without any complications[23,24]. Even for short-term, high-dose administration of vitamin E no adverse effects have been described[22]. However, persistent high-dose supplementation has been shown to interfere with blood clotting and has been associated with increased risk of hemorrhagic stroke in animal studies[22]. In the past, TOH was considered to be a safe food additive[25]. But, Miller *et al*[26] reported an increase of total mortality

after high-dose vitamin E intake for at least one year. Since adverse effects, such as increased tendency to hemorrhage, have been observed at high vitamin E intake, the tolerable upper intake level for adults was set at 1000 mg/d α-TOH[8].

Life-style and age influence the bioavailability of vitamin E

Concentrations of serum nutrients such as vitamin E are influenced by age and lifestyle factors (*e.g.,* obesity, tobacco smoking, alcohol consumption)[27]. Age-related differences or changes in vitamin E levels in serum and tissue have been discussed in numerous studies. Campbell *et al*[28] showed that vitamin E decreased after the age of 80 years and argued that this finding is possibly connected with reduced food intake of elder people. In contrast, hepatic levels of vitamin E have been found to not be significantly affected by age[29]. Other studies have reported enhanced serum concentrations of vitamin E in the elderly from 60 years[27,30,31], which may be attributed to the age-dependent increases in serum cholesterol/lipoprotein concentrations[30]; this may be protective against increased lipid peroxidation during aging[29,32]. Another explanation was given by Succari *et al*[31], who suggested that life-style and age-associated changes independent of serum cholesterol/lipoprotein concentrations are responsible for the increased vitamin E levels in aged French women.

Numerous clinical trials have demonstrated a gender-independent inverse relationship between human obesity and serum α-TOH concentrations[33,34]. However, clear correlations between serum α-TOH concentrations and general obesity [defined by body mass index (BMI) or body fat percentage] have not been shown, but other parameters for obesity, such as waist circumference and waist-to-hip ratio, were positively associated with α-TOH serum concentrations in both men and women[35-37]. Indeed, Wallström *et al*[35] reported from the Malmö Diet and Cancer Study cohort that serum levels of α-TOH were associated with central adiposity after adjustment for body fat. In contrast, Gunanti *et al*[34] found inverse associations between BMI and serum α-TOH concentrations adjusted for total cholesterol, *i.e.,* the α-TOH:total cholesterol ratio in Mexican-American children of the United States NHANES study. In addition to the association between BMI and α-TOH, BMI has also been positively associated with serum γ-TOH concentrations[32].

It has been observed in several studies that smoking affects serum levels of antioxidants, such as vitamin E. For example, Al-Azemi *et al*[33] and Shah *et al*[38] observed that smokers had lower serum concentrations of α-TOH compared to non-smokers. These findings were supported by Miwa *et al*[39] and Galan *et al*[27] analyzing the female participants of a Japanese study and the cohort of the SU.VI.MAX study, respectively. It is suggested that the dietary patterns of smokers differ from that of non-smokers ultimately leading to differences in vitamin E intake. Interestingly, others have not found differences in vitamin E plasma concentrations between smokers and non-smokers[40,41]. Whereas Mezzetti *et al*[42] observed no differences in plasma vitamin E concentrations between the two groups, vitamin E arterial tissue content was significantly lower in the group of smokers compared to that of non-smokers. This observation may indicate that vitamin E acts as a lipid-soluble antioxidant for the prevention of oxidative damage in the arterial wall. Increased serum concentrations of γ-TOH have also been found both in active and passive smokers by Dietrich *et al*[41]. This finding suggests that excretion of non-α-TOH forms of vitamin E may be decreased to mobilize additional anti-oxidative agents, such as γ-TOH, to increase the body's anti-oxidative capacity. Chronic alcohol consumption also leads to decreased serum levels of α-TOH, partly due to malnutrition[43]. Animal studies have suggested that chronic administration of alcohol results in significantly lower hepatic α-TOH concentrations, probably due to decreased amounts of α-TOH in the mitochondria of hepatocytes[43,44].

INTESTINAL ABSORPTION

When vitamin E is consumed, intestinal absorption is an important factor that limits vitamin E bioavailability. It is known that vitamin E, as a fat-soluble vitamin, follows the intestinal absorption, hepatic metabolism and cellular uptake processes of other lipophilic molecules and lipids[45]. Therefore, intestinal absorption of vitamin E requires the presence of other lipid-rich foods.

In the gastro-intestinal system the absorption rate of vitamin E varies interindividually between 20%-80%[43,45], and is lower than for other fat-soluble vitamins, such as vitamin A[46]. Reasons for individual differences in the absorption rate are diverse. Increased administration of α-TOH with parallel intake of additional food ingredients can decrease the absorption of α-TOH and non-α-TOH forms of vitamin E[47]. For example, retinoic acid[48], plant sterols[49], eicosapentaenoic acid[43], chronic alcohol consumption[43], and dietary fiber[50] are natural food components known to compete with the absorption of vitamin E. How vitamin E is presented to the intestinal surface is crucial for its absorption[19]. In addition, the supplied form of α-TOH, either as a free molecule or, for example, as α-TOH acetate, a common food additive, is of particular importance for its bioavailability[51].

First, triacylglycerols and esterified fat-soluble compounds are partly processed enzymatically in the stomach by gastric lipase[46]. Digestive enzymes including pancreatic lipase, carboxyl esterase and phospholipase A, secreted into the intestinal lumen, continue the digestion of dietary lipids[52]. As vitamin E in the human diet is mostly not esterified, the importance of the lipolytic degradation in the digestion system is likely limited[46], but the need for gastric hydrolysis of the stabilized

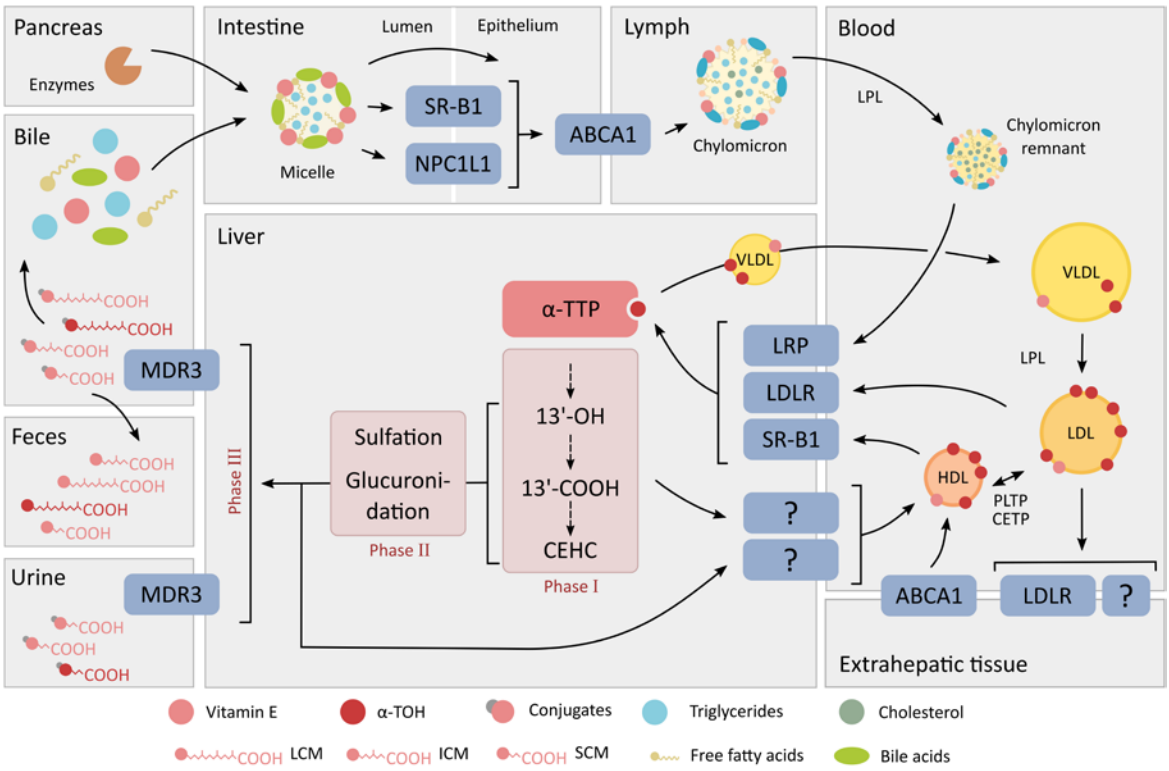


Figure 2 Absorption, transport and metabolism of vitamin E. The route of vitamin E after oral intake follows in general the pathway of other lipids. Pancreatic and intestinal enzymatic digestion followed by the circulation and distribution to the liver and non-hepatic tissues is the same for all vitamin E forms. Discrimination between the different forms of vitamin E in favor of α-TOH occurs mainly in the liver by α-TTP, which protects α-TOH from excessive degradation and excretion. The figure was modified from[43,46,56,60]. SR-B1: Scavenger receptor class B type 1; LPL: Lipoprotein lipase; NPC1L1: Niemann-Pick C1-like 1; VLDL: Very low density lipoproteins; HDL: High density lipoproteins; α-TOH: α-tocopherols; α-TTP: α-TOH transfer protein; LDL: Low density lipoproteins; LRP: LDL receptor-related proteins; LDLR: LDL receptor; 13'-OH: 13'-hydroxychromanol; 13'-COOH: 13'-carboxychromanol; CEHC: Carboxyethylhydroxychromanols; HDL: High density lipoproteins; PLTP: Phospholipid transfer protein; CETP: Cholesteryl ester transfer protein; LCM: Long-chain metabolites; ICM: Intermediate-chain metabolites; SCM: Short-chain metabolites.

forms of vitamin E, such as α-TOH acetate, has not been studied in detail so far[46]. The more stable α-TOH acetate, however, requires further hydrolysis by the bile acid-dependent lipase in the pancreas or an intestinal mucosal esterase[43]. Subsequent absorption of vitamin E in the duodenum is characterized by the transfer from emulsion fat globules to water-soluble multi- and unilamellar vesicles and mixed micelles comprised of phospholipids and bile acids. This is the fundamental step in the gastric digestion and uptake of lipids, and also a crucial phase in the absorption of vitamin E (Figure 2). Since the uptake of vitamin E into enterocytes is less efficient compared to other types of lipids, this may explain the relatively low bioavailability of vitamin E[46]. As mentioned by Desmarchelier *et al*[53], α-TOH acetate is embedded in matrices where its hydrolysis and its uptake by intestinal cells are markedly less efficient than in mixed micelles. The intestinal cellular uptake of vitamin E from mixed micelles follows in principle two different pathways across enterocytes, as has been shown *in vitro* and *in vivo*: (1) passive diffusion; and (2) receptor-mediated transport. Receptors facilitating α-TOH transport across the enterocyte membrane, similar to the intestinal uptake of cholesterol[54], are the class B type 1 scavenger receptor class B type 1 (SR-B1)[55] and the Niemann-Pick C1-like protein 1 (NPC1L1), an apical membrane receptor of the small intestine[56]. The ATP-binding cassette (ABC) transporters ABCG5/ABCG8, at the luminal site, and ABCA1, at the apical site, are responsible for steroid efflux into the intestinal lumen and transport into the lymph system, respectively[57,58]. It has been shown in rats that vitamin E up-regulates these transporters, highlighting the contribution of vitamin E to cholesterol absorption and release[59]. Finally, ABCA1 is directly involved in the export of vitamin E from cells[60]. Presently, an increased intestinal absorption of *RRR*-α-TOH as compared to other stereoisomers[61], or other vitamin E forms such as γ-TOH[62], has not been uncovered. Thereby the efficiency in absorption, and of the integration of α-TOH or γ-TOH into chylomicrons, appears to be comparably equivalent.

VASCULAR TRANSPORT

No specific plasma transport protein for α-TOH has yet been described[43,63]. The transport of vitamin E in blood circulation follows largely that of cholesterol within lipoprotein metabolism[64]. Lipoproteins serve as carriers for lipophilic molecules, such as cholesterol, triglycerides and vitamin E, to distribute them between the liver and different organs and tissues[43]. In principle, the transport of vitamin E is independent of the type of

stereoisomer[65,66]. Under normal physiological conditions, α-TOH is mostly transported *via* chylomicrons, very low density lipoproteins (VLDL) and high density lipoproteins (HDL), whereas under fasting conditions low density lipoproteins (LDL) take on this task[67]. The distribution ratio of α-TOH between the different types of lipoproteins is difficult to define. In a study on fasting normolipemic volunteers, Kostner *et al*[68] found a ratio of 1.0:1.9:1.4 of α-TOH distribution between VLDL, LDL and HDL, respectively. In contrast, Behrens *et al*[69] calculated a ratio of 1.0:9.4:8.4. These variations are most likely due to individual differences in α-TOH distribution in the lipoproteins caused by differing dietary α-TOH intake, metabolic states[68], and other factors influencing bioavailability and the status of vitamin E. In addition, results obtained from human studies regarding the distribution of α-TOH to lipoproteins differ from studies performed on rodents. As shown by Bjørneboe *et al*[67], under non-fasting conditions in rats 51% of serum α-TOH were associated with chylomicrons and VLDL whereas 47% were found within HDL. These differences across species highlight clearly the complexity of this topic.

The concentrations of vitamin E in chylomicrons are independent of the vitamin E form or stereoisomers. Vitamin E is secreted *via* chylomicrons by enterocytes into the intestinal lymph system to begin systemic circulation. The triglycerides in chylomicrons are subjected to hydrolysis by lipoprotein lipase (LPL)[56,70]. During lipolysis, vitamin E remains in the lipoprotein particle and its further transport through circulation occurs *via* chylomicron remnants. Vitamin E is then imported into the liver *via* LDL receptor (LDLR)-related proteins - and LDLR-mediated uptake of chylomicron remnants[60,71].

In the liver, vitamin E undergoes several sorting steps [see section "α-TOH transfer protein (α-TTP)" in the following chapter "intracellular binding proteins"] or metabolic processes (see section "metabolism of vitamin E"). α-Tocopherol is the form of vitamin E that is almost exclusively secreted into circulation *via* VLDL. Whereas VLDL have the highest capacity to carry α-TOH, these particles represent the smallest fraction of lipoproteins involved in vitamin E transport in the circulation in the fasting state[67]. Unfortunately, the mechanisms by which VLDL are enriched with α-TOH are poorly understood[72] (for further details, see the section on α-TTP in "intracellular binding proteins"). Alternatively, analogous mechanisms for α-TOH and free cholesterol have been discussed to explain the intracellular incorporation of α-TOH into nascent VLDL[72]. In support of this hypothesis, Bjornson *et al*[73] found that α-TOH and unesterified cholesterol translocate spontaneously from cellular membranes to lipoproteins. Further, ABCA1 has been shown to enrich HDL with α-TOH, which can be then transfered spontaneously or *via* the plasma phospholipid transfer protein (PLTP) to VLDL[68,74,75]. A similar mechanism has been suggested for the transfer of α-TOH to HDL *via* ABCA1[75].

Similar to chylomicrons, triglycerides in VLDL particles are enzymatically hydrolyzed by LPL resulting in the stepwise formation of LDL. Kono *et al*[76] suggested recently that LDL particles carry the major portion of plasma α-TOH and that LDLR-mediated endocytosis contributes significantly to the uptake of α-TOH into cells[77]. However, studies performed on apolipoprotein B (Apob)-knockout mice and heritable hyperlipidemic Watanabe rabbits lacking the LDLR showed discrepancies in circulating α-TOH levels and tissue distribution, thus questioning the importance of LDL for α-TOH transport[78,79]. Cohn *et al*[79] therefore concluded that α-TOH in LDL can be taken up by tissues *via* LDLR but is also independent of this lipoprotein receptor. Uptake transporters and specific intracellular transport proteins involved in α-TOH trafficking are handled in more detail in a following section.

High-density lipoproteins provides the means for α-TOH to be secreted out of the extrahepatic tissues and enter into circulation to be transported back to the liver. High-density lipoprotein particles contain the lowest concentration of vitamin E per particle but HDL is the most potent donor of vitamin E to several target tissues[64,74,80], despite the larger amounts of LDL in plasma. One possible explanation for this observation was given by Clevidence *et al*[81], who speculated that HDL contain more components for binding α-TOH besides serum ApoAI[69]. The *in vivo* importance of HDL in providing α-TOH to the central nervous system was highlighted by Goti *et al*[80]. In agreement with this finding, Kolleck *et al*[64] found that HDL might be the primary source of vitamin E for type Ⅱ pneumocytes. Furthermore, it has been suggested that the supply of vitamin E by HDL could be more important under pathophysiological conditions, such as oxidative stress, most likely independent from the HDL-mediated uptake of cholesterol[64]. The HDL-interacting scavenger receptor SR-B1 controls the uptake and accumulation of α-TOH in specific tissues[82]. *In vitro* experiments using type Ⅱ pneumocytes identified SR-B1 as a potential α-TOH uptake promoter, whereas the role of SR-B2 in this process is still speculative[64].

Vitamin E or rather α-TOH has been shown to move actively between lipoproteins of different density classes[74]. As previously mentioned, triglycerides in lipoproteins are catabolized *via* LPL[56,70]. During this lipolysis step, vitamin E remains incorporated either in chylomicron remnants (in the case of chylomicrons) or in LDL (in the case of VLDL). PLTP mediates the transfers of α-TOH between different classes of lipoproteins and also the exchange of α-TOH between HDL and cell membranes[68]. Another member of the group of lipid transfer proteins, the cholesteryl ester transfer protein, has also been suggested as having a role in vitamin E transport and metabolism[60] (Figure 2).

Deficiencies due to absorption and vascular transport disorders

As already outlined, Ulatowski and Manor[16] categorized

metabolic deficiencies of vitamin E into primary and secondary deficiencies, where the latter includes disorders of lipid absorption and transport as well as impaired lipoprotein metabolism, such as cholestatic liver disease, short bowl syndrome, Crohn's disease, abetalipoproteinemia and Niemann-Pick disease type C as well as Tangier disease. Depending on the disease, the severity of vitamin E deficiency varies[83]. The causes for fat malabsorption are diverse and usually result in vitamin E deficiency presenting already in early childhood[19]; these include, *inter alia* cholestatic liver diseases, cystic fibrosis[84], Crohn's disease, thrombosis, intestinal pseudoobstruction and chronic pancreatitis. The Marinesco-Sjögren syndrome and chylomicron retention disease, also known as Anderson's disease, are further causes of severe vitamin E deficiency. The symptoms of Anderson's disease are much milder compared to that of the Marinesco-Sjögren syndrome[85,86]. However, both diseases are caused by the inability of enterocytes to assemble or deliver chylomicrons leading to disturbed intestinal fat transport. Abetalipoproteinemia is caused by mutations in the microsomal triglyceride transfer protein that result in impaired intestinal absorption of lipids and severe vitamin E deficiency. In addition, the transport of vitamin E *via* VLDL to extrahepatic tissues is disturbed. Reduced levels of vitamin E are also found in patients with homozygous hypobetalipoproteinemia[83], which is caused by mutations in the *APOB* gene.

Finding a cure for vitamin E deficiency has remained elusive. Administration of α-TOH in micellar form in moderate doses[43] and supplementation of vitamin E are recommended but should be performed without any interfering components, such as some food ingredients. Intramuscular injection or oral uptake as water-soluble vitamin E ester has also been considered[19].

Afamin

As outlined above the vascular transport of vitamin E is performed by lipoproteins. However, in body fluids with low lipoprotein concentrations, such as follicular fluids, an alternative carrier protein for vitamin E, namely afamin, has been described[87-89]. Afamin is a liver-derived plasma glycoprotein and a member of the albumin protein family[90-92]; therefore it is also called α-albumin[93]. Afamin is partly (13%) bound to plasma lipoproteins[88], but circulates mostly in free form. Since afamin has 18 predicted binding sites for vitamin E and shows binding affinity for both α-TOH and γ-TOH, it has been suggested to be an alternative vitamin E transporter in body fluids under conditions where the lipoprotein system is not sufficient for vitamin E transport[89]. Originally, vitamin E was discovered as a resorption-gestations factor in female rats[2,94]. Afamin was therefore suggested as playing a role in female fertility[92], and indeed it has been shown to bind vitamin E and to increase in maternal serum during pregnancy[92]. Yet the precise role of afamin in pregnancy or for infertility is still not known[95]. On the one hand, afamin increases during pregnancy and decreases after childbirth to baseline levels, but on the other hand, a pilot study of Hubalek *et al*[92] showed that women suffering from pregnancy complications had significantly higher median afamin concentrations than women with uncomplicated pregnancy. In addition to being found in follicular fluids, afamin has also been detected in other extravascular fluids, such as cerebrospinal fluids, although the concentration is tenfold lower than in follicular fluids[88]. Since vitamin E deficiency is also known to cause cerebral complications[96], the detection of afamin in cerebrospinal fluids may indicate a role of afamin in neuroprotection[88]. Supporting the suggestion that afamin plays a potential role in fertility and neuroprotection, Jerkovic *et al*[88] found significant correlations of afamin and vitamin E concentrations in follicular and cerebrospinal fluids.

INTRACELLULAR BINDING PROTEINS

α-Tocopherol transfer protein

In contrast to the lipoprotein-mediated transport of vitamin E in the vascular system, cellular vitamin E is specifically bound to intracellular transport proteins, such as α-TTP in the liver where this protein is highly expressed[97]. As α-TTP is also abundantly expressed in the placenta, the importance of α-TOH in preventing fetus resorption is evident[98]. Furthermore, α-TTP is also expressed in several other tissues[76], such as rat brain, spleen, lung and kidney[99], the pregnant mouse uterus[100], retina[101] and central nervous system[21], suggesting an ubiquitous role for α-TTP in intra-organ trafficking[102].

Hosomi *et al*[103] estimated the relative affinities of α-TTP to the different vitamin E forms and stereoisomers starting from *RRR*-α-TOH set to 100%: β-TOH (38%), α-T3 (12%), *SRR*-α-TOH (11%), γ-TOH and trolox (9%) followed by δ-TOH, α-TOH acetate and α-TOH quinone with 2%. Requirements for the binding of vitamin E forms and derivatives to α-TTP are: Three methyl groups at the chromanol ring system (especially at position C5), one free hydroxyl group and the phythyl side-chain[103]. As α-TOH fulfills all of these criteria best within the group of vitamin E forms, it binds efficiently to α-TTP into a deep cavity lined with hydrophobic residues[16], while α-TTP does not readily bind[104] to or transfer γ-TOH[103]. Since the affinity of different vitamin E forms to α-TTP reflects the biological activity from rat resorption-gestations assays[94], the hypothesis is supported that α-TTP is responsible for the discrimination of α-TOH. However, a current study from Grebenstein *et al*[105] raises the suggestion that the metabolism of vitamin E, but not α-TTP, is responsible for discrimination against mainly non-α-TOH forms, as α-TTP protects the side-chain of the different vitamin E forms from ω-hydroxylase-induced degradation. High levels of expression of α-TTP were found to lead to higher intracellular concentrations of γ-TOH in combination with a reduced production of

γ-CEHC, which confirms the concept that binding to α-TTP protects from metabolic degradation[105].

While α-TTP binds both TOHs and T3s, the binding affinity is modulated by the presence of α-TOH, leading to decreased binding of non-α-TOH forms and T3s[106] and, in turn, to improved metabolism of the non-α-TOH forms. Several studies support the observation that α-TOH supplementation depletes plasma and tissue γ-TOH because of the enhanced metabolism of non-α-TOH forms[107,108].

The main function of α-TTP is to maintain normal α-TOH concentrations in the plasma and extrahepatic tissues[72]. This function is ensured by facilitating the transport of α-TOH from the lysosomes to the plasma membranes[109], followed by the continuous export of α-TOH from the liver to the plasma[110]. It is assumed that α-TTP is required for the incorporation of α-TOH into VLDL particles, but the underlying mechanisms are still not understood[72]. Traber *et al*[72] have systematically reviewed the enrichment of VLDL with α-TOH in the ribosomal endoplasmic reticulum, the Golgi apparatus, and the plasma membrane. It is suggested that α-TTP transfers α-TOH into nascent VLDL from the endosome, multi-vesicular bodies and lysosome[72]. Further, Kono and Arai suggested that α-TTP translocates from the cytosol to late endosomes/lysosomes to acquire α-TOH, which has been taken up by endocytosis or released from lipoproteins[76]. The outer membrane of the endocytic vesicles is enriched with both *RRR*-α-TOH and *SRR*-α-TOH by ABCA1, followed by the selective uptake of *RRR*-α-TOH *via* α-TTP[72]. Non-α-TOH forms are not protected against ω-hydroxylation after endocytosis and are further transported to the endoplasmic reticulum and the late endosomal compartment[105]. The α-TTP/α-TOH complex moves to the plasma membrane where it is targeted by phosphatidylinositol bisphosphates (PIP2), which are essential interaction partners of α-TTP[76]. In this case, α-TTP has been shown to transfer α-TOH between membranes through direct protein-membrane interactions[111-113]. In brief, experiments with liposomes have revealed that α-TTP acts as an α-TOH/PIP2 exchanger[76]. A flippase has been suggested to be involved in the transfer of α-TOH to the outer leaflet of the plasma membrane[114]. Next is the spontaneous transfer of α-TOH to nascent VLDL particles in the perisinusoidal space[72]. Interestingly, the effectiveness of the enrichment of nascent VLDL and pre-VLDL with the different stereoisomers *RRR*-α-TOH and *SRR*-α-TOH is similar, although α-TTP is known to be more specific for *RRR*-α-TOH[72]. Hence, the enrichment of VLDL seems to be more complex. However, the Golgi apparatus is probably not involved in this process. As shown by Arita *et al*[115], suppression of the endoplasmic reticulum/Golgi secretory pathway using brefeldin A did not affect the release of α-TOH. These findings suggest that α-TOH can be secreted by the liver independently from VLDL, although post-secretory nascent VLDL has not yet been eliminated as a physiological α-TOH acceptor[72].

Familial isolated vitamin E deficiency, which is also known as ataxia with isolated vitamin E deficiency (AVED), is categorized as a primary vitamin E deficiency. Symptoms of AVED are ataxia, dysarthria, reduced or absent tendon jerks, and impaired vibration sense. AVED is an autosomal recessive disorder caused by mutations in the *TTPA* gene[76], which result in the inadequate distribution of vitamin E to peripheral tissues[116,117]. Many of the 20 different mutations known to underlie AVED encode for truncated and therefore defective α-TTP proteins[76]. While absorption and vascular transport of vitamin E is normal in these patients, the release of α-TOH from the liver into circulation is disturbed[118]. Due to the dysfunctional α-TTP, AVED patients show impaired selectivity between α- and γ-TOH[119] and no differentiation between *RRR*- and *SRR*-α-TOH[72]. As a consequence of the lack of α-TTP, lower α-TOH plasma concentrations are found in AVED patients, likely due to increased rates of metabolic degradation of α-TOH, measureable as significantly increased urinary excretion of α-CEHC, the end-product of vitamin E catabolism, compared to healthy individuals[116]. It was therefore concluded that the capacity of α-TTP rather than the plasma concentration of α-TOH regulates the rate of vitamin E degradation[120].

Kono *et al*[76] found in mice with different genetic α-TTP backgrounds, with respect to the *Ttpa* gene encoding α-TTP, such as $Ttpa^{+/+}$, $Ttpa^{+/-}$ and $Ttpa^{-/-}$, that α-TOH plasma levels correlate with the number of functional *Ttpa* alleles, which supports the aforementioned conclusion. Similarly to human AVED patients, *Ttpa* knockout mice are "characterized by vitamin E deficiency, oxidative stress, late-onset ataxia, and female infertility, all of which can be prevented by timely supplementation with α-tocopherol"[16]; the *Ttpa* knockout mouse seems to be therefore a suitable animal model to study the role of α-TTP in vitamin E homeostasis.

Other binding proteins

In addition to α-TTP, further intracellular α-TOH binding and transport proteins are known, namely the tocopherol-associated protein (TAP) and the tocopherol-binding protein. In humans, three highly homologous TAP proteins, namely TAP1/SEC14-like 2 protein (SEC14L2; synonymously, supernatant protein factor, SPF), TAP2/SEC14L3 and TAP3/SEC14L4, have been described[121,122], but the contribution of TAP2 and TAP3 to the transport and metabolism of vitamin E has not yet been investigated. TAP 1 and α-TTP belong to a family of ligand-binding proteins that have a *cis*-retinal binding motif sequence, the so-called CRAL-TRIO domain. All members of this protein family bind α-TOH, but to a lesser extent than α-TTP, and the physiological relevance is unknown[19]. It has been suggested that TAP1 is involved in intracellular trafficking of α-TOH[56,123]; TAP1 mediates anti-proliferative effects in LNCaP and DU-145 prostate cancer cell lines by promoting vitamin E uptake

but also by effects independent of α-TOH[1,124]. TAP 1 is involved in cholesterol synthesis and forms complexes with *RRR*-α-TOH quinone, the oxidation product of α-TOH; however, for a better understanding of the function of TAP1, further studies are needed[19,125].

In addition to the binding and transport proteins with affinity and specificity for α-TOH, the cytosolic protein saposin B has a specific binding site for γ-TOH[126]. Saposin B binds γ-TOH more effectively than α-TOH when these vitamin E forms are competing for binding to saposin B *in vitro*[126]. However, *in vivo* the concentration of α-TOH is tenfold higher than of γ-TOH, leading to widely differing conditions which characterize the *in vitro* binding affinity studies in which a ratio of γ-TOH to α-TOH of 1:5 was used.

METABOLISM OF VITAMIN E

Simon products

Many decades ago it was thought that the metabolism of vitamin E was initiated by a radical attack on the chroman structure resulting in a ring opening and the building thereby of TOH quinone[127]. Subsequent side-chain degradation would lead to α-tocopheronic acid and its lactone, α-tocopheronelactone (α-TL), the so-called Simon metabolites described in conjugated and non-conjugated forms in urine of mice and humans[120,128-130]. This pathway has since been questioned because: (1) urinary TOH metabolites with an intact chroman ring system, the carboxyethylhydroxychromanols (CEHC), have been discovered[131-133]; and (2) an almost complete conversion of α-CEHC to α-TL is possible at least *in vitro* by bubbling oxygen through a solution of 70 mmol/L α-CEHC in 0.1 mol/L HCl for 24 h at room temperature[132,134]. In contrast, a recent study by Sharma *et al*[135] provided evidence that conjugates of α-TL are indeed real metabolites and not methodological artefacts. In addition, the study characterized α-TL as a biomarker of oxidative stress in children with type 1 diabetes, as the mean concentrations of the glucuronides and sulfate conjugates of α-TL were all significantly increased in these children[135]. Further research is required to answer the question of whether α-TL is a definite marker or an analytical artefact.

Hepatic metabolism of vitamin E

Degradation processes in the hepatic metabolism of vitamin E remain poorly understood. Initial mechanisms are generally accepted, *i.e.,* all vitamers are degraded to vitamer-specific physiological metabolites with an intact chromanol ring (therefore the nomenclature as α-, β-, γ- and δ-metabolites is used), leading to changes in the side-chain. The metabolites have been found in different tissues and body fluids conjugated similarly to xenobiotics and in non-conjugated form. For a detailed overview, see Figure 3.

Metabolism of TOH

Hepatic metabolism of TOH is initiated by CYP4F2/CYP3A4 (for details, see the sections on CYP3A4 and CYP4F2 in chapter "metabolism of vitamin E") dependent ω-hydroxylation of the aliphatic side-chain, forming 13'-hydroxychromanol (13'-OH), which can be analyzed using GC-MS (γ-, δ-13'-OH[139,140]). Next, ω-oxidation leads to 13'-carboxychromanol (13'-COOH), which can be detected *via* LC-ESI-MS (γ-, δ-13'-COOH[139,136]), HPLC-FD or HPLC-ECD (γ-, δ-13'-COOH[136,141]) or GC-MS (γ-13'-COOH[140]). An overview of all metabolites of TOH, analytical methods and identified matrices is provided in Table 1. Subsequent β-oxidation steps shorten the side-chain, thus forming carboxydimethyldecylhydroxychromanol (CDMDHC, 11'-COOH) followed by carboxymethyloctyl-hydroxychromanol (CDMOHC, 9'-COOH), both of which can be analyzed by LC-ESI-MS (γ-, δ-11'-COOH and γ-, δ-9'-COOH[139,136,141]), HPLC-FD or HPLC-ECD (γ-, δ-11'-COOH and γ-, δ-9'-COOH[136,141]) or GC-MS (γ-11'-COOH and γ-9'-COOH[140]). These metabolites with a side-chain length of between 13 to 9 carbon units can be summarized as long-chain metabolites (LCM) of vitamin E. According to their hydrophobicity, the LCMs are not excreted into urine and have been found in human and rat liver microsomes (α-, γ-13'-OH, and α-, γ-13'-COOH[139,140]), murine serum (γ-, δ-11'-COOH[136]), as well as human serum (α-13'-COOH[142]), in human and mice feces (α-13'-COOH; γ-, δ-11'-COOH; γ-, δ-9'-COOH[136]), and *in vitro* in cell culture supernatants of human lung epithelial A549 cells (γ-, δ-13'-OH and γ-, δ-13'-COOH[139]), as well as in HepG2 cells (γ-, δ-13'-OH; γ-, δ-13'-COOH; γ-11'-COOH; γ-9'-COOH[140,143]).

Intermediate-chain metabolites (ICM) are the products of two further β-oxidation steps. Carboxymethylhexylhydroxychromanol (CDMHHC, 7'-COOH) and carboxymethylbutylhydroxychromanol (CMBHC, 5'-COOH) have been detected *via* GC-MS (γ-7'-COOH and α-, γ-5'-COOH[134,140,144,137]), HPLC-ECD (γ-, δ-7'-COOH and α-, γ-, δ-5'-COOH[136,137]), and LC-ESI-MS (γ-, δ-7'-COOH and α-, γ-, δ-5'-COOH[136]) in murine liver (γ-, δ-5'-COOH[136]), in murine serum (γ-, δ-5'-COOH[136]), as well as in human serum or plasma (α-, γ-5'-COOH[136,116]), in murine and human feces (γ-, δ-7'-COOH; α-, γ-, δ-5'-COOH[136]), and in murine urine (γ-, δ-5'-COOH[136]), as well as in human urine (α-, γ-, δ-5'-COOH[134,136,144]). The ICMs have also been found in cultured HepG2 cells (α-, γ-7'-COOH and α-, γ-5'-COOH[120,140,144]).

The catabolic end-products of vitamin E metabolism are the CEHC (CEHC, sometimes also referred to 3'-COOH or short-chain metabolites, SCM), which were an early focus of research on vitamin E metabolism[145,146]. In the 1980s and 1990s different CEHCs were identified as the first known metabolites of vitamin E degradation: α-CEHC[132,146], γ-CEHC[147] and δ-CEHC[131]. Shortly after the discovery of the SCMs, it was shown that not only γ-TOH metabolism but also the degradation of γ-T3 results in γ-CEHC[148]. Meanwhile, this has been confirmed for all T3s and their corresponding CEHCs[136]. Many different analytical procedures and detection

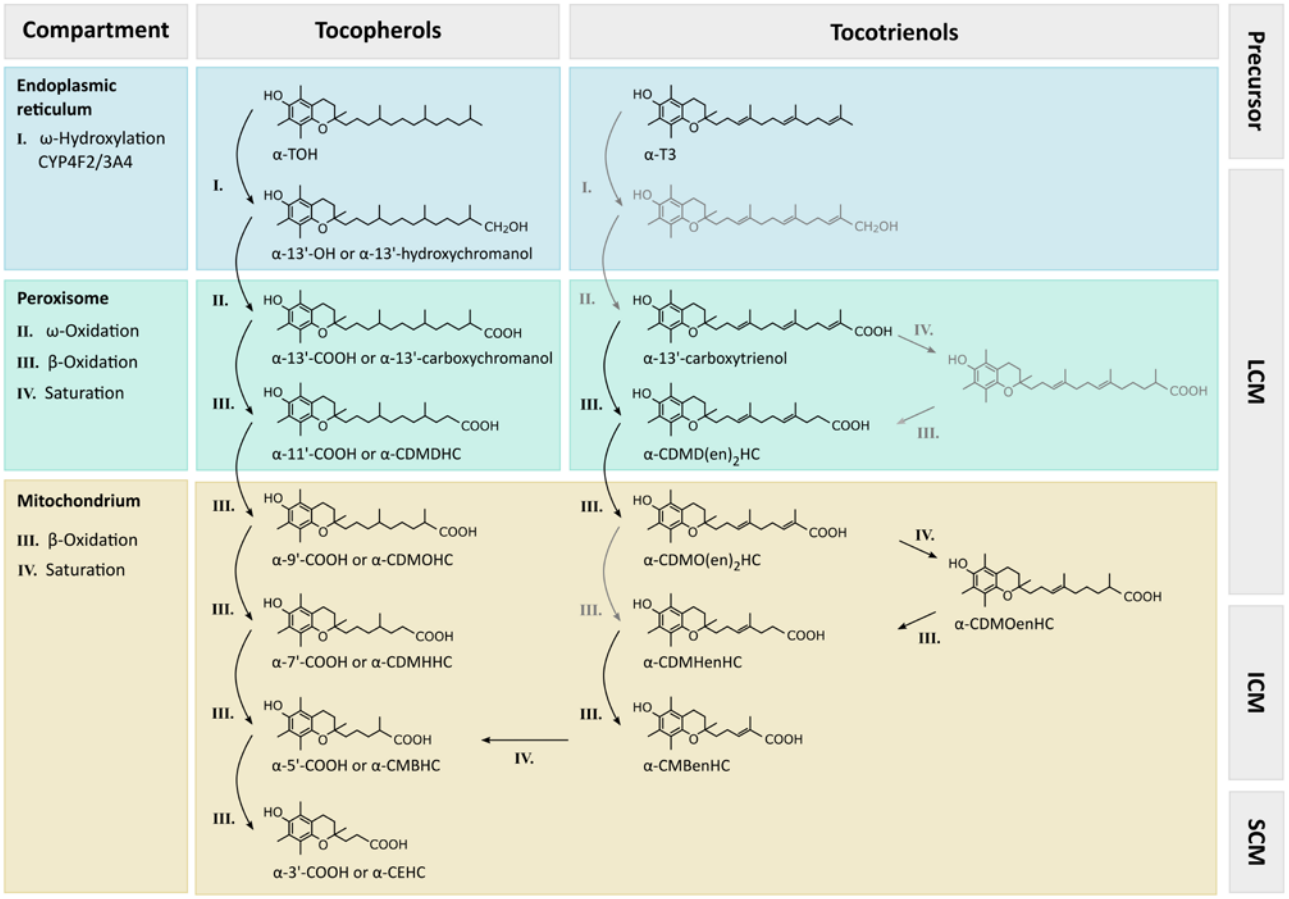


Figure 3 Metabolism of vitamin E. The metabolism of vitamin E starts with one cycle of ω-hydroxylation followed by five cycles of β-oxidation. The principal catabolic pathway is independent of the saturation of the side-chain[136,137] or the substitution of the chromanol ring system[136], whereas the rate of metabolic degradation is modulated mostly by these two factors[136] (for further details, see the sections on α-TTP in chapter "Intracellular binding proteins" as well as CYP4F2 and kinetics in chapter "Metabolism of vitamin E"). While the intracellular compartmentation of the different reaction steps is known for TOHs[138], it remains unresolved for T3s. The same applies to the structures and arrows marked in grey; they reflect molecules or principle steps which fit into the general concept so far but need further experimental confirmation and molecular characterization. α-TOH: α-tocopherols; α-T3: α-tocotrienols; 13'-OH: 13'-hydroxychromanol; 13'-COOH: 13'-carboxychromanol; LCM: Long-chain metabolites; ICM: Intermediate-chain metabolites; SCM: Short-chain metabolites; CDMD(en)2HC: Carboxydimethyldecadienylhydroxychromanol; CDMOenHC: Carbodimethyloctenylhydroxychromanol; CDMHenHC: Carboxymethylhexenylhydroxychromanol; CMBenHC: Carboxymethylbutadienylhydroxychromanol; CDMOHC: Carboxymethyloctylhydroxychromanol; CDMHHC: Carboxymethylhexylhydroxychromanol; CMBHC: Carboxymethylbutylhydroxychromanol; CEHC: Carboxyethylhydroxychromanols.

methods for the SCMs have been described: LC-ESI-MS (α-, γ-, δ-CEHC[139,136,141]), HPLC-ECD (α-, γ-CEHC[136,137,149-152]), and GC-MS (α-, γ-, δ-CEHC[134,144,137,153]). Usually, trolox or 1-naphthol are used as internal standards for the analysis of SCMs[150,151,154]. The CEHCs have been analyzed in rat and murine liver (α-, γ-, δ-CEHC[136,154]), in rat plasma and in mice serum (γ-, δ-CEHC[136,141]), as well as in human plasma and serum (α-, γ-, δ-CEHC[140,136,150,152,155]), in human and mouse urine (α-, γ-, δ-CEHC[134-136,144,151,155,156]) and in human and mouse feces (α-, γ-, δ-CEHC[136]) as well as in rat bile (α-, γ-CEHC[149]) and *in vitro* in A549 and HepG2 cells (γ-CEHC[140,141,144,137]).

Recently, two new metabolites, namely 12'-hydroxychromanol (12'-OH: γ- and δ-12'-OH) and 11'-hydroxychromanol (11'-OH: γ- and δ-11'-OH) have been identified using GC-MS in fecal pellets of mice fed a diet rich in γ-TOH[157]. These products provide evidence for ω-1 and ω-2 hydroxylation activity and that 12'-OH is not able to undergo oxidation followed by side-chain truncation. These metabolites are therefore excreted *via* bile and are found in the feces of mice and humans[157].

Metabolism of T3

The metabolism of T3s was first analyzed by Birringer *et al*[137] in HepG2 cells; it is in principal comparable to the metabolism of TOHs starting with an ω-hydroxylation followed by five cycles of β-oxidation and resulting in the end-product CEHC. Meanwhile almost all expected metabolites have been found in mouse and human samples after supplementation with their respective metabolic precursors, TOHs and T3s[136]. The corresponding metabolites were found: Carboxytrienol (13'), carboxydimethyldecadienylhydroxychromanol [CDMD(en)$_2$HC; 11'], carbodimethyloctenylhydroxychromanol (CDMOenHC; 9'), as well as carboxydimethyloctadienylhydroxychromanols [CDMO(en)$_2$HC; 9'], carboxymethylhexenylhydroxychromanol (CMHenHC; 7'), and carboxymethylbutadienylhydroxychromanol (CMBenHC; 5'). This leads to the conclusion that the side-chain of T3s is saturated before shortening. We

Table 1 Overview on metabolites of vitamin E

Metabolite	Methods	Matrix
13′-Hydroxychromanol (13′-OH)	GC-MS[139,140]	Human and rat liver microsomes[139,140] Cell culture medium of A549 cells[139] HepG2 cells[140,143]
13′-Carboxychromanol (13′-COOH)	LC-ESI-MS[136,139] HPLC-FD or HPLC-ECD[136,141] GC-MS[140]	Human liver microsomes, serum, feces[136,140,142] Rat liver microsomes[139,140] Mouse feces[136] Cell culture medium of A549 cells[139] HepG2 cells[140,143]
Carboxydimethyldecyl-hydroxychroman (CDMDHC) (11′-COOH)	LC-ESI-MS[136,139,141] HPLC-FD or HPLC-ECD[136,141] GC-MS[140]	Human feces[136] Mouse serum and feces[136] HepG2 cells[140,143]
Carboxymethyloctyl-hydroxychroman (CDMOHC) (9′-COOH)	LC-ESI-MS[136,139,141] HPLC-FD or HPLC-ECD[136,141] GC-MS[140]	Human and mouse feces[136] HepG2 cells[140,143]
Carboxymethylhexyl-hydroxychroman (CDMHHC) (7′-COOH)	GC-MS[134,137,140,144] HPLC-ECD[136,137] LC-ESI-MS[136]	Human and mouse feces[136] HepG2 cells[120,140,144]
Carboxymethylbutyl-hydroxychroman (CMBHC) (5′-COOH)	GC-MS[134,137,140,144] HPLC-ECD[136,137] LC-ESI-MS[136]	Human serum or plasma, urine and feces[116,134,136,144] Mouse liver, serum, urine and feces[136] HepG2 cells[120,140,144]
Carboxyethyl-hydroxychroman (CEHC) (3′-COOH)	LC-ESI-MS[136,139,141] HPLC-ECD[136,137,149-152] GC-MS[134,137,144,153]	Human plasma or serum, urine, feces[134-136,140,144,150-152,155,156] Rat liver, plasma, bile and urine[141,149,154] Mouse serum or plasma, urine, feces and liver[134-136,144,151,155,156] A549 and HepG2 cells[137,140,141,144]

speculate that auxiliary enzymes also needed for the degradation of unsaturated fatty acids, *e.g.,* 2,4-dienoyl-CoA reductase and 3,2-enoyl-CoA isomerase, are therefore required for the saturation of the double bonds in the side chain of T3 metabolites, as was originally suggested by Birringer *et al*[137].

Intracellular compartmentation of vitamin E metabolism

Mustacich *et al*[138] were the first to identify the intracellular compartmentation of vitamin E metabolism. Based on their work, the ω-hydroxylation catalyzed by alcohol dehydrogenase (resulting in 13′-OH) and the following reaction catalyzed by aldehyde dehydrogenase (forming 13′-COOH), take place at the endoplasmic reticulum. The carboxylated side-chain of TOHs are similar in structure to 2-methyl branched-chain fatty acids, which are subsequently β-oxidized in peroxisomes by their activation to an acyl-CoA ester[138]. This pattern seems to also characterize degradation of LCMs as two cycles of peroxisomal β-oxidation (resulting in 11′-COOH and 9′-COOH) with separation of propionyl-CoA or acetyl-CoA were suggested for TOHs[138]. Cho *et al*[156] noted that the sterol carrier protein-x (SCP-x) is involved in peroxisomal oxidation of branched-chain lipids, serves as a peroxisomal 3-ketoacyl-CoA thiolase, and shows reduced expression in response to pregnane X receptor (PXR) activation (for further details on PXR and vitamin E interactions, see the section on CYP3A4 in chapter "metabolism of vitamin E"). Based on their data the authors suggested that SCP-x is involved in the formation of vitamin E metabolites. It is not known yet whether LCMs are transported into peroxisomes as "free"molecules or as CoA esters, as it is known for long-chain, very long-chain or branched-chain fatty acids[158]. Based on knowledge of the transport of very long-chain acyl-CoA into the peroxisomes *via* ABCD[159], an involvement of related proteins for the import of vitamin E metabolites could be possible. As the last degradation steps are located in the mitochondria[138], the transport mechanisms out of peroxisomes and the subsequent import into mitochondria also needs to be unraveled. The import into mitochondria might occur through carnithin-acyl-transferases, as reported for metabolites of vitamin K[160]. Grebenstein *et al*[105] suggested that the LCMs are possible ligands for α-TTP and/or other hepatic TOH binding proteins. However, the importance of these proteins for the transfer of LCMs to mitochondria needs to be investigated further[105]. The mitochondrial production of ICMs and SCMs (three β-oxidation steps) is indicated by the analysis of CEHCs solely in mitochondria but not in peroxisomes[138]. It was emphasized by Mustacich *et al*[138] that these data do not exclude a model of exclusive mitochondrial β-oxidation.

Enzymatic degradation of vitamin E

As mentioned above, catabolism of TOHs and T3s begins with an ω-hydroxylation of the side-chain which is catalyzed by cytochrome P450 (CYP) enzymes, namely CYP4F2 or CYP3A4. This oxidation is the rate-limiting step in vitamin E metabolism[161]. CYP enzymes are heme-thiolate proteins that differ in substrate selectivity but catalyse monooxygenation reactions *via* activation of molecular oxygen[162]. In the following sections, we provide an overview of the two CYP enzymes known to be involved in vitamin E metabolism.

CYP3A4: The enzyme CYP3A4 is the most important CYP enzyme in humans, as the majority of administrated drugs are metabolized *via* CYP3A4 due to its wide range of substrates[162]. The first evidence for the involvement of CYP3A4 in vitamin E metabolism was provided by Parker *et al*[163]. Ketoconazole, an inhibitor of CYP3A4, blocked the metabolism of TOHs [α- and γ-TOH in primary rat hepatocytes or γ- and δ-TOH in HepG2/C3A (25 μmol/L each)] to their corresponding SCMs by approximately 90% after incubation with 1 mmol/L or 0.25 mmol/L for 48 h[163]. Similar effects were seen for γ-TOH when 1 mmol/L sesamin was used, a major sesame seed lignin and natural inhibitor of CYP3A4[163]. When 50 mg/kg body weight of ketoconazole were applied by oral gavage to rats simultaneously with a mixture of 10 mg/kg body weight of α-TOH, 10 mg/kg body weight of γ-TOH or 29.5 mg/kg body weight of T3 the catabolism of the vitamin E forms to their respective SCMs and excretion *via* urine was clearly reduced compared to the controls[164]. Using 50 μmol/L rifampicin, an inducer of CYP3A4 activity, Birringer *et al*[120] demonstrated an up to fivefold increase in *all-rac*-α-TOH degradation in HepG2 cells. It should be noted that preconditioning of the cells with 100 μmol/L α-TOH for 10 d was necessary, as the standard cell culture medium is deficient for α-TOH[120]. This might be the reason why Parker *et al*[163] were not able to detect α-TOH metabolism in HepG2 cells, as they did not perform a preconditioning and instead incubated the cells only for 24 h to 48 h with 0.25 μmol/L or 0.5 μmol/L α-TOH. Further evidence is given by the α-TOH-dependent regulation of CYP3A4[165]. Feeding mice with 200 mg/d of α-TOH for nine months resulted in 1.7-fold higher Cyp3a11 (*i.e.,* the murine orthologue of human CYP3A4) mRNA expression levels compared to after three months, while γ-T3 did not increase Cyp3a11 mRNA levels[166]. Similar effects on Cyp3a protein levels were observed when 10 mg/100 g body weight of α-TOH was injected subcutaneously into rats[167]. Traber *et al*[165] showed that C57BL/6 mice receiving a diet sufficiently enriched with α-TOH (31 mg α-TOH per kg diet) for five weeks have increased hepatic Cyp3a levels compared to mice fed an α-TOH-deficient diet with less than < 2 mg α-TOH per kg diet.

Several CYP enzymes, including CYP3A4, are regulated by structurally diverse xenobiotics *via* PXR, a nuclear receptor that regulates the expression of metabolic enzymes and transporters involved in the metabolism of xenobiotics and endobiotics[156,168]. Landes *et al*[169] showed that vitamin E acts as an agonist of PXR. Thereby, the inductive effect of vitamin E on chloramphenicol acetyl transferase activity was dependent on the vitamin E form in the following order: γ-T3 approximately equal α-T3 > rifampicin > δ-TOH > *RRR*-α-TOH ⩾ γ-TOH. The treatment of HepG2 cells with γ-T3 led to an up-regulation of CYP3A4 and CYP3A5 mRNA levels[169] and a dose-dependent activation of chloramphenicol acetyl transferase at 1 μmol/L to 10 μmol/L, concentrations which can be reached also in human plasma[23]. These striking contrary effects of γ-T3, up-regulation of CYP3A4 expression *in vitro*[168] and no effect[166] or even a reduction of Cyp3a expression[165] *in vivo* might be explained by different availabilities of the individual vitamin E forms at the site of action. Whereas the substance *in vitro* is applied onto the cells directly, all physiological processes of vitamin E handling, especially α-TTP dependent sorting of non-α-TOH forms in combination with the high metabolic degradation and elimination rates for γ-T3[166], may interfere *in vivo*, thus resulting in contradictory effects.

However, the induction of Cyp3a11, the mouse homologue of human CYP3A4, by α-TOH and the involvement of PXR was confirmed in wild-type mice by Johnson *et al*[170]. In this study, mice kept vitamin E-deficient were fed daily for two weeks with 500 mg/kg *DL*-α-TOH acetate, which is equivalent to a typical supplementation with 400 - 600 mg/d α-TOH for a 70 kg human. Expression of Cyp3a11 was induced in wild-type mice, but remained unchanged in Pxr-null mice and in humanized PXR mice as well, while dosing with known murine and human PXR-specific agonists up-regulated expression of Cyp3a11 in both the wild-type and the humanized PXR mice, but not the Pxr-deficient mice. This led to the conclusion that α-TOH is a partial agonist of mouse Pxr and that Pxr is required for the induction of Cyp3a11 by α-TOH in mice[170].

Apart from these, there are further contradictory findings. Parker *et al*[171] emphasized that the hypothesis of involvement of CYP3A4 in the metabolism of vitamin E is only based on the assumption of ketoconazole specificity, which proved incorrect[172]. Testing recombinant human CYP3A4 in insect cell derived microsomes revealed no activity towards α- or γ-TOH[140], whereas a systematic screening of other CYP enzymes showed tocopherol-ω-hydroxylase activity only for CYP4F2[171] (for further details, see the section on CYP4F2 in chapter "metabolism of vitamin E"). In addition, Birringer *et al*[120] showed that production of γ-CEHC from γ-TOH was not affected by rifampicin in HepG2 cells, leading to the conclusion that either CYP3A4 may not degrade all vitamin E forms to the same extent or other CYP enzymes may be involved in γ-TOH metabolism. Furthermore, Schuetz *et al*[173] reported in 1993 that HepG2 cells do not express CYP3A4 but CYP3A7. With respect to PXR, more discrepancies have been reported. Cho *et al*[156] used *Pxr*-deficient *vs* wild-type mice both treated with and without pregnenolone 16α-carbonitrile (an activator of rodent Pxr) to analyze the impact of Pxr on vitamin E degradation. The study revealed that urinary excretion of α-CEHC glucuronide was significantly decreased down to 16% and γ-CEHC glucoside was attenuated down to 40% in pregnenolone 16α-carbonitrile-treated wild-type mice compared with control wild-type mice, while urinary excretion of both metabolites were unaffected in the *Pxr*-null mice. Johnson *et al*[170] suggested that these findings

Table 2 Experimentally validated conjugates of vitamin E metabolites

Metabolite	Conjugate	Organism
LCM	Sulfates	Rats and *in vitro*[139,141]
ICM	Ether glucuronides	Human and mouse urine[176]
	Sulfates	Human urine[177]
SCM	Glucosides, glutamine	Mouse[156,176]
	Taurine, glycine or glycine glucuronides	Human and mouse urine[176]
	Sulfates	Rats[178]

LCM: Long-chain metabolites; ICM: Intermediate-chain metabolites; SCM: Short-chain metabolites.

are the result of a down-regulation of β-oxidation by pregnenolone 16α-carbonitrile. To sum up, the role of CYP3A4 in the metabolism of vitamin E remains unclear.

CYP4F2: The CYP4 subfamily of CYP enzymes catalyzes the ω-hydroxylation of saturated, branched-chain fatty acids as well as unsaturated fatty acids, whereas members of the CYP4F subfamily metabolize long-chain and very long-chain fatty acids[158]. As mentioned above, Sontag and Parker[140] reported the involvement of CYP4F2 in vitamin E metabolism using reporter-gene assays combined with a systematical screening of CYP enzymes in this context. Among the CYP enzymes tested, only CYP4F2 exhibited tocopherol-ω-hydroxylase activity, which was higher for γ-TOH than for α-TOH. In a subsequent study, Sontag and Parker[108] characterized the substrate specificity of CYP4F2. According to this study, the unsubstituted carbon at position C5 of the chromanol ring system induces activity of CYP4F2, so that γ- and δ-TOH are metabolized more efficiently than α-TOH, which in turn stimulates the metabolism of other vitamin E forms. The authors found higher V_{max} values for T3s than for their corresponding TOHs, suggesting that CYP4F2 contributes to the preferential physiological retention of α-TOH compared to other vitamin E forms. This finding supports the central role of this pathway in modulating the vitamin E biopotencies of TOHs and T3s[140,108]. Bardowell *et al*[161] identified Cyp4f14 as the murine orthologue of human CYP4F2 and analyzed vitamin E homeostasis in Cyp4f14 knock-out mice. Cyp4f14-deficient mice had higher tissue concentrations of non-α-TOH forms, such as γ-TOH in plasma and tissues and δ-TOH in fat tissue, whereas tissue and plasma levels of α-TOH remained unchanged (except for lower concentrations in heart tissue). In line with these findings, reduced elimination of γ-, δ-, and α-TOH metabolites *via* urine and feces was found, but increased fecal excretion of γ- and δ-TOH[161]. Due to the reduction of vitamin E metabolism instead of a complete abolishment, Bardowell *et al*[161] suggested the involvement of other enzymes in murine vitamin E catabolism.

While expression of CYP3A4 is regulated by α-TOH, CYP4F2 levels are not influenced by α-TOH as reported by Mustacich *et al*[167] in rats that were injected subcutaneously daily with 10 mg/100 g body weight α-TOH for up to 18 d. In these rats, protein levels of Cyp3a, Cyp2b, and Cyp2c were increased. Johnson *et al*[170] found that only the mouse orthologue of CYP4F2, Cyp4f13, but not Cyp4f14 was upregulated by α-TOH in wild-type mice, while in *Pxr*-null or humanized PXR mice no influence on the expression of Cyp4f13 and Cyp4f14 was observed. In *Ttpa*$^{-/-}$ and wild-type mice, Traber *et al*[165] revealed no influence of γ-TOH on the expression of the Cyp4f13 protein. However, a synthetic inhibitor of CYP4F2/Cyp4f13, namely (R)-2-[9-(1H-imidazol-1-yl)nonyl]-2,5,7,8-tetramethylchroman-6-ol, decreased formation of γ-CEHC from γ-TOH in HepG2 cells in culture[174]. The stable expression of CYP4F2 *in vivo* regardless of elevated vitamin E intake or availability, as indicated by higher concentrations of the metabolites, calls into question the suggestion that CYP4F2 alone is responsible for the initial step of vitamin E degradation. Taken together, the evidence for the involvement of CYP4F2 in vitamin E metabolism is convincing, but the participation of other CYP enzymes such as CYP3A4 cannot be excluded yet.

Conjugation of metabolites: Non-α-TOH forms of vitamin E are preferentially handled in the human body as xenobiotics involving phase I enzymes, and further degradation steps seem to also follow the track of xenobiotic metabolism, as vitamin E degradation products are found as sulfate and glucuronide conjugates. See Table 2 for an overview on the conjugates of vitamin E metabolites identified thus far.

Sulfates are thought to be the main conjugation products of the LCMs (γ- and δ-LCMs: 13′-COOH, 11′-COOH and 9′-COOH in rats and *in vitro*)[139,141], as an unknown peak occurring only after supplementation with γ-TOH in rats had the theoretical weight of sulfated γ-CEHC. In agreement with this finding, the peak decreased after treatment with sulfatase[139] or a combination of β-glucuronidase and sulfatase[141]. As the LCMs were found in both conjugated and non-conjugated form in cell culture medium of human A549 cells, it was suggested that conjugation and subsequent β-oxidation are parallel processes. This was confirmed in studies by Hashiguchi *et al*[175]. α-5′-COOH ether glucuronides[176] in urine of both humans and mice as well as α-5′-COOH sulfate in human urine[177] are known conjugates of ICMs. More is known about the conjugates of the SCMs. In humans, the majority of CEHCs are excreted *via* urine as glucuronides[136,147,152,155], whereas sulfates were found only in trace amounts[177] or in significant amounts[135,136,176]. In addition, other conjugates are known for α-CEHC, such as taurine, glycine or glycine glucuronide conjugates[176]. In mice, glucuronides and sulfates of α-, γ-, δ-CEHC have been found[136,156,176]. While glucoside conjugates of γ-CEHC[156] and α-CEHC glutamine[176] have been only found in mice, taurine, glycine or glycine glucuronides conjugates were found

in murine urine as well as in urine of humans[176]. The glucoside conjugate of γ-CEHC was found by Cho *et al*[156] and appeared to be the main conjugated form of γ-CEHC in mice. A comparative experiment revealed that β-glucuronidase treatment hydrolyzed not only glucuronide conjugates but also glucose-conjugated metabolites; it has been therefore suggested that glucosides originally contained in the samples were not detectable when β-glucuronidase treatment was applied[156]. As this procedure is common in vitamin E metabolite analysis, it is possible that glucosides remained mostly undetected. Tanabe *et al*[178] found sulfated γ-CEHC to be the main excretion product in rats, when γ-CEHC was applied; a conjugation product with the expected weight of sulfated γ-CEHC was detected and was also sensitive to β-glucuronidase/sulfatase treatment. This finding was confirmed by others, when sulfated γ-CEHC in rat urine or plasma was found[139,141,179].

To address the ratio of conjugated to non-conjugated metabolites in different body fluids, parallel analyses with or without enzymatic hydrolysis were used. Most CEHCs in urine are conjugated, as several groups have reported. Zhao *et al*[136] found a six- to tenfold increase of free CEHC in mouse urine after treatment with β-glucuronidase and sulfatase. Freiser and Jiang[141] suggested that between 88% to 98% of γ-CEHC is conjugated in the plasma of rats, and Leonard *et al*[154] found that between 30% to 40% of the tested α- and γ-CEHCs are conjugated in the liver. Lodge *et al*[151] suggested analysis of the portion of non-conjugated SCMs, as γ-CEHC was shown to be a powerful natriuretic factor in human urine[133].

The conjugates of vitamin E metabolites identified so far indicate an involvement of phase Ⅱ enzymes, mainly UDP glucuronosyltransferases (UGT) and sulfotransferases (SULT). UDP glucuronosyltransferase enzymes are involved in the conjugation of CEHCs with glucuronic acid or glucose[156], whereas SULT catalyze the transformation to sulfate conjugates. Interestingly, the different vitamin E forms have no effect on the expression of phase Ⅱ enzymes, as *in vitro* none of the eight vitamin E forms showed an altered expression of UGT1A1 mRNA in human primary hepatocytes[180]. Furthermore, the majority of UGT isoforms were not regulated in mice fed α-TOH deficient or enriched diets[170], and hepatic UGT activity was not influenced by feeding rats daily with 200 mg/kg body weight α-TOH for two weeks. It is of note that expression of UGT1A1 mRNA was increased 1.7-fold after treatment of wild-type mice with the PXR agonist pregnenolone 16α-carbonitrile compared to untreated control mice[156]. As UGTs are regulated *via* PXR[181,182], the effect of pregnenolone 16α-carbonitrile is in line with the latter finding, while the lack of UGT activation by α-TOH was unexpected. In a comparative screening of 14 members of the SULT enzyme family, Hashiguchi *et al*[175] found evidence for the involvement of SULT1 enzymes in vitamin E metabolism. Sulfotransferase 1 showed a stronger preference for γ-TOH than for α-TOH and for γ-CEHC over all other CEHCs. Contradictory results were obtained in mice where expression of Sult2a mRNA was increased 10.8-fold by α-TOH compared to the control mice[183]. Mustacich *et al*[138] noted that the presence of sulfated metabolites is difficult to clarify with respect to the exclusive peroxisomal degradation of vitamin E and the cytosolic localization of phase Ⅱ enzymes. However, when metabolites are generated in peroxisomes and mitochondria, they must be transported through the cytoplasm and can be subjected as substrates to the cytosolic SULTs[138]. Jiang *et al*[139] noted that sulfated metabolites of vitamin E may not only contribute to detoxification but could also perform regulatory functions. As mentioned before, in humans and mice, the concentration of glucuronides is higher than that of sulfates and a ratio of glucuronide:sulfate of approximately 8 was reported by Sharma *et al*[135]. The authors argued that this is in line with the fact that humans have higher capacity for glucuronidation than sulfation because of the high activity of hepatic UGT.

Another enzyme involved in the xenobiotic metabolism is glutathione S-transferase (GST). Van Haaften *et al*[184,185] found that TOHs and T3s inhibit isolated human GST P1-1 with IC_{50} values of 0.7 mmol/L for α-TOH, 0.8 mmol/L for δ-TOH, 1.8 mmol/L for α-T3 and 0.7 mmol/L for γ-T3. Contradictory findings were reported by Podszun and Frank[186] in rats fed high doses of α-TOH (2500 mg/kg diet for ten days), in which GST activity was increased 2- to 3-fold. When mice were fed with 1000 mg/kg diet of *all rac*-α-TOH acetate for four months expression of hepatic Gstm3 mRNA, responsible for the detoxification of electrophilic compounds, increased about twofold compared to the controls (35 mg *all rac*-α-TOH acetate/kg diet)[183].

Excretion: Due to their polarity, ICMs and SCMs, namely 5'-COOH and CEHCs, are excreted *via* urine, mostly as glucoside conjugates[136]. Feces contain the whole set of vitamin E metabolites, including precursors (TOHs and T3s) and water-soluble SCMs in humans[136] and mice[157,161,187]; LCMs (especially 13'-COOH) are the main fecal metabolites with > 60% of total metabolites[187]. Zhao *et al*[136] reported that the LCMs are unconjugated in feces. Because of the lack of β-glucuronidase treatment, Jiang *et al*[187] could not distinguish between conjugated and non-conjugated metabolites in their fecal samples, which in turn could support the results from Zhao *et al*[136]. Wu and Croft[188] concluded that part of the total vitamin E undergoes enterohepatic circulation (estimated to be about 60% in rats) and that the remaining is likely lost *via* the fecal route[189]. However, the fecal portion of metabolite excretion was estimated to be about 80%[161]. As mentioned before, intestinal absorption was reported to be between 20%-80% for α-TOH, leaving a considerable portion that remains in the intestinal tract[188]. Zhao *et al*[136] tested whether intestinal flora is able to degrade

TOHs by incubating fecal extracts with TOHs, but this experiment failed to produce vitamin E metabolites. To summarize, SCMs are transported *via* blood and are excreted through urine, while TOH, T3 and all other metabolites circulate through the vascular system (shown for α-13'-COOH in humans by our group[142]) and are secreted into bile and eliminated *via* feces.

Thus far, no specific transport or binding proteins for CEHCs or CEHC conjugates have been reported. However, some phase Ⅲ transporters are involved in the elimination of vitamin E, or their expression is regulated by vitamin E[63]. Early findings by Bjørneboe *et al*[190] in rats injected with radioactively labeled α-TOH revealed that 14% of the radioactivity was recovered during 24 h of bile draining, thus indicating an involvement of biliary excretion pathways. Since then, the underlying molecular mechanisms have been of particular interest. Apob seems to be involved in the secretion of α-TOH from liver cells into blood, as in *Apob*-knockout mice the biliary secretion of α-TOH is significantly decreased after a single *i.v.* injection of 25 mg/kg α-TOH compared to the controls[191]. Mardones *et al*[82] provided evidence that SR-B1 encoded by the *Scarb1* gene contributes to biliary excretion of α-TOH, as the hepatic concentration of α-TOH is normal compared to the controls but biliary excreted α-TOH was 74%-81% lower in *Scarb1*-knockout mice. Expression of SR-B1 is also reported to be regulated by α-TOH. Mice kept vitamin E-deficient showed elevated hepatic SR-B1 protein levels, which were reversible by feeding them a vitamin E-enriched diet, whereas HepG2 cells cultured in the presence of vitamin E-loaded HDL showed decreased SR-B1 levels[192]. According to Takada and Suzuki[193], SR-B1 might be responsible for the import of vitamin E into hepatocytes when located at basolateral membranes and also for the export into bile when expressed at the canalicular site. Therefore, many studies have aimed to clarify the differing expression patterns of SR-B1 between sinusoidal and canalicular membranes[194]. Comparison of human and mouse liver tissues as well as HepG2 cells have revealed pronounced differences. While *ex vivo* SR-B1 was highly enriched in sinusoidal membranes and was also found in canalicular membranes, HepG2 cells clearly showed enrichment of SR-B1 in bile canalicular-like structures[194].

Multidrug resistance proteins (MDR) are also involved in the elimination of α-TOH, as inhibition or deletion of canalicular Mdr2 (also named Ppg or p-glycoprotein) leads to a decrease in the basal release of α-TOH into bile in rats and mice[189]. Results from *in vitro* studies on MDR1 did not reveal clear results. Primary human hepatocytes showed no response in the expression of MDR1 to T3 treatment, whereas intestinal LS180 cells reacted with a clear increase of MDR1 expression (depending on the type of T3)[180]. In line with the latter finding, rats daily injected subcutaneously with 10 mg/100 g body weight α-TOH had increased levels of hepatic Mdr1 protein beginning at day 9 and reaching a peak at day 15 of injections[167]. Further, mice fed with 1000 IU/d *all-rac*-α-TOH acetate for four months showed elevated levels of Mdr1a protein, the mouse orthologue of human MDR1, compared to the controls, while expressions of Mdr1b, Mdr2, Abcc2, Abcc6 and breast cancer resistance protein 1 (Bcrp1/Abcg2) remained unchanged[183]. When rats were daily injected subcutaneously with 100 mg/kg body weight α-TOH, the hepatic efflux transporters Abcb1b and Abcg2 were upregulated, while the organic anion transporting polypeptide 2 (Oatp), a liver influx transporter, was downregulated[195]. Traber *et al*[195] noted that Abcg2 transports sulfates and glucuronides and suggested that this protein is involved in the excretion of conjugated vitamin E metabolites, such as CEHCs, while Oatp might be involved in the uptake of CEHCs into liver cells. Summarizing the current knowledge on excretion of vitamin E metabolites, it has to be emphasized that many aspects regarding the involvement of transporters in vitamin E and vitamin E metabolites still lack clarity. It remains to be resolved whether and which specialized proteins for the regulated excretion of vitamin E metabolites exist.

Kinetics of vitamin E degradation: All vitamin E forms are in principle degraded *via* the same pathways independent from their substitution pattern with methyl groups at the chromanol ring system. However, the rate of degradation depends on the methylation pattern of the chromanol ring, the saturation of the side-chain, and on the source of vitamin E (*i.e.,* natural *vs* synthetic).

Natural forms of vitamin E, *i.e.,* enantiopure vitamin E forms with the *RRR* configuration, are partially protected from degradation compared to synthetic forms, *i.e.,* vitamin E forms with the *all-rac* configuration, as was shown by Traber *et al*[196], who supplemented humans with 150 mg d_3-*RRR*-α- or d_6-*all rac*-α-TOH acetates. While d_3-*RRR*-α-TOH accumulated in plasma, d_6-α-CEHC derived from d_6-*all rac*-α-TOH was found almost only in urine. Vitamin E forms with unsaturated side-chains are degraded faster than the saturated forms. T3 were reported to appear in human plasma with half-lives of 4.3, 4.4, and 2.3 h for α-, γ- and δ-T3, respectively[23], whereas for *RRR*-α-TOH a half-life of 45 h[110] to 60 h[197] was found. When humans were supplemented with a single dose of 125 mg or 500 mg γ-T3, urinary excretion levels of γ-CEHC rose about 4- to 6-fold with a maximum at 9 h after ingestion and a decline to baseline by the following day[198]. An increase of urinary α-CEHC was only observed after ingestion of a very high dose of α-tocotrienyl acetate (500 mg compared to 125 mg)[198]. This study found 1%-2% of α-tocotrienyl acetates and 4%-6% of γ-tocotrienyl acetates as urinary metabolites, suggesting alternative elimination routes for T3s[198].

As outlined above, the elimination of vitamin E forms depends on the methylation pattern of the chromanol ring system. Zhao *et al*[136] analyzed human serum after

one-time supplementation with a mixture of different vitamin E forms (2400 mg γ-TOH, 1596 mg α-TOH, 936 mg δ-TOH and 24 mg T3s) and found concentrations of α-, γ- and δ-TOHs of 21.1, 6.19, and 0.5 μmol/L, respectively, as well as ICM and SCM concentrations after enzymatic hydrolysis of 0.03 μmol/L (α-5'-COOH), 0.21 μmol/L (γ-5'-COOH), 0.08 μmol/L (δ-5'-COOH), 0.02 μmol/L (α-CEHC), 0.35 μmol/L (γ-CEHC), 0.09 μmol/L (δ-CEHC) 12 h post dose. In support of these findings the estimated half-life of γ-TOH is, at 12 ± 4 h[197], shorter than that of α-TOH at 45 h[110] to 60 h[197]. Leonard *et al*[197] administrated humans orally with about 50 mg of an equimolar mixture of d_6-α-TOH and d_2-γ-TOH acetates and found no increase of α-CEHC (detection limit in this study: 1 nmol/L), while γ-CEHC plasma concentrations doubled (129 ± 20 to 258 ± 40 nmol/L in women) after 12 h. Schuelke *et al*[116] concluded that α-CEHC excretion follows α-TOH plasma levels only when a threshold of 30-40 μmol/L α-TOH in plasma is exceeded. Rats treated orally with an oil containing 10 mg of γ-TOH or a combination of α- and γ-TOH (10 mg each) had urinary γ-CEHC levels which reached their highest levels 24 h to 30 h post application in both groups, but the concentration of γ-CEHC was 20%-50% higher in the α- and γ-TOH treated group compared to the group receiving only γ-TOH[149]. In addition, a shift from biliary to urinary excretion of γ-CEHC was observed, with γ-CEHC concentrations being higher for the combined treatment with α- and γ-TOH than for the γ-TOH-treated group[149]. Analyzing the absolute contents of γ-CEHC in bile and urine, they found 130 μg or 190 μg for γ-TOH or α- and γ-TOH in bile, respectively, and 250 μg γ-CEHC in the γ-TOH treated group and 280 μg in the α- and γ-TOH treated group. The authors therefore suggested that the major excretion pathway for γ-CEHC is *via* urine[149]. Mustacich *et al*[167] injected rats daily with 10 mg/100 g body weight α-TOH and analyzed the hepatic concentrations of LCM α-13'-OH and ICM α-5'-COOH. The study revealed an increase in both metabolites for the first measurement after three days post first application, with higher concentrations for α-13'-OH (up to 6.4 ± 0.7 nmol/g; 20-fold higher than prior to injection) than for α-5'-COOH (1.0 ± 0.3 nmol/g at day 3; undetectable prior to injection). None of the other known metabolites were found in the analyzed livers. The levels of α-13'-OH decreased to 1.2 ± 0.2 nmol/g at day 18 and levels of α-5'-COOH decreased to 0.4 ± 0.1 nmol/g at day 12; both values remained unaffected by the subsequent injections of α-TOH. Bardowell *et al*[157] speculated that mechanisms other than ω-hydroxylation might contribute to the elimination of non-α-TOH forms of vitamin E. They proposed formation and fecal excretion of ω-1 and ω-2 metabolites γ-TOH, namely γ-12'-OH and γ-11'-OH, as well as fecal elimination of non-metabolized TOH, as these metabolites and their precursor TOHs were found in human feces. Zhao *et al*[136] found several vitamin E metabolites in mouse feces (see Table 1) after supplementation with a diet enriched with 0.3% of a mixture of different vitamin E forms (20.2% α-T3, 4.0% β-T3, 16.1% γ-T3, 9.9% δ-T3, 14.8% α-TOH, and 3.1% γ-TOH) for four weeks and reported increases in the concentrations of almost all of these metabolites in human fecal samples over time after a single dose supplementation of 2400 mg γ-TOH, 1596 mg α-TOH, 936 mg δ-TOH und 24 mg T3s. To sum up, elimination of non-α-TOH forms is not only greater than of α-TOH, but α-TOH also increases elimination rates of non-α-TOH forms of vitamin E. This can be explained by the physiological action of α-TTP (see the corresponding section on α-TTP in chapter "Intracellular binding proteins") and vitamin E ω-hydroxylase CYP4F2 (see the section on "Enzymatic degradation of vitamin E").

Parker *et al*[171] published a hypothesis to explain the underlying physiological importance of the different rates of metabolism and elimination of the different forms of vitamin E. When murine macrophages (RAW264.7 cells) were incubated with α-TOH, γ-TOH, δ-TOH or δ-T3, different cell viabilities were found: Cell viability for α-TOH was not impaired but was intermediately reduced for γ-TOH and cell viabilities for δ-TOH or δ-T3 were substantially lower. The catabolism rates of these vitamin E forms in HepG2 cells, however, were inverse, as almost no metabolites for α-TOH but increasing amounts of metabolites for the other vitamin E forms investigated were found, with highest amounts for δ-T3. Therefore, the authors suggested an inverse correlation between the different cytotoxicities of the different vitamin E forms and their elimination rates.

Gender-specific bioavailability of vitamin E: Several years ago, differences in the bioavailability of vitamin E between genders were described. Feingold *et al*[199] observed in rats that α-TOH levels were higher in adrenal glands and livers as well as in all subcellular fractions derived from female rats compared to male rats. However, no sex differences in the subcellular distribution of α-TOH were observed. Notable differences in the kinetics of vitamin E metabolism were observed also in humans orally administered with a single dose of 50 mg deuterium-labeled d_6-α-TOH acetate and 50 mg d_2-γ-TOH acetate. Women showed higher maximum plasma concentrations of d_2-γ-CEHC and excreted four times more d_2-γ-CEHC in urine as men[197]. However, in both sexes urinary d_2-γ-CEHCs concentrations were decreased within 24 h after consumption of sesame oil, containing sesamine, a known inhibitor of vitamin E metabolism[200]. In line with this, urinary excretion of non-deuterated γ-CEHC was also higher in women compared to men. But, in contrast to d_6-α-TOH, urinary excretion of non-deuterated α-CEHC was also higher in women than in men. This suggests that women have faster γ-TOH disappearance rates in plasma than men[200], possibly because of their higher HDL TOH levels[197]. In contrast to γ-TOH, no differences in plasma disappearance rate have been found for α-TOH. But,

serum concentrations of α-TOH are higher in females compared to men[33,39].

As postulated by Miwa *et al*[39], the observed gender-specific differences in α-TOH/lipids ratio in serum may be due to effects of the female hormone estrogen. Unfortunately, studies measuring serum vitamin E concentrations in humans have revealed contradictory results. In the SU.VI.MAX cohort of middle-aged French participants, lower serum vitamin E concentrations in women than in men were found[27]. But, when serum vitamin E levels were compared to energy intake or serum cholesterol concentrations, which are both lower in women than in men, vitamin E density per kcal intake was higher in women[27]. Reasons for the gender-specific differences in vitamin E serum levels in humans are most likely: (1) the hormonal differences; and (2) gender-dependent differences in the activation of the CYP enzymes involved in vitamin E metabolism[201]. In many species, expression of liver-expressed genes depends on hormones and growth factor profiles, which show sexual dimorphism[202-206]. For example, the transcription factor Stat5b is a key factor in maintaining the sexual dimorphic response of CYP gene expression[202,207]. As a consequence, protein levels of CYP enzymes, such as Cyp4f1, Cyp4f4, Cyp4f5 and Cyp4f6, are usually higher in liver, kidneys, lungs and brain of female rats compared to male rats[208]. In the case of the CYP4F family, Kalsotra *et al*[208] suggested the importance of estrogen in regulating the sex-specific expressions of Cyp4f1, Cyp4f4 and Cyp4f6 in the kidneys and liver of rats. It is questionable whether the sex-specific differences in hepatic CYP expression profiles can be directly transferred to the case of humans. Whereas sex differences in drug metabolism have been shown in humans, too, larger interindividual differences in CYP activity have been also discussed[201,202]. Indeed, in general studies have shown that CYP3A activity is higher in women than in men[201]. In line with the observation that estradiol and testosterone regulate the expression of CYP3A isoforms in the liver[209], Parkinson *et al*[210] and Wang *et al*[204] demonstrated that women metabolize some CYP3A4 and CYP3A9 substrates faster than men do.

It is known that vitamin E levels change under metabolic challenges, such as age-associated diseases or oxidative stress (see the section "Life-style and age influence the bioavailability of vitamin E" in the chapter on the bioavailability of vitamin E). Furthermore, sex-related differences occur under these conditions, as observed by Aryamanesh *et al*[211]. This group described increased serum levels of α-TOH in women compared to men under ischemic preconditioning. Cavalca and colleges[212] found interactions between coronary artery disease (CAD) status, gender and α-TOH concentrations; in this study α-TOH concentrations were lower in female CAD patients than in male patients. Further analysis revealed that women with CAD, but not men, had lower serum levels of α-TOH compared to women with no CAD. Interestingly, no gender-specific differences were observed for γ-TOH[212].

Extrahepatic vitamin E metabolism: In addition to the established importance of hepatic metabolism of vitamin E, there is evidence for extrahepatic, namely intestinal vitamin E catabolism. First hints were obtained from studies by Abe *et al*[164], in which ketoconazole decreased γ-CEHC concentrations in jejunum after oral gavage of γ-TOH. A preferential degradation of γ-TOH and γ-T3 over α-TOH was also found. Further evidence was provided by Bardowell *et al*[157], as mice lacking hepatic microsomal CYP enzyme activity (including Cyp4f14) had only 70% reduced vitamin E metabolism after supplementation with γ- and δ-TOH instead of the expected total absence of metabolites. Supporting this observation, Cyp4f14 is expressed in the small intestine[213] and ω-hydroxylase activity for several forms of vitamin E was found by Bardowell *et al*[157]; this activity comprised about 10% of the ω-hydroxylase activity found in the liver. Mice deficient for Cyp4f14 had no ω-hydroxylase activity, leading to the conclusion that the small intestine, in contrast to the liver, may have only one enzyme capable of hydroxylating vitamin E[157].

GENETIC POLYMORPHISMS

Interindividual differences in vitamin E metabolism are known and have been reported for, for example, γ-CEHC excretion after γ-TOH supplementation[198], as well as for α-TOH metabolism[176]. Both studies suggest that differences in the absorption and metabolism of vitamin E are responsible for these effects. In agreement with this suggestion, genetic polymorphisms are known for genes involved in vitamin E homeostasis, which could cause such interindividual differences. An overview on known polymorphisms in genes encoding for proteins involved in vitamin E homeostasis is provided in Table 3.

Döring *et al*[214] were the first to systematically evaluate single nucleotide polymorphisms (SNPs) in genes encoding for proteins involved in vitamin E homeostasis. They analyzed *TTPA, TAP, LPL, ABCC3/MRP2, PXR* and *CYP* genes such as *CYP3A4, CYP3A5* and *CYP4F2* for the frequency of SNPs in coding regions and found *LPL*, *ABCC3/MRP2, PXR, CYP3A4* and *CYP4F2* to be highly polymorphic in their coding region. Recently, a new screening for candidates was published by Borel *et al*[215]. The postprandial chylomicron α-TOH response was positively correlated to fasting plasma α-TOH levels and 28 SNPs in eleven genes were identified that may contribute to 82% of the postprandial chylomicron α-TOH response, namely *ABCA1, ABCG1, APOB, BET1, IRS1, LIPC, NAT2, PNLIP, SLC10A2, SREBF2* and *ZNF664*. Among these genes are seven which are involved in chylomicron metabolism and are therefore indirectly responsible for vitamin E transport in serum. Borel *et al*[215] noted that *TTPA, CD36, SCARB1* and *SEC14L* were not found to be associated with the response of serum α-TOH levels in this analysis.

Table 3 Genetic polymorphisms of genes encoding for proteins involved in vitamin E homeostasis

Gene	Polymorphism	RefSNP ID	Functional consequence	Ref.
ABCA1	NM_005502.3:c.2828+426A > G	rs4149314	Intron variant	[215]
	NM_005502.3:c.814-1304T > C	rs4149297	Intron variant	[215]
	NM_005502.3:c.161-170G > A	rs11789603	Intron variant	[215]
	NM_005502.3:c.936C > A	rs2274873	Synonymous codon	[215]
	NM_005502.3:c.936C > T			
ABCG1	NC_000021.9:g.42167452C > T	rs468320		[215]
APOA5	NM_052968.4:c.-644C > T	rs662799	Upstream variant 2KB	[219]
APOB	NC_000002.12:g.20937665C > T	rs4643493		[215]
	NM_000384.2:c.12541G > A	rs1042031	Intron variant	[215]
			Missense: E4181K	
	NC_000002.12:g.21048451A > G	rs1713222		[215]
BET1	NC_000007.14:g.94352727G > A	rs10464587	Intron variant	[215]
CD36	NM_001289911.1:c.-108-13288T > C	rs1527479	Intron variant	[220]
CYP4F2	NM_001082.4:c.1297G > A	rs2108622	Missense: V433M	[217,225]
	NM_001082.4:c.34T > G	rs3093105	Missense: W12G	[225]
IRS1	NC_000002.12:g.226683594A > G	rs1316328		[215]
LIPC	NC_000015.9:g.58688186A > C	rs4238329		[215]
	NM_000236.2:c.89-47937A > G	rs8041525	Intron variant	[215]
	NC_000015.10:g.58394555C > T	rs7164909	Intron variant	[215]
	NC_000015.10:g.58353522T > C	rs8035357	Intron variant	[215]
	NM_000236.2:c.88+49790T > C	rs12591216	Intron variant	[215]
	NM_000236.2:c.89-50891C > A	rs12593880	Intron variant	[215]
NAT2	NC_000008.11:g.18439195T > C	rs4921920		[215]
NPC1L1	NM_013389.2:c.1184C > T	rs62001882	UTR variant 5 prime	[216]
			Missense: A395V	
	NM_013389.2:c.1204G > A	rs141973731	UTR variant 5 prime	[216]
			Missense: G402S	
	NM_001300967.1:c.1249C > T	rs139659653	UTR variant 5 prime	[216]
			Missense: R417W	
	NM_013389.2:c.1300G > A	rs114375162	UTR variant 5 prime	[216]
			Missense: G434R	
PNLIP	NM_000936.2:c.930+205T > C	rs2915775	Intron variant	[215]
	NC_000010.11:g.116569152G > T	rs3010494		[215]
SCARB1	NM_001082959.1:c.127-4800C > T	rs11057830	Intron variant	[217]
SEC14L2	NM_033382.2:c.32G > A	rs757660	UTR variant 5 prime	[224]
			Missense: R11K	
	NM_016498.4:c.-2240T > C	rs1061660	Upstream variant 2KB, UTR variant 3 prime	[224]
	NM_016498.4:c.-2110G > A	rs1061664	Upstream variant 2KB, UTR variant 3 prime	[224]
SLC10A2	NC_000013.11:g.103037119C > T	rs1571513		[215]
	NC_000013.11:g.103898490C > T	rs9558203		[215]
	NC_000013.11:g.103040492G > A	rs16961116		[215]
	NC_000013.11:g.103444248G > A	rs12874168		[215]
	NC_000013.11:g.103452026G > A	rs2065550		[215]
SREBF2	NM_004599.3:c.2208+3245C > T	rs2839715	Intron variant	[215]
	NM_004599.3:c.1761+160G > A	rs4822062	Intron variant	[215]
TTPA	NM_000370.3:c.421G > A	rs397515524	Intron variant	[222]
			Missense: E141K	
	NM_000370.3:c.175C > T	rs397515522	Missense: R59W	[222]
	NM_000370.3:c.-1753G > A	rs12056582	Upstream variant 2KB	[223]
	NM_000370.3:c.-1410A > T	rs6472071	Upstream variant 2KB	[223]
	NM_000370.3:c.-1410A > C			
	-345C/T[1]	rs75371508		[223]
	NM_000370.3:c.-981T > A	rs6994076	Upstream variant 2KB	[223,224]
	NM_000370.3:c.-945G > A	rs34358293	Upstream variant 2KB	[223]
	NM_000370.3:c.-675G > A	rs80169698	Upstream variant 2KB	[223]
	NM_000370.3:c.-440C > T	rs73684515	Upstream variant 2KB	[223]
	-344C/T[1]	rs74684018		[223]
ZNF664	NM_001204299.1:c.-234+53341T > C	rs7296124	Intron variant	[215]
	NM_152437.2:c.*2065G > A	rs1048497	Intron variant,	[215]
			UTR variant 3 prime	
ZPR1	NM_003904.3:c.*724C > G	rs964184	Downstream variant 500B	[217]

[1]This variant has been described by Ulatowski *et al*[223] but was not found in the NCBI dbSNP database (http://www.ncbi.nlm.nih.gov/SNP/).

The importance of variants of a transporter involved in the intestinal uptake of lipids and lipophilic nutrients, namely NPC1L1, was analyzed *in vitro* and four variants, located on the predicted extracellular loop of NPC1L1

protein (*i.e.,* A395V, G402S, R417W, and G434R) showed lower transport efficiencies for both cholesterol and α-TOH[216]. Borel *et al*[217] analyzed polymorphisms in genes involved in lipid metabolism, namely *APOA4, APOB, APOE, LPL* and *SRBI*, and found α- or γ-TOH levels to be different in subjects with SNPs in *APO4, APOE* and *SRBI* or *APO4* and *SRBI*, respectively. Major *et al*[218] found three SNPs to be associated with circulating α-TOH serum levels, rs964184 near *APOA1*, *APOA4*, *APOA5* and *APOC3*, rs2108622 in *CYP4F2* as well as rs11057830 in *SCARB1*. As the importance of proteins involved in lipoprotein metabolism for vitamin E transport and serum levels has been highlighted by these findings, Sundl *et al*[219] emphasized that vitamin E serum levels should be adjusted for plasma or serum lipids. The authors reported that the association of the APOA5 -1131T>C variant (rs662799) with vitamin E serum concentrations was no longer statistically significant, when the vitamin E values were adjusted for serum triglyceride or total serum cholesterol concentrations. The scavenger receptor cluster of differentiation 36 (CD36) might also be involved in cellular vitamin E uptake[220], and indeed a SNP (rs1527479) was identified to be associated with lower α-TOH serum concentrations[221].

As already mentioned, hepatic α-TTP plays a central role in vitamin E homoestasis. As some genetic variants in the *TTPA* gene encoding α-TTP cause reduced circulating α-TOH levels in serum, Bromley *et al*[222] analyzed two SNPs in the *TTPA* gene, uncovering the variants E141K and R59W, which are known to be associated with AVED. These variants are located in or close to the proposed ligand-binding domain of α-TTP, and the authors showed that the variants are responsible for reduced binding of α-TOH to α-TTP *in vitro*. Ulatowski *et al*[223] analyzed SNPs in the promotor region of the *TTPA* gene and found that the substitutions -1752C/T (rs12056582), -1408G/T (rs6472071), and -345C/T (rs75371508) increased promotor activity about 3-fold, whereas the SNPs -1408A/T (rs6472071), -980A/T (rs6994076), -943A/G (rs34358293), -674C/T (rs80169698), -439A/G (rs73684515), and -344C/T (rs74684018) repressed promoter activity. To support the physiological relevance of their findings the authors noted that Wright *et al*[224] found reduced vitamin E plasma levels to be associated with the SNP -980A/T in the promotor region of the *TTPA* gene. In addition, three SNPs in the coding region of the *SEC14L2* gene encoding TAP1 are associated with increased serum vitamin E concentrations[224]. Major *et al*[218] identified a new SNP in the *CYP4F2* gene (rs2108622) which encodes the vitamin E ω-hydroxylase. The physiological relevance of this SNP and the SNP rs3093105 has been characterized by Bardowell *et al*[225]. The CYP4F2 W12G variant leads to a 2.3 to 2.8-fold increased specific enzyme activity for both TOHs and T3s, whereas the CYP4F2 V433M (rs2108622) variant causes reduced enzyme activity for TOHs, without affecting the activity for T3s. Athinarayanan *et al*[226] analyzed the impact of these two genetic variants on vitamin E plasma levels in children and adults with non-alcoholic fatty liver disease. The study revealed moderate effects on plasma vitamin E levels during vitamin E treatment only for the V433M variant but showed no effects on histological parameters, such as fibrosis, ballooning or steatosis.

In summary, genes involved in vitamin E homeostasis are polymorphic and some genetic variants, in particular in coding and promotor regions, are associated with vitamin E plasma/serum levels.

METABOLIC INTERACTIONS WITH VITAMIN E CATABOLIC PATHWAY

As the pathways responsible for vitamin E metabolism are not restricted to vitamin E, interactions with other metabolic pathways can occur that make use of: (1) the same enzymes; (2) the vitamin E-dependent regulation of gene expression; or (3) the enzyme activity of xenobiotic pathways. Two possible categories of interactions are discussed here; firstly, interaction with vitamin K metabolism and secondly, with xenobiotics and drugs.

Interaction with vitamin K metabolism

The interference of vitamin E with vitamin K metabolism and, as a result, with blood coagulation has been known for decades[227]. As shown by several supplementation studies with vitamin E in rats[228] or humans[229] an increased risk of bleeding was noted. The underlying molecular mechanisms are still unclear, but recent research has shed new light on this aspect of vitamin E metabolism.

Vitamin E and vitamin K share the same metabolic pathways, as the degradation of both vitamins is initiated with an ω-hydroxylation followed by subsequent β-oxidation of the aliphatic side-chain, thus resulting in urinary and biliary excretion of the respective carboxylic acids or conjugates with shortened side-chains[230]. When vitamin E increases the expression or activity of enzymes involved in its own degradation, it is possible that vitamin K metabolism is also enhanced under elevated vitamin E status; this may lead to higher rates of vitamin K excretion and in turn to vitamin K deficiency with enhanced bleeding risk[160]. Evidence for this hypothesis was provided by Hanzawa *et al*[231], as rats treated with a diet consisting of 20% sesame seeds, containing sesamin as an inhibitor of vitamin E metabolism, for 40 d had increased vitamin K tissue concentrations. It should be noted that the vitamin E form used in this diet was solely γ-TOH. Farley *et al*[232] investigated the influence of α-TOH on phylloquinone or menadione metabolism and excretion. They fed rats with either a phylloquinone or a menadione containing diet (2 μmol/kg body weight) for 2.5 wk and injected after ten days a daily dose of 100 mg/kg body weight α-TOH for a further seven days. Tissue levels of menaquinone-4, a tissue-specific metabolite of phylloquinone and menadione, were decreased in brain, lung, kidney and heart and levels of phylloquinone were

decreased in lung independent of the type of diet. The authors observed a downregulation of the expression of CYP enzymes CYP3A, CYP4F4 and CYP4F1, which was explained by an alternative mode of interference of vitamin E with vitamin K metabolism apart from the induced degradation of vitamin K. The induction of the xenobiotic exporters ABCB1/MDR1 and ABCG2/BCRP1 provided the first hints for an increased excretion of vitamin K metabolites into bile. This concept is supported by the 100-fold increased urinary excretion of α-CEHC in response to the application of α-TOH, whereas urinary excretion of vitamin K metabolites remained unchanged. In another study, Farley *et al*[233] focused on CYP4F2, as this protein is involved in the degradation of both vitamin E[140] and vitamin K[234]. The catalytic efficiency of CYP4F2 for the hydroxylation of phylloquinone is higher than for α-TOH, while the co-incubation of phylloquinone and α-TOH had no influence on the metabolism of phylloquinone. Thus, this study indicates that the activity of CYP4F2 is not enhanced by high concentrations of α-TOH. In their most recent study, Farley *et al*[235] concluded that α-TOH, or even α-CEHC, interferes with an intermediate step in the formation of tissue-specific menaquinone-4 from phylloquinone, such as alterations in the transport *via* chylomicrons or other lipoproteins or reductions of the cellular uptake of phylloquinone or menadione, an intermediate product of phylloquinone in the menaquinone formation pathway. This hypothesis is supported by findings of Hanzawa *et al*[236] who found that rats fed a diet with 0.75 mg/kg phylloquinone for six weeks have decreased phylloquinone tissue concentrations with supplementation of 100 mg α-TOH while in rats fed with 0.75 mg/kg menaquinone diet the tissue concentrations of menaquinone remained unchanged with an increased application of α-TOH.

Finally, the form of vitamin E also contributes to the interference with vitamin K metabolism, as γ-TOH is not as potent as α-TOH in decreasing extrahepatic phylloquinone concentrations[236]. Taken together, vitamin K metabolism is disturbed by vitamin E at the level of extrahepatic synthesis of menaquinone from phylloquinone or menadione and the different vitamin E forms also lead to differing effects on the metabolic interactions.

Interactions with pharmaceutics

As CYP3A4 is responsible for the metabolism of more than 50% of xenobiotics[237], the upregulation of CYP3A4 expression by α-TOH may be of concern regarding the interactions of α-TOH with drug metabolism. Clarke *et al*[238] investigated the effect of α-TOH on midazolam plasma concentrations and found no influence. When the interaction with cyclosporine A was analyzed, Bárány *et al*[239] found reduced plasma levels after six weeks of supplementation with 800 IU/d α-TOH. These results indicate possible interactions of α-TOH with drug metabolism that need further investigation.

Vitamin E was a promising candidate as a supplement to prevent atherosclerosis and its complications. Data obtained from *in vitro* studies have been very promising, yet large intervention studies have revealed inconsistent results (for an overview, see Wallert *et al*[240]). There may be many reasons to explain this finding, but the details still need to be unraveled. With respect to patients who take medications, the situation may be even more complicated. For example, in patients treated with statins to reduce LDL cholesterol levels, interactions with simultaneously ingested vitamin E supplements may occur. A combined treatment with atorvastatin and α-TOH in stable, non-diabetic dialysis patients revealed a reduced *in vitro* oxidizability of LDL[241]. Podszun *et al*[242] found no interactions in guinea pigs fed with high-dose α-TOH (250 mg/kg diet) for six weeks when atorvastatin (300 mg/kg diet) was simultaneously applied. In 2001, however, two studies were published which questioned the benefit of the combined therapies. Patients treated with simvastatin, niacin and a combination of different antioxidants (400 IU *bis in die* (BID; twice a day) vitamin E, 500 mg BID vitamin C, 12.5 mg BID β-carotene and 50 mg BID selenium) profited less than the simvastatin and niacin groups as the protective increase in HDL_2 with simvastatin plus niacin was attenuated by concurrent therapy with antioxidants[243,244]. Tousoulis *et al*[245] treated patients suffering from ischemic heart failure with low dose atorvastatin (10 mg/d) alone or in combination with 400 IU/d vitamin E and found that vitamin E impairs the positive effects of atorvastatin, on, for example, endothelial function or inflammatory response.

Many studies focusing on the effects of statins on human lipid levels also analyzed other plasma parameters, such as vitamin E status. The results depend, among others, on the evaluation of the data. When vitamin E serum levels were measured without any corrections most researchers found a decrease[241,246-250]; however, when vitamin E serum levels were normalized to plasma lipids, such as LDL cholesterol, total serum cholesterol or serum triglycerides, increased or unchanged ratios of vitamin E and serum lipids were observed[247,249-252]. The underlying processes are still unclear but on the one hand, the decrease of circulating lipoproteins could directly cause reduced levels of vitamin E (in terms of absolute concentrations), whereas the ratio of vitamin E to serum lipids could at the same time be increased, as statins mostly decrease specific lipid fractions, namely LDL cholesterol and total cholesterol. On the other hand, Werba *et al*[253] suggested an inhibitory effect of some statins, such as simvastatin, on CYP3A4.

VITAMIN E STATUS

When assessing vitamin E status, analytical parameters which have proven the most reliable must be primarily considered. As noted by Traber[18] in 2014, circulating α-TOH concentrations are not necessarily a reliable marker for an adequate vitamin E status in humans.

Supplementation of α-TOH, for example, increases serum levels but leads simultaneously to increased amounts of α-CEHC in urine. Hence, α-CEHC in urine can be used as a marker for α-TOH status in healthy humans[132] or at a minimum as a marker for an adequate level of α-TOH[116]. In addition to serum α-TOH levels and urinary α-CEHC, the quest for another more reliable marker for adequate supply of vitamin E would be helpful for determining vitamin E status. Our group has been the first to reveal preliminary evidence for the systemic bioavailability of the first hepatic metabolites, α-13'-OH and α-13'-COOH, and their extrahepatic presence[142]. We suggest that the serum concentrations of these α-LCMs may be promising candidates in the search for new reliable markers of α-TOH levels. Both LCMs increase after supplementation with 1000 IU *RRR*-α-TOH for a few days. It should be also noted that the assessment of absolute food and energy intake is needed when vitamin E concentrations are measured and correlated to gender, age and life-style[118]. Determining the right analytical parameters for evaluating vitamin E status is critically important; however it is also crucial that new analytical parameters and procedures be validated, optimized and standardized for ensuring optimal diagnosis and comparability.

Analytics

The analytics here involve numerous elements which should be considered before beginning measurements. Yu *et al*[254] demonstrated nicely that the reproducibility of the measurement of vitamin E metabolites is better in plasma than in serum samples, while the concentrations measured in serum are higher. The sample matrix also matters in the determination of conjugated metabolites. Freiser and Jiang[141] showed that conjugated metabolites are more difficult to hydrolyze in plasma compared to other biological fluids, such as urine. In principle, there are two methods for analyzing conjugated metabolites, the simultaneous analysis of conjugated and non-conjugated metabolites or the pre-treatment of samples with the respective enzymes glucuronidase and sulfatase[139]. With respect to the simultaneous analysis, Jiang *et al*[139] stated that "neither electrochemical detection nor GC-MS can be used to detect sulfated carboxychromanols because of their lack of redox activity or the inertness of the conjugated phenolic group to chemical derivatization, respectively". Pope *et al*[177] developed the first tandem MS-MS approach for the reliable and simultaneous detection of conjugated and non-conjugated metabolites. Since then, the analytical procedures for measuring the metabolites in both conditions were adopted for LC-MS[135] and UPLC-TOF-MS[176].

Treatment of samples with deconjugation enzymes must be performed carefully. Only a cleanup-procedure with methanol/hexane extraction for the removal of proteins and lipids in the sample and an enzymatic hydrolysis overnight ensures complete deconjugation; otherwise the conjugated CEHCs are significantly underestimated[141]. It must be considered that the length of the side-chain may also influence the efficiency of deconjugation, because sulfatase treatment of relatively hydrophilic compounds (*e.g.*, sulfated 9'-COOH) is more efficient than the treatment of more hydrophobic compounds (*e.g.*, sulfated 13'-COOH)[141]. Freiser *et al*[141] optimized deconjugation of vitamin E metabolites and found that only an overnight treatment with β-glucosidase/sulfates ensures optimal recovery. Although enzymatic hydrolysis is a standard procedure, the type of enzymes used differs[255]. In some studies recombinant enzymes with distinct activities are used, such as β-glucuronidase from *E. coli*[144,147,157], whereas others[149,151,154-156,177] used enzymes with the combined activity of β-glucosidase and sulfatase. In these cases, glucuronidase activity is usually higher than sulfatase activity. Here it is important to consider the study by Freiser *et al*[141] who reported that type B-1 β-glucuronidase from bovine liver has the lowest sulfatase activity of all enzymes tested, which is consistent with the common use of type H-1 enzymes from *H. pomatia*. Pope *et al*[177] noted that deconjugation of CEHCs may produce artefacts, such as a conversion of CEHC to tocopheronolactone. In addition, extraction conditions are important in terms of yield and recovery. According to Lodge *et al*[151], acidification and ether extraction ensure the highest recovery rates for α- or γ-CEHC from urine. To sum up, no standardized analytical method is available today which is suitable for use on all metabolites and conjugates. However, the establishment and careful evaluation of standardized analytics enables the reliable validation of biomarkers. This in turn offers the opportunity to obtain an accurate impression about the distribution and importance of vitamin E metabolites.

PERSPECTIVE

The coordinated mechanisms involved in the metabolism of vitamin E provide an effective but complex physiological pathway to protect the body against critical imbalances, *i.e.,* an excessive accumulation of non-α-TOH forms to unphysiological levels. However, vitamin E metabolism is complex and large gaps remain in the understanding of crucial processes which are involved. Thus far, the contribution of enzymes other than CYP enzymes and the mechanisms regulating the intracellular and interorgan trafficking and metabolism are still not understood in sufficient detail.

In addition to mechanistic insights into vitamin E metabolism, the physiological functions of the different vitamin E metabolites remains incompletely understood. While many researchers have focused in the past on the SCMs, there is a significant lack of knowledge on the physiological importance of the ICMs and, in particular, the LCMs. As of yet only a few studies have investigated the principle modes of action of the LCMs *in vitro*, while studies *in vivo* have not yet been performed as LCMs have not been available as pure compounds until

recently. As absolute concentrations of LCMs in the blood circulation of mammals are far too low for isolation, a natural compound, called garcinoic acid, isolated from the African bitternut *Garcinia kola* has proven to be a usefull resource to synthesize the α- and δ-LCMs of α- and δ-TOH[143]. Recent studies on α- and δ-LCMs have provided first preliminary insights[142,143,256-258] into the molecular modes of action of the LCMs, which seem to be specific and distinct from that of their metabolic precursors. We therefore hypothesize that at least some of the discrepancies in the results obtained from *in vitro* and *in vivo* studies on vitamin E might be explained by individual differences in the physiological metabolism of vitamin E, and in particular under pathological conditions. We suggest that the LCMs comprise a new class of regulatory metabolites, which must be investigated in more detail to unravel their proposed physiological relevance. In the meantime, a promising new approach may be to analyze serum concentrations of the LCMs in human intervention studies on vitamin E supplementation, to determine whether correlations exist to vitamin E intake and to the incidence of defined diseases.

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REVIEW

New insights into sodium transport regulation in the distal nephron: Role of G-protein coupled receptors

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Abstract

The renal handling of Na^+ balance is a major determinant of the blood pressure (BP) level. The inability of the kidney to excrete the daily load of Na^+ represents the primary cause of chronic hypertension. Among the different segments that constitute the nephron, those present in the distal part (*i.e.*, the cortical thick ascending limb, the distal convoluted tubule, the connecting and collecting tubules) play a central role in the fine-tuning of renal Na^+ excretion and are the target of many different regulatory processes that modulate Na^+ retention more or less efficiently. G-protein coupled receptors (GPCRs) are crucially involved in this regulation and could represent efficient pharmacological targets to control BP levels. In this review, we describe both classical and novel GPCR-dependent regulatory systems that have been shown to modulate renal Na^+ absorption in the distal nephron. In addition to the multiplicity of the GPCR that regulate Na^+ excretion, this review also highlights the complexity of these different pathways, and the connections between them.

Key words: Kidney; Sodium excretion; Blood pressure; G-protein coupled receptors; Peptide hormone

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Core tip: The maintenance of the blood pressure depends partly on the ability of the organism to match the daily intake and excretion of Na^+. The kidney, which is the main organ involved in Na^+ excretion, is the target of multiple regulatory pathways that contribute to the fine-tuning of secretion/reabsorption processes occurring all along the nephron. In this review we described "classical" and "novel" G-protein coupled receptor (GPCR)-mediated pathways that impact trans-

epithelial Na^+ transport in the distal nephron. This detailed inventory of the GPCR-mediated pathways that affect renal Na^+ handling gives a broad overview of the complexity of this integrated system.

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INTRODUCTION

Sodium is the main osmotic component of extracellular compartments and its concentration (140 mmol/L) must be kept within a narrow range to maintain extracellular volume and blood pressure (BP) at a normal levels. According to Guyton's model, dysfunctional renal sodium excretion is involved in the development of salt-sensitive hypertension[1]. Daily, Na^+ consumption may varies between 50 to 150 mmol and water consumption is about 1.5 L. Nearly all the Na^+ and water are transferred to the extracellular compartment, which may induce changes in the circulating volume if Na^+ and water are not correctly excreted. The kidneys regulate the balance of Na^+ and water balances (extra-renal losses are negligible under normal circumstances). In humans, about 25000 mmoles of Na^+ in 180 L of fluid are delivered daily to the glomerular filtrate. These very large amounts of Na^+ and fluid are almost entirely reabsorbed by the kidney, as urinary excretion is calibrated to match dietary intake. The bulk of the filtered load of sodium (around 60%) is reabsorbed in the proximal tubule; 25% is reabsorbed by the thick ascending limb (TAL); approximately 5% to 7% is reabsorbed along the distal convoluted tubule (DCT), and 3% to 5% along the connecting tubule (CNT) and the collecting duct (CD); the latter is composed of a cortical segment (CCD), an outer medullary part and an inner medullary part (IMCD). The distal part of the nephron as defined in this review encompasses the segments from the cortical TAL (cTAL) to the IMCD since these are all involved in the fine-tuning Na^+ excretion. In these segments are found all sodium transporters in which mutations induce alterations of systemic BP. Not surprisingly, numerous hormones regulate the transport of Na^+ in these segments. These regulatory systems involve the activation of various receptors among which the G-protein coupled receptors (GPCRs) represent an important component.

In this review we 1/rapidly summarize the mechanisms of sodium transport in the distal nephron and 2/describe the well-established regulatory mechanisms mediated by "classical" GPCRs as well as more recently described systems involving "novel" GPCRs.

SODIUM TRANSPORT MECHANISMS IN THE DISTAL NEPHRON

Transepithelial sodium reabsorption can take place either accoss tubular epithelial cells (transcellular route) or between cells (paracellular pathway). Transcellular Na^+ reabsorption requires the successive crossing of the apical and basolateral membranes. At the basolateral membrane, the Na, K-ATPase generates the electro-chemical gradient that drives Na^+ entry at the apical membrane *via* different channels, cotransporters or exchangers. The specificity of ion transport in different nephron segments largely stems from the nature of the Na^+-coupled transport systems, that are expressed at the apical membrane (Figure 1). Transcellular transport generates a transepithelial potential difference, which, can drive the paracellular transport of solutes and/or water depending on the water and ion permeability of intercellular junctions (for review see[2,3]).

Transport mechanisms in the cTAL

The TAL is a tight epithelium that actively reabsorbs about 25% of filtered NaCl. The apical entry of Na^+ is mainly mediated by the electroneutral cotransporter Na/K/2Cl (NKCC2), which is sensitive to furosemide (Figure 1). The transcellular reabsorption of Na^+ in this segment is coupled to the recycling of K^+ at the apical membrane through a K^+ channel (ROMK) and the reabsorption of Cl^- at the basolateral membrane through Cl^- channels (CLCKb). Altogether, these moves generate a positive transepithelial potential difference (PDte) of about +15 mV, that allows the reabsorption of monovalent and divalent cations through the paracellular pathway. The tight junctions of TAL cells are poorly permeable to anions, which limits chloride back-flux through the paracellular route[3]. Moreover, the transport of Na^+, K^+ and Cl^- in the TAL cells is coupled and interdependent: when one of the carriers (NKCC2, ROMK or CLCKb) is inhibited or carries a loss-of-function mutation, transport across other transcellular and paracellular pathways is impaired. In humans, these mutations are responsible for Bartter syndrome, an inherited disease characterized by excessive loss of Na^+, Cl^-, water and accompanied by an abnormally high urinary excretion of Mg^{2+} and Ca^{2+}[4]. Moreover, certain loss-of-function mutations of NKCC2 have been shown to protect against the development of hypertension[5].

Transport mechanism in the DCT

The DCT retain 5% to 10% of the filtered NaCl, have tight waterproof junctions and reabsorb Ca^{2+} and Mg^{2+} through the transcellular pathway. In mice and rats, the DCT is divided into two parts, each of which expresses different apical Na^+ transporters the DCT1, expresses only the apical NaCl cotransporter NCC. As for the DCT2, it expresses both NCC and the epithelial sodium channel ENaC. As opposed to the rodent DCT, the human DCT is uniform; it has the same expression profile as the

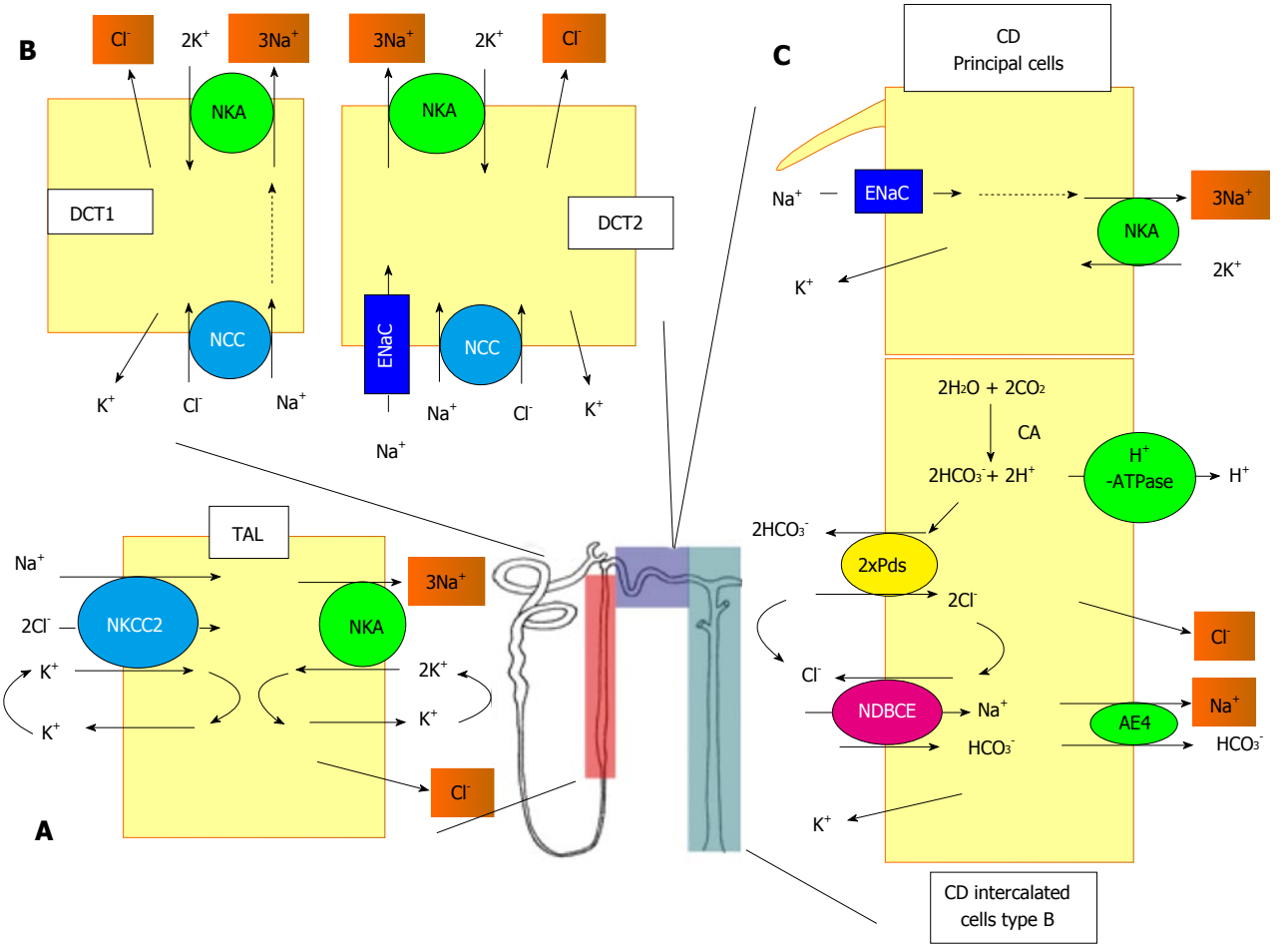


Figure 1 Schematic representation of the different sodium transport systems along the nephron. A: In TAL, the apical entry of Na^+ is mediated by the NKCC2. At the basolateral side, the Na^+ exits the cell through the NKA and the Cl^- through a channel of the CLC-K family; B: DCT consists of two different sub-structures, the DCT1 and the DCT2. In DCT1, the entry of Na+ and Cl- is mediated by the NCC and in DCT2, a ENaC is also involved. In both structures, the exit of Na^+ and Cl^- is mediated by the NKA and a CLC-K conductance; C: In the CD, the Na^+ enters the principal cells through ENaC and exits through the NKA. The system is more complex in the B-intercalated cells. The apical entry of of Na^+ and Cl^- is mediated by the functional association between the pendrin, bicarbonate/Cl^- exchanger (Pds), and NDBCE. The system is energized by the vacuolar proton pump (H^+-ATPase) and the generation of bicarbonate mediated by a CA. The exit of Na^+ and Cl^- is mediated by an AE4 and a Cl^--conductance, respectively. TAL: Thick ascending limb; NKCC2: $Na^+/K^+/2Cl^-$-cotransporter; NKA: Na,K-ATPase; DCT: Distal convoluted tubule; NCC: Na^+/Cl^--cotransporter; ENaC: Na^+ channel; CD: collecting duct; Pds: Bicarbonate/Cl^- exchanger; CA: Carbonic anhydrase; NDBCE: Na^+-driven bicarbonate/Cl^- exchanger; AE4: Anion exchanger.

murine DCT1[6]. Sodium reabsorption through NCC is electroneutral and sensitive to thiazide diuretics such as hydrochlorothiazide (HCTZ), whereas sodium reabsorption through ENaC is electrogenic and sensitive to amiloride.

In humans, NCC mutations inducing loss of function are responsible for Gitelman syndrome, which features hypokalemic metabolic alkalosis, hypomagnesemia and hypocalciuria[7]. In contrast, "gain-of-function" mutations of kinases regulating NCC induce an increase in NaCl reabsorption and hypertension, as encountered in Gordon's hypertension syndrome[8].

Transport mechanisms in CNT and CD

Unlike upstream nephron segments, the epithelia of the CNT and the CD are made of two cell types, known as principal cells (PC) and intercalated cells (IC) (Figure 1) with specific and different functions. Until recently, the reabsorption of Na^+ was attributed to PC whereas IC were thought to be involved in acid/base excretion. This dogma has been revisited these last few years, as discussed below.

Transport mechanisms in the PC: PC constitute about 60% of CNT/CCD cells and express ENaC at their apical membrane. Na^+ reabsorption across PC is electrogenic and necessary for K^+ secretion (Figure 1). The terminal part of the CNT and the CCD can reabsorb water owing to the osmotic gradient generated in part by the tubular fluid dilution that occurs in the TAL as well as Na^+ reabsorption in the CNT/CCD through ENaC. The transcellular reabsorption of water is mediated by apical aquaporin 2 channels (AQP2) and the basolateral aquaporin 3 and 4 channels (AQP3 and AQP4). It is generally accepted that there is a decreasing longitudinal gradient of Na^+ reabsorption and K^+ secretion along the CNT and CCD. Under basal condition, in *in vitro* microperfusion experiments, rodent CCDs do not ex-

hibit significant net Na^+ or K^+ transport[9,10]. This does not mean that there is no functional ENaC or ROMK at the apical surface of PC in the CCD. In fact, both channels have been found to be active by patch clamp experiments[11,12]. The channel activity is either not sufficient to trigger a net flux that can be measured by *in vitro* microperfusion experiments or is compensated by secretion processes that remain to characterized. Regarding chloride transport, it has to be noted that such a secretion pathway has been partially described; it involves the basolateral transporter NKCC1 localized in the B-type intercalated B cells of the CCD[13]. Finally, significant net fluxes of Na^+, Cl^- and K^+ are measured in rat or mouse *in vitro* perfused CCD when animals are either submitted to a sodium-depleted diet or chronically treated with corticosteroids[9,10]. Therefore the CNT/CCD represents a functional "transport" reserve that can be recruited when Na^+ reabsorption (hypovolemia) and/or K^+ secretion (hyperkalemia) is needed[9,10].

In humans, a gain-of-function mutation of ENaC induces a constitutively active Na^+ reabsorption in CNT and CD[14]. The continual passage of Na^+ through the apical membrane then drives excessive K^+ secretion. Thus, patients with this mutation (which causes Liddle's syndrome) develop severe early hypertension and hypokalemia[15]. Conversely, a loss-of-function mutation of the human ENaCα-subunit results in pseudo-hypo-aldosteronism 1 and is associated with urinary Na^+ wasting, low BP (at least in children) and hyperkalemia[16].

Transport mechanisms in the type B IC: Based upon immuno-histochemical studies, there are at least four types of IC: Type A, type B, type non A-non B cells and bipolar cells. Types A (A-IC) and type B (B-IC) cells are traditionally involved in the regulation of acid/base balance. However, recent results demonstrate that B-IC play a role in the reabsorption of Na^+ and Cl^-[10]. These cells, which are found only in the CNT and the CCD and express the H^+-ATPase pumps and Cl^- channels (CLCK) at the basolateral membrane and the Cl^-/HCO_3^- exchanger pendrin (PDS) at the apical membrane. B-IC are known to secrete a high quantity of bicarbonate in response to metabolic alkalosis. Moreover, they are the only cells that reabsorb Cl^- in the CNT/CCD. Even though the CNT and CCD do not express NCC, these two segments can mediate electroneutral, thiazide-sensitive sodium chloride absorption. Recently, Leviel *et al*[10], demonstrated a functional coupling between pendrin and a Na^+-driven Cl^-/HCO_3^- exchanger (NDCBE), using transgenic mice models and *in vitro* microperfusion experients. This coupling results in net and electroneutral NaCl reabsorption in the CCD (Figure 1). More recently, Chambrey *et al*[17] showed that this system is not energized by Na, K-ATPase as it is the case in most cells but by the H^+-ATPase. Na^+ enters B-ICs *via* NDCBE and exits through the basolateral anion exchanger AE4, which is specifically expressed in this cell type[18].

SODIUM TRANSPORT REGULATION IN THE DISTAL NEPHRON BY CLASSICAL GPCRS

In the distal nephron, the sodium transport is subject to numerous regulatory factors (endocrine, neuroendocrine and paracrine) that adjust the excretion of the cation to homeostatic needs, maintaining constant blood volume and BP. Among the different regulatory systems orchestrating the fine-tuning of the Na^+ balance, those that entails the stimulation of GPCR are numerous. Some of them have been known for many years to interfere with Na^+ reabsorption and their ligands are "classical" hormones of the Na^+ homeostatic system. Thus, it is well known that angiotensin 2 and vasopressin have a stimulatory effect on sodium absorption all along the nephron through their respective GPCRs, whereas bradykinin, dopamine, endothelin, and PGE2 down-regulate sodium absorption (for review see[2]). More recently, novel pathways regulating Na^+ absorption have been reported. They involve new types of ligands (such as proteases, lipids, and small metabolites), and receptors that have not all been fully characterized. In the following part, we will describe both "classical" and "novel" regulatory pathways of the Na^+ reabsorption in the distal nephron (Table 1).

"Classical" GPCRs that activates sodium absorption in the distal nephron

Vasopressin receptor V_2: Vasopressin (AVP) is a peptide secreted by the hypothalamic nuclei cells and stored in the posterior pituitary. It is released into the blood stream under dehydration and cellular volume shrinkage. AVP acts on three subtypes of GPCRs: V_1 (ex V_{1a}), V_2 and V_3 (ex V_{1b}). In the kidney, AVP triggers an increase in water and/or sodium reabsorption through V_2 receptors. V_2 receptors are located at the basolateral cell membrane of the ascending limb and DCT segments. In the CD, they are specifically present in PC[19-21]. In all these cells, the V_2 receptor is coupled to the G_s protein, which rapidly activates adenylyl cyclase (AC6 and AC3 isoforms, chiefly), increases the formation of cAMP and induces the activation of PKA[22]. By this way, the activation of V2 receptor stimulates numerous Na^+ transporters by phosphorylation. For instance, the Na, K-ATPase activity is enhanced by PKA activation[23]. In TAL and DCT cells, NKCC2 and NCC are also targets of PKA, which increases their membrane expression after AVP activation[24,25]. The stimulation of NKCC2 and NCC by AVP also involved intermediary kinases, such as STE20/SPS1-related proline-alanine-rich kinase (SPAK) and oxidative stress responsive kinase 1 (OSR1), and may be not solely due to a direct action of PKA on the transporters[26]. Recent studies have shown that AVP increases the abundance of phosphorylated SPAK and phosphorylated OSR1 in mTAL and DCT cells[21,27]. Thus, both SPAK and OSR1 could be important vasopressin second messengers. Interestingly, using $SPAK^{-/-}$ mice,

Table 1 Recapitulation of the G-protein coupled receptors families present in the the distal nephron

Receptors	G-proteins	Cell location	Final effector	Effect on Na^+ transport
Vasopressin receptor (V2R)	G_s	Basolateral	Na, K-ATPase - AQP2 - NKCC2 - NCC - ENaC	Increase
Angiotensin Ⅱ receptors (AT)	$G_{q/11}$	Apical/basolateral	Na, K-ATPase - NKCC2 - NCC - ENaC - pendrin	Increase
Bradykinin receptor (B2R)	G_q	Basolateral	Na, K-ATPase - NKCC2 - NCC - ENaC (??)	Decrease
Endothelin receptors (ETA-ETB)	G_i, G_q, G_s, $G_{\alpha 12/13}$	Apical/basolateral	NKCC2 - ENaC - pendrin	Decrease
α-adrenergic receptor (α-AR)	G_i - $G_{q/11}$	Basolateral	Na, K-ATPase (?) - NKCC2 - ENaC	Increase (α1-AR) Decrease (α2-AR)
β-adrenergic receptor (β-AR)	G_s	Apical/basolateral	NKCC2 - NCC - ENaC - pendrin	Increase
Prostanoïd receptors	G_s – G_i	Apical/basolateral	NKCC2 - ENaC - pendrin	Decrease (PGE2) Increase
Dopamine receptors	G_s – G_i	Basolateral	Na, K-ATPase	Decrease
Proteinase-activated receptor (PAR)	$G_{q/11}$	Basolateral	Na, K-ATPase - NKCC2 - ENaC - pendrin/ NDCBE	Increase
Sphingosine-1 phosphate receptor	$G_{12/13}$ - G_i- G_q	?	ENaC	Decrease
Nucleotide-like receptors (P2X-P2Y)	G_q (P2X) G_i (P2Y)	Apical/basolateral	NKCC2 (?) - NCC - ENaC	Decrease
Oxaloacetate receptor (OXGR1)	G_q	Apical	Pendrin/NDCBE	Increase

NDBCE: Na^+-driven bicarbonate/Cl^- exchanger; OXGR1: 2-oxoglutarate receptor 1; NKCC2: Na/K/2Cl.

Cheng *et al*[28] showed that SPAK was important for NKCC2 activity in both mTAL and cTAL cells but was not essential for vasopressin-induced stimulation of sodium reabsorption. In the CCD, AVP activation of V_2 elicits the phosphorylation of aquaporin-2 (AQP2) leading to its translocation to the apical membrane[22]. It also increases Na^+ reabsorption by activating ENaC[29,30], as PKA phosphorylation leads to the inhibition of Nedd4-2, the ubiquitin ligase that promotes the endocytosis of ENaC[31,32]. Sodium reabsorption across the CD epithelium is essential for water absorption through aquaporins[33].

Angiotensin Ⅱ receptor AT1: Angiotensin Ⅱ (Ang Ⅱ) is a hormone peptide of the renin-angiotensin-aldosterone system (RAAS) that regulates BP and other physiological processes (for review see[34]). Circulating Ang Ⅱ is produced by the proteolytic cleavage of angiotensinogen (which is continously produced by the liver) and involves two steps: (1) the production of angiotensin Ⅰ by renin, which is released by juxtaglomerular apparatus cells; and (2) the conversion of Ang Ⅰ into Ang Ⅱ by the angiotensin converting enzyme (ACE) that is produced by the lungs. Renin is the rate-limiting factor that determines the levels of circulating Ang Ⅱ such that the production and release of Ang Ⅱ respond rapidly to hypovolemia[34]. Ang Ⅱ can also be produced in various tissues like the kidneys and have different effects than systemic AngII (for review see[2]).

The blood level of Ang Ⅱ is increased in some form of hypertension where it is involved in its development (see review[35]).

AngII binds to 2 subtypes of GPCRs called AT1 (AT1a and AT1b in rodents) and AT2. AT1 isoforms are encoded by homologous genes, have the same affinity to different ligands, trigger the same signaling pathways and are located all along the distal nephron but AT1a is the predominant renal form. AT1 receptors are expressed at both apical and basolateral membrane[36-38] and mediate the known physiological and pathological actions of Ang Ⅱ. These receptors are coupled to $G_{q/11}$, $G_{12/13}$ and $G_{i/o}$ proteins in rodents[39]. The main pathway triggered by Ang Ⅱ involved $Gq_{/11}$-mediated inositol phosphate/Ca^{2+} signaling. In the cTAL, AT1a receptors activate both the PLC/PKC/ERK and protein tyrosine kinase (PTK) Src pathways thereby enhancing calcium signaling and an increase in the activity of NKCC2[40,41]. Here too, the intermediary action of SPAK has also been proposed since Ang Ⅱ infusion increased abundance of the phosphorylated forms of SPAK and NKCC2[42].

In the DCT, Ang Ⅱ increases sodium reabsorption in both the DCT1 and DCT2 by binding to its AT1 receptor. Recent experiments have shown that the AT1a receptor induces an increase in NCC phosphorylation, abundance at the apical membrane and activity through the activation of WNK4 (with no lysine kinase 4), SPAK, and OSR1[41,43].

Both the apical and basolateral addition of Ang Ⅱ can induce an increase in sodium reabsorption through ENaC in the rabbit CCD[44]. Using patch clamp on split open CCD, Mamenko *et al*[45], showed that Ang Ⅱ activation of AT1 receptors had a double effect on ENaC: A rapid increase in the open probability of ENaC and a slower increase in the abundance of ENaC at the apical membrane. Interestingly, AT1 receptor-induced activation of ENaC was not dependent on calcium signaling in these cells. Instead, the AT1 receptor activated a Ca^{2+}-independent PKC and triggered the NADPH oxidase pathway[45] (see for review[35]).

In the type B IC, activation of AT1 receptors enhances Cl^- uptake by pendrin[46]. Pendrin activation and the subsequent increase in Cl^- reabsorption are necessary for the full pressure effects of Ang Ⅱ since pendrin knockout mice exhibit an attenuated BP response to this peptide hormone[46,47]. The signaling pathways between AT1 receptors and pendrin remain to be discovered, and AT1 receptors capacity to induce

sodium reabsorption through NDBCE remains unknown. In summary, through the activation of AT1 receptors, Ang Ⅱ induces an increase in Na^+ reabsorption all along the distal nephron by activating apical Na^+ entry through NKCC2, NCC, ENaC and the pendrin/NDCBE functional complex[35].

Catecholamine receptors: Catecholamines [adrenaline, also called epinephrine (E), and noradrenaline, also called norepinephrine (NE)] mediate the sympathetic regulation of BP[48]. E and NE are synthetized by neurons and the adrenal gland[49]. Both cathecholamines act by binding to 9 different subtypes of adrenergic receptors (ARs) that belong to 3 different groups (α1-AR, α2-AR or β-AR). All three classes of receptors contribute to the control of BP by acting on the central nervous system (CNS), the cardio-vascular system, and the kidney. In the latter organ, all segments of the nephron as well as juxtaglomerular granular cells are in contact with sympathetic nerve terminal extremities releasing noradrenaline[50]. Renal nerve activation enhances sodium reabsorption all along the nephron and promotes renin secretion. Conversely, renal denervation inhibits sodium retention and renin secretion (for review, see[51]).

a/α1-AR and α2-AR: Three molecular species of receptors α1-AR have been cloned: $\alpha1_A$-AR, $\alpha1_B$-AR, and $\alpha1_D$-AR[52]. In the TAL only $\alpha1_B$-AR and $\alpha1_D$-AR are expressed, and they are coupled to $G\alpha_{q/11}$ proteins that activate PLC-β[53,54] and trigger the IP3/DAG/PKC pathway[55,56]. In the CNT/CCD, all three $\alpha1_A$-AR are expressed[57] where their effects have not been determined.

The α2-AR receptors ($\alpha2_A$-AR, $\alpha2_B$-AR, and $\alpha2_C$-AR)[52], are coupled to G_i proteins and inhibit the formation of cAMP[58-61]. In the distal nephron, α2-AR is expressed in the TAL and CD with a higher abundance in the medulla[62]. In the rat cTAL, α2-AR activation has recently been shown to induce NO production *via* a PI3K/NOS pathway and to inhibit chloride absorption[63]. In the rat CCD, α2-AR activation inhibits AVP-induced cAMP production[64,65] and blunts the AVP-dependent stimulation of Na^+ and water transport[66].

In the CCD, the activation of the $\alpha2_A$- and/or $\alpha2_C$-receptors by catecholamines has a dose-dependent effect on ENaC activity[67]. At low concentrations, NE triggers the inhibition of Na^+ and water transport, whereas higher concentrations have the opposite effects. Since renal NE concentrations have never been measured precisely, one can only speculate that depending on the intensity of sympathetic activity, sodium transport could be either activated or inhibited along the nephron.

b/β-AR: Three subtypes of the β-AR receptors are known (β_1-AR, β_2-AR and β_3-AR). In the distal nephron, β_1-AR is expressed in the cTAL, at the apical pole of DCT cells, in all CNT cell types and preferentially in the IC of the CCD[68,69]. Using β_1-AR or β_2-AR knock-out mice models, Mu *et al*[70] demonstrated that the activation of β_2-AR by isoproterenol but not that of β_1-AR induces salt-sensitive hypertension. β_2-AR is expressed in the DCT and in the CD where its expression increases along the cortico-medullar axis[71]. No staining of β_2-AR has been found in the rat TAL[71]. β-AR receptors are coupled to G_S, they stimulate cAMP production by the adenylyl cyclase and induce the activation of PKA[72]. In the cTAL, β-AR activation elicits an increase in the expression of NKCC2 at the apical membrane[73]. This increase is dependent on cAMP formation and PKA activation[73].

Using β_1-AR knockout mice and β_2-AR knockout mice, Mu *et al*[70] showed that in DCT NE induces an increase in NCC expression and phosphorylation through the activation of β_2-AR. Terker *et al*[74], recently demonstrated that β-AR activation enhances NCC phosphorylation through the activation of oxidative stress-response kinase 1 OSR1 rather than through SPAK.

In the CCD, we found that β-AR activation leads to increased pendrin activity by triggering its translocation to the cell surface[75]. Furthermore, application of isoproterenol on *in vitro* microperfused mice CCD elicited rapid NaCl reabsorption. These results suggest that the CCD participates in adrenergic-dependent Na^+ retention. The finding that β-AR activation enhances electroneutral Na^+ reabsorption in both the DCT and CD is consistent with the results of Mu *et al*[70] showing that HCTZ, a specific inhibitor of NCC in the DCT and of the electroneutral pathway in the CNT/CCD, blunts the BP elevation observed under isoproterenol infusion and salt loading. Interestingly, in a more distal segment of the CD (*i.e.*, the IMCD) β_2-AR stimulation activates sodium, chloride and fluid secretion through a cAMP dependent pathway[76]. To our knowledge, the underlying mechanisms remain unknown.

Altogether, these studies show that renal sympathetic nerves can deliver NE to the basolateral membrane of distal tubule cells. Interestingly, depending on the concentration of NE and on the type of receptors (α-AR or β-AR) that are activated, the sympathetic nervous system could increase sodium absorption either in all distal tubule cells or preferentially in a few cell types. More experiments are needed to define the exact mechanisms by which of NE acts in the distal nephron. Regarding all the SNS effects on kidney, it is now evident that renal sympathetic nerves play an important role in sodium balance (for review see[77]).

Indeed, increased salt intake stimulates sympathetic activity in the kidney and in brain,. Moreover, the renal NE metabolism is modified in hypertensive rats[78] as well as in in obese patients with hypertension[79]. Renal denervation has been shown to prevent or attenuate hypertension in a wide variety of animal models of hypertension (for review see[51]). In humans, following successful clinical trials, the techniques of renal nerve ablation are now spreading in Europe[80]. However, recent clinical investigations on randomized renal denervation trials known as "symplicity HTN-3" have shown that renal denervation has limited efficacy[81,82]. These investigations, however, are subject to criticism

and more studies will be needed to assess the benefits of renal denervation[83].

"Classical" GPCR that inhibits sodium absorption in the distal nephron

Bradykinin B2 receptor: Bradykinins are autocrine and paracrine hormones that are generated in different tissues by the cleavage of kininogen by kallikrein (for review see[84]). Bradykinins, *via* their actions on the heart, blood vessels, and kidneys, play an important role in cardiovascular homeostasis (reviewed in[85]). Along with bradykinin receptors B1 and B2 (which are two GPCRs), bradykinins and kallikrein form the kinin/kallilrein system (KKS).

In the kidney, all components of the KKS are produced in the distal nephron[86-88]. Kallikrein is a serine protease that is produced by DCT and CNT cells and secreted into the tubular lumen and extracellular space. Kininogen is produced by all distal nephron cells, which all express B2 receptors at their basolateral membrane

In vitro experiments on rodent CCDs showed that kallikrein activates ENaC by cleaving its regulatory subunit γ[89] and that it directly inhibits the H,K-ATPase type 2[90]. Therefore, kallikrein that is secreted apically could simultaneously activate ENaC and inhibit the H, K-ATPase type 2[89,90]. Kallikrein that is secreted on the basolateral side is thought to act through bradykinin formation and B2 receptors coupled to G_q proteins. In the cTAL, B2 activation in turn activates the MAPK/$ERK_{1,2}$ signaling pathway and increases intracellular calcium concentrations like AT1 activation[40]. Surprisingly, NaCl reabsorption is inhibited following B2 receptor activation but enhanced following AT1 receptor activation[40]. The molecular mechanisms that allow the same transduction pathway to yield two opposite responses need to be determined in this segment. In CCDs perfused *in vitro* from DOCA treated rat, activation of B2 receptor sby 10^{-9} mol/L bradykinin inhibits the electroneutral and thiazide sensitive NaCl reabsorption[91]. Meanwhile, neither the transepithelial potential, nor the K^+ secretion is inhibited by B2 receptor activation, which clearly indicates that ENaC activity is not diminished under these conditions[91]. A higher concentration of bradykinin (10^{-7} mol/L) has been shown to lower the open probability (Po) of ENaC *via* the activation of the B2/$G_{q/11}$/PLC pathway[92]. A role of B2 receptors in inhibiting salt reabsorption is confirmed by the phenotype of B2 knockout mice, which exhibit salt-sensitive hypertension[93].

KKS is a complex system having the ability to either stimulate or to inhibit Na^+ reabsorption. To understand these opposite effects, it is necessary to consider the physiological context in which this system is triggered. For instance, KKS is known to be strongly upregulated by a high dietary K^+ intake, a condition under which the levels of AngII and renin are low. The overall action of KKS in the distal nephron would then favor K^+ secretion and limit excessive Na^+ retention: Firstly, the inhibition of Na^+ reabsorption in the cTAL would increase the delivery of fluid and Na^+ to the distal nephron; secondly, the direct role of luminal kallikrein on ENaC and the H, K-ATPase would favor K^+ secretion by stimulating its excretion (through both ROMK and MaxiK channels) and inhibiting its retention. In other situations, for instance when salt intake is increased, the stimulated KKS stimulation would inhibit the electroneutral (at low concentrations of bradykinin) or the electrogenic (at high concentration of bradykinin) Na^+ reabsorption pathways. This may explain why the absence of one component of the KKS induces salt-sensitive hypertension (for review see[94]). More investigations are needed to understand how the renal effects of KKS are orchestrated to favor either K^+ secretion or Na^+ secretion, two apparently antagonistic situations.

Endothelin receptors ETA and ETB: Endothelin 1 (ET-1) is an endothelial cell-derived peptide that is the most potent vasoconstrictor of the body[95]. ET-1 plays important roles in the physiology and physiopathology of all organs and is an important factor in the control of sodium homeostasis and BP (reviewed by[96]). There are three different endothelins (ET-1, ET-2 and ET-3), the first of which, (ET-1) has been the most studied. The plasma levels of ET-1 are lower than its tissue levels. Moreover, ET-1 that is produced by renal tubular cells is essentially secreted at the basolateral pole. This is why ET-1 is considered to be a paracrine/autocrine hormone.

ET-1 binds to two GPCRs known as the ET_A and ET_B receptors[97,98]. Almost all cells express ET_A and/or ET_B receptors: In the nephron, ET_B is the predominant endothelin receptor. These receptors are coupled to many different G proteins: G_i, G_q, G_s, and $G_{\alpha 12/13}$[99,100].

In the rat cTAL, ET-1 activates basolateral ET_B receptors, which triggers the production of NO and the subsequent inhibition of NKCC2[101,102], leading to a reduction in transepithelial Cl^- and Na^+ fluxes. However, ET-1 does not inhibit the AVP-stimulated cAMP accumulation in the rat cTAL[103,104]. To our knowledge, the roles of ET-1 and ET_B in the DCT have not been investigated. In contrast, the effects of ET-1 and ET_B in the CD have been extensively studied. CD cells, particularly those in the inner medulla produce more ET-1 than any other nephron cell. ET-1 decreases Na^+ reabsorption in the isolated CCD and the IMCD[105,106]. As opposed to finding in the cTAL (see above), ET-1 triggers the inhibition of the AVP-stimulated chloride transport in the CCD through PKC activation[105]. In addition to counteracting the effects of AVP, ET-1 also inhibits ENaC activity through both MAP kinase-dependent and NO-dependent pathways[107]. ET_B activation has functional consequences on IC. Indeed, in IC-A, it activates H^+-ATPase and in IC-B, it reduces pendrin activity (for review see[108]).

Interestingly, ET-1 production by CD cells seems to be regulated by the extracellular fluid volume (ECFV) status (for review see[96]). Mice with a specific deletion of the ET-1 gene in the CD are hypertensive under normal conditions and develop salt-sensitive-hypertension[96]. These observations could reflect an increase in sodium reabsorption in the CD but further experiments are need-

ed to confirm this hypothesis. Several animal models of hypertension are, in fact, associated with increased vascular ET-1 synthesis (for review see[109]), which may be understood as a compensatory mechanism to try to reduce BP[96].

Prostanoid receptors: Prostaglandins are lipid mediators generated by cyclooxygenase (COX) that metabolizes arachidonic acid. The cellular concentration of COX-derived prostaglandins is modulated by several extracellular and intracellular factors. Prostaglandins have many effects in pain, inflammation, cell proliferation and body homeostasis. Signaling prostaglandins are PGE_2, PGI_2, PGF_2, PGD_2 and thromboxane A_2 (TxA_2). Prostaglandins are rapidly degraded which limits the scope of their action to autocrine and paracrine functions[110]. Prostaglandins act on nine specific prostaglandin receptors encoded by nine different genes. All are lipid-like GPCRs: Prostaglandin D2 receptor 1 and 2 (DP1 and DP2); Prostaglandin E receptor 1 to 4 (EP1-EP4); Prostaglandin F receptor (FP); Prostaglandin I2 receptor (IP) and thromboxane A2 receptor (TxA2R). The kidney expresses all four EP receptors, the FP receptor and TxA_2R (for review see[111]).

In the cTAL, EP receptors that bind PGE2 have been reported to antagonize the action of AVP[112]. In this segment, EP3 is coupled to G_i and the presence of PGE2 was shown to inhibit AVP-induced cAMP accumulation induced by AVP and therefore to blunt the NaCl reabsorption[112]. Moreover, EP3 activates a Rho-kinase/PKC/ERK pathway that inhibits the basolateral potassium channels. Since potassium recycling at the basolateral membrane is necessary for Na,K-ATPase function, inhibition of these channels is sufficient to reduce Na^+ reabsorption all along the cTAL[113]. As for the CNT/CD, these segments express all four EP receptors. In the rabbit CCD, the use of specific agonists and antagonists demonstrated that PGE2 activation of EP1 inhibits ENaC through a calcium signaling pathway[114]. In other studies, PGE2 has been shown to induce the inhibition of Na^+ reabsorption by decreasing the amount of cytosolic cAMP through a G_i coupled receptor, thought to be EP3[115]. Recent studies have shown that increases in luminal flow rate activate the release by epithelial cells of PGE2 in the lumen of rabbit CCD perfused *in vitro*. The prostaglandin secretion is dependent on calcium and MAP-kinase signaling. The flow-dependent production of PGE2 activates luminal EP receptors and triggers the inhibition of Na^+ reabsorption[116]. In the rabbit CCD, Chabardès *et al*[112] also reported that PGE2 antagonized the effects of AVP, as seen in the cTAL. However, this effect was not observed in the rat CCD[112].

Investigation of the regulatory system involving prostaglandin and their receptors has led to the identification of a cross-talk between the intercalated and PC of the CCD (see below)[117].

FP receptors are present in rabbit DCT cells and CCD PC where their activation was shown to inhibit sodium and water reabsorption[118]. However, nothing is known regarding the effect of FP activation in mice or rats.

Finally, the thromboxane A2 receptor is localized in the TAL segment where it is activated by a peroxidized derivative of PGF2, 8-iso-prostaglandin-F2α (8-iso-PGF2α)[119]. In the kidney, 8-iso-PGF2α induces vasoconstriction, which may impact renal blood flow[120]. In the TAL, TxA2R activation stimulates PKA and induces an increase in chloride reabsorption across NKCC2[121]. Increased concentrations of 8-iso-PGF2α and other isoprostanes have been observed in both the urine and plasma of hypertensive subjects. Enhanced NaCl retention in response to 8-iso-PGF2α may contribute to the pathogenesis of hypertension[121].

Dopamine receptors: Dopamine is a catecholaminergic neurotransmitter necessary for the function of neurons. It is involved in many different physiological functions related to CNS as well as peripheral organs or tissues[122]. In the kidney, proximal cells expressing dopamine decarboxylase produce dopamine from the precursor L-dihydroxyphenylalanine. Dopamine is then released by cells at the apical and basolateral poles possibly by multiple amino acid transporters[123]. Dopamine is subsequently eliminated either by urine excretion or methylation and deamination. Dopamine concentrations are 1000-fold higher in kidneys (nmol/L range) than in plasma (pM range)[124,125], and increase even more after sodium loading[126,127]. As described below, dopamine modulates the renal Na^+ transport through an autocrine or paracrine pathway[128].

Dopamine binds to five different receptors (D_1R to D_5R) encoded by five different genes classified into D1-like coupled to G_s proteins (D1 and D5) and D2-like coupled to $G_{i/o}$ proteins (D2, D3, and D4) subtypes[129]. Since the two receptor subtypes signal through different G proteins and are present in the kidney, one might expect dopamine to have opposite effects depending on the receptor subtypes it binds to. However, dopamine has been reported to inhibit Na^+ transport at multiple sites along the renal tubule by acting on major Na^+ transporters[130].

In the distal nephron, D3 is the only dopamine receptor expressed in the cTAL but its role in this segment is not known. In fact, D1-like receptors agonists have been shown to down-regulate Na, K-ATPase activity in the mTAL, but the cTAL has not been proven to be sensitive to dopamine[131]. The DCT and CNT/CCD express all 5 dopamine receptors (see[132] for review). In the CCD, D1-like receptors reduce activity of Na, K-ATPase through a cAMP/PKA pathway. Moreover, phospholipase A2 (PLA2) stimulation is necessary to inhibit the pump[133]. This inhibitory pathway has been described in the proximal tubule[134,135].

Vasopressine and aldosterone effects have been shown to be antagonized by the D4 receptor in the CD[136]. More recently, a D2-like receptor agonist was reported to induce the inhibition of basolateral potassium transporters Kir4.1 and Kir5.1. Given that these channels are essential for K^+ recycling and maintaining the

basolateral membrane resting potential, their inhibition induces a decrease in sodium reabsorption[137].

Recent reviews clearly delineate the antihypertensive role of dopamine and dopamine receptors[124,138]. For instance, renal dopamine production is decreased in some cases of hypertension and some human populations (African-American, Japanese) do not raise their renal production of dopamine in response to a NaCl or protein load. This may contribute to the development of salt-sensitive hypertension in these particular ethnic groups[139]. The observation that $D3^{-/-}$ mice have a diminished ability to excrete an acute or chronic NaCl load, leading to the expansion of ECFV support the idea that the renal dopamine pathway regulate BP (see[140] for review). However, in patients with essential hypertension or in hypertensive animal models, D1-like receptor agonists fail to induce natriuresis[141].

REGULATION OF SODIUM TRANSPORT IN THE DISTAL NEPHRON BY "NOVEL" GPCRS

"Novel" GPCR that activate sodium absorption in the distal nephron

Proteinase-activated receptors: The example of proteinase-activated receptors 2: Proteases play important roles in the regulation of sodium transport in the distal nephron and BP, by stimulating the maturation of hormones or through their direct interactions with ion transporters (see above). In addition, proteases act by activating specific receptors of the so-called protease-activated receptor family (PAR).

PARs are activated trough the proteolytic cleavage of their N-terminal domain. This cleavage unmasks a peptide sequence that binds to the inner binding site and activates the receptor. It is possible to "artificially" activate these receptors by using agonist peptides that have the same sequence as the masked ligand[142,143]. For instance, the design of human- and mouse-specific agonists of PAR2[144] has enabled investigators to elucidate the particular role of PAR2 in systems that express many PARs.

The PAR family consists of 4 members (denoted PAR1 to PAR4) that are activated by different proteases. PAR1 and PAR3 are exclusively activated by thrombin[145,146]. PAR4 can be activated by thrombin and trypsin[145,147]. PAR2 is fully insensitive to thrombin but activated by trypsin and other serine proteases[148]. It can also be activated by cysteine proteases such as Derp-1[149] and be disabled by certain serine proteases (*e.g.*, cathepsin-G) witch cleave the N-terminal domain of the receptor beyond the peptide ligand[142]. PAR2 is expressed in all tissues tested so far. In the kidney, PAR2 is expressed in epithelial, mesangial, and endothelial cells (ECs) but also in interstitial fibroblasts and inflammatory cells that invade the kidney[150].

PAR2 is involved in inflammatory responses, and it plays a coordinating role in all inflammation steps. PAR2 activation triggers a specific response in each cell type it is expressed in: The relaxation of blood vessels, increased vascular permeability, granulocyte infiltration and leukocyte adhesion, the activation of the production of cytokines IL-6 and IL-8, and finally the transmission of pain from nerve cells[151]. In the kidney, PAR2 is involved in the renal inflammation that is observed in IgA nephropathy[152] and crescent glomerulonephritis[153]. In addition, PAR2 activates monocyte chemo-attractant protein-1[154], which stimulates the production of IL-6 and IL-8 in renal tubular cells[155]. In addition, PAR2 has been shown to modulate substrate and ion transport in many epithelia (lungs, gastro-intestinal tract, lacrimal and salivary glands)[156,157].

PAR2 is expressed along the entire nephron but at higher levels in the distal nephron[158,159]. Using *in vitro* microperfusion of rat and mouse renal tubules, we first reported that PAR2 may activate Na^+ reabsorption in the cTAL (Figure 2). We showed that PAR2 is expressed at the basolateral pole of cTAL cells and is probably coupled to a $G_{q/11}$ protein, since it induced calcium signaling and activated a PLC/PKC/ $ERK_{1,2}$ pathway. This latter pathway induces in turn the stimulation of Na, K-ATPase activity and an increase in the paracellular permeability to sodium. Interestingly, the PAR2-induced increase in sodium reabsorption was partly related to a rise in the apparent affinity of Na, K-ATPase to sodium. The latter effect was induced by another signaling pathway involving PLC and a calcium-insensitive PKC that did not activate $ERK_{1,2}$ phosphorylation[158]. We next characterized the role of PAR2 in mouse and rat CCDs, where it is expressed at the basolateral membrane of ICs and PCs (Figure 3). PAR2 triggered calcium signaling in both types of cells and induced an increase in the maximum Na, K-ATPase transport rate (Vmax) through ERK phosphorylation. Under our experimental conditions, PAR2 increased sodium reabsorption through ENaC only slightly. This could be explained by the fact that the ERK pathway inhibits the apical sodium channel[160]. The increase in sodium reabsorption induced by PAR2 activation stemmed mostly from the thiazide-sensitive, electroneutral NaCl reabsorption pathway of the B-IC, which was found to be dependent on ERK phosphorylation. Using Na-depleted $PAR2^{-/-}$ mice, we showed that PAR2 activation was necessary to induce transport across the electroneutral NaCl reabsorption pathway of the B-IC. Indeed, compared to control mice, $PAR2^{-/-}$ mice exhibited excessive and rapid renal Na^+ loss during sodium depletion and developed hypotension[159].

This hypotension cannot result from the absence of vascular PAR2 effects because in ECs, PAR2 triggers the production of NO, thereby inducing vasodilation and decreasing BP[161-163]. Proteases that cleave PAR2 at the surface of distal nephron cells in response to sodium depletion have not yet been identified. They could include serine proteases that are secreted close to PAR2. A membrane-bound serine protease such as matriptase MT-SP1 (which is encoded by the gene *St14*) could be a good candidate since it is one of the rare serine

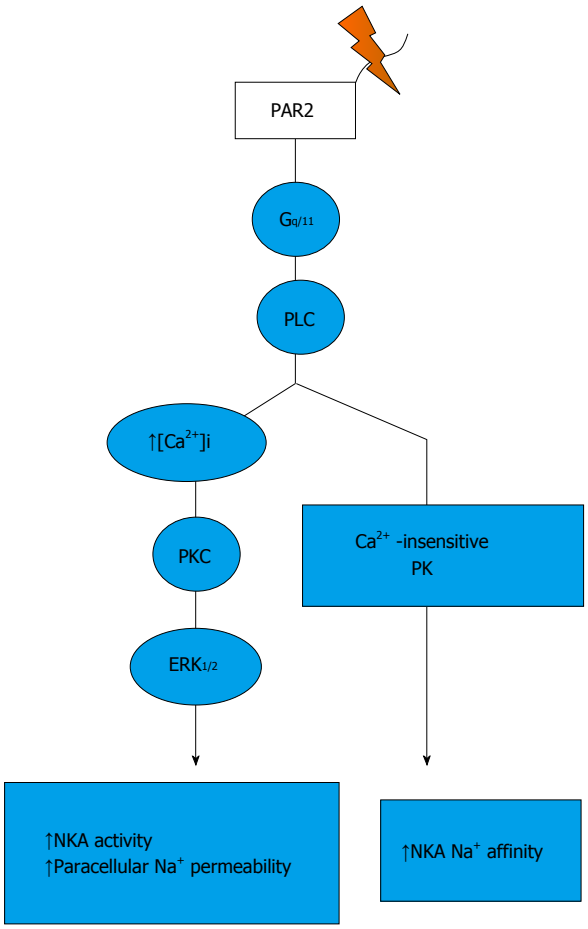


Figure 2 Signalling pathways trigger by protease-activated receptor 2 activation in the thick ascending limb. PAR: Protease-activated receptor.

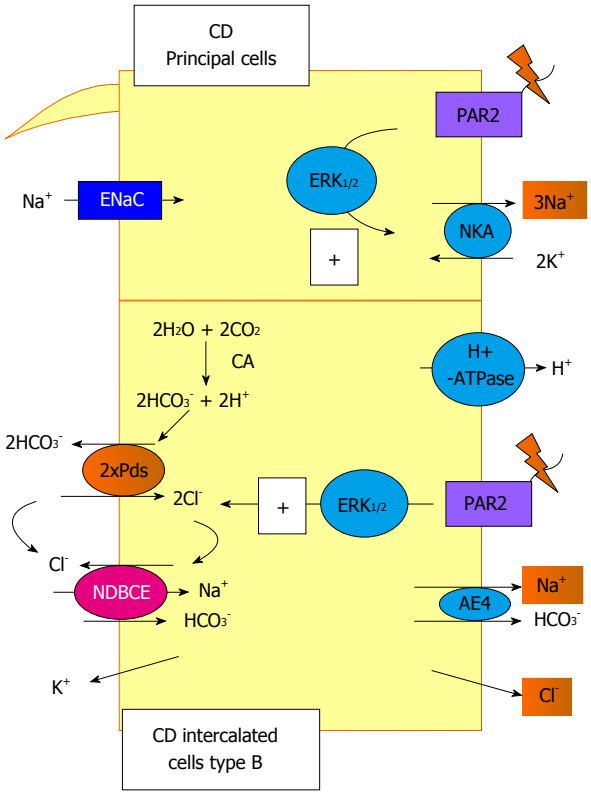


Figure 3 Signalling pathways trigger by protease-activated receptor 2 activation in the collecting duct. In this segment, protease-activated receptor (PAR) 2 activation increases the NKA activity in the principal cells and the Pds/Na^+-driven bicarbonate/Cl^- exchanger (NDBCE) system in the B-IC. Both processes involved the activation of the ERK1/2 pathway. CD: Collecting duct; PAR: Protease-activated receptor.

proteases that has been shown to activate PAR2 *in vivo*, at the surface of ectoderm cells during neural tube closure[164]. Moreover, matriptase is expressed in human CD cells[165]. Further investigations have to be carried out to identify the protease(s) that activate(s) renal PAR2 *in vivo*.

In light of its effects on Na^+ reabsorption, over-activation of PAR2 in the kidney could lead to inappropriate sodium retention and hypertension. Two observations strengthen this hypothesis. First, the PAR2-encoding gene (coagulating factor Ⅱ receptor like 1; F2RL1) is localized in a region of the genome associated with low renin hypertension in humans (q13 position on the fifth chromosome). Interestingly, f2rl1 is localized in a region rich in polymorphisms in the Lyon rat (q12 region on the 2nd chromosome), which is a model of low renin hypertension[166,167].

As mentioned above, PAR2 is also involved in the inflammatory response, a condition known to be related to the development of hypertension[168]. Macrophages and other inflammatory cells secrete numerous proteases that can activate or disable PAR2: Granzyme B, Elastase, tPA, plasmin, Cathepsin G and kallikrein 2, 12 and 4[169]. PAR2 could therefore play a role in inflammation-induced hypertension. Lohman *et al*[170] designed the first bio-compatible molecule capable of inhibiting PAR2. This molecule, called GB88, has been shown to reduce inflammation and cardiovascular damage triggered by a high fat diet in rats. Moreover, GB88 significantly diminishes BP in these animals[171]. Further investigations are needed to determine whether renal PAR2 is implicated in the BP-lowering effects of GB88.

α-Ketoglutarate receptor OXGR1: α-Ketoglutarate (αKG) is a metabolite of the Krebs cycle and may serve as a cofactor for many enzymes. Moreover, αKG is the ligand of the 2-oxoglutarate receptor 1 (OXGR1), a GPCR coupled to G_q and mainly expressed in renal distal tubules, in the pendrin-positive cells (type B IC)[172]. αKG is reabsorbed in the proximal nephron and TAL after glomerular filtration. This reabsorption is stimulated by acidosis, which, therefore, decrease αKG urinary excretion. Conversely, base loading stimulates αKG secretion by the proximal segments, leading to an increase of its urine excretion[173-175]. Since αKG is not reabsorbed after the TAL segments[173], modifications of its urine amount are proportional to variations occurring in the lumen of distal nephron. By comparing wild type mice and OXGR1$^{-/-}$ mice, we[176] showed that apical but not basolateral αKG acts through OXGR1 in

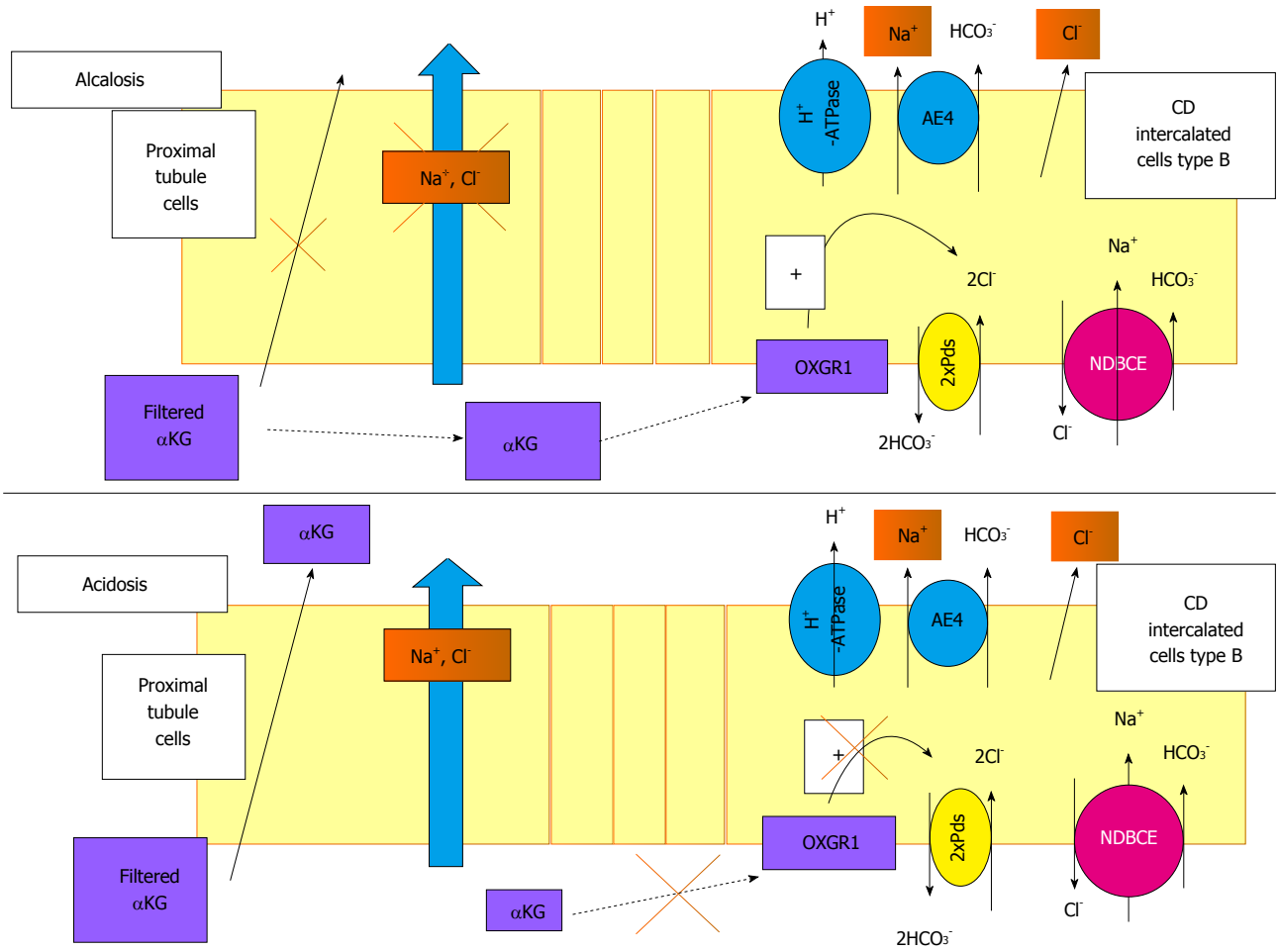


Figure 4 Role of α-ketoglutarate and its receptor, OXGR1, in the regulation of renal Na^+ transport. α-ketoglutarate (αKG) is freely filtered by the glomerulus. During alkalosis, Na^+ reabsorption in the proximal segment is reduced and the reabsorption of αKG is blunted, increasing its concentration in the lumen. This high level of αKG delivered to the distal tubule may activate its receptor in the B-intercalated cells. The signalling pathway triggered by this activation is still unknown but activates the Pds/NDBCE system, leading to an increased Na^+ reabsorption. In case of acidosis, the filtered pool of αKG is reabsorbed by the proximal tubule, decreasing its luminal concentration and blunting the activation of OXGR1 in the CD. In this case, the reabsorption of Na^+ in the distal nephron is not stimulated. CD: Collecting duct; NDBCE: Na^+-driven bicarbonate/Cl^- exchanger; OXGR1: 2-oxoglutarate receptor 1; αKG: α-ketoglutarate.

CNT/CCD type B IC to stimulate HCO_3^- secretion and NaCl reabsorption. Our metabolic studies indicate that urinary αKG levels change very rapidly under acute acid-base stress. It is well known that acute acidosis activates, whereas alkalosis inhibits, NaCl reabsorption in the proximal tubule and ascending limb[177]. Since αKG concentration in the CNT/CCD decreases in acute acidosis and increases in acute alkalosis, αKG/OXGR1 could be considered as a paracrine system allowing proximal and distal parts of the nephron to communicate (Figure 4). Similarly, this communication system between proximal and distal tubule may serve to set the bicarbonate excretion by B-type IC under either acute acidosis or alkalosis. This system is sufficiently sensitive and rapid to respond to the daily variations in the dietary acid-base load as well as modifications of the metabolic production of acid and base.

Another recent study highlights the importance of the αKG/OXGR1 paracrine system[178]. This study reports that αKG/OXGR1 mediates the adaptation of the CNT/CCD to the down-regulation of NCC in the DCT in SPAK-null mice. In these mice, Oxgr1 expression is increased but remains exclusively localized in pendrin-positive cells, the number of these cells being strikingly increased in the CNT/CCD. Interestingly, in the proximal tubule of these mice, sodium transporter expression is augmented but the expression of OAT1 (Slc22a6, that transports αKG), is down-regulated. Moreover, the expression of glutaminase and glutamate dehydrogenase, which are essential for ammoniagenesis and αKG production, is up-regulated in the proximal tubule. This should raise αKG production and secretion by proximal tubule cells and elevate luminal αKG concentration in SPAK-null mice, which is actually observed. Alkalosis is one of the consequences of NCC down-regulation. SPAK-null mice develop alkalosis, which could stimulate the αKG/OXGR1 paracrine system. The investigators postulated that the αKG/OXGR1 paracrine system may be activated by AngII, since this hormone stimulates the metabolic pathways that enhance ammoniagenesis and αKG formation in proximal tubule cells[179]. This system may be of particular importance to help restoring Na^+ balance

and BP in hypovolemic conditions[178].

"Novel" GPCR that inhibit sodium absorption in the distal nephron

Sphingosin-1-phosphate receptors: The first step in the synthesis of sphingosine-1-phosphate (S1P) is the hydrolysis of sphingomyelin (a component of cell membranes) into ceramide by sphingomyelinases. Ceramide is then hydrolyzed by ceraminidases to form sphingosine, which is in turn phosphorylated within the cell by sphingosine kinase SphK, an enzyme that comes in two isoforms (SphK1, SphK2), to form S1P. The intracellular concentration of S1P is maintained constant by the recycling of S1P into sphingosine by two sphingosine phosphatases and/or the degradation of S1P by a sphingosine lyase[180]. The major source of blood S1P is thought to be the red blood cells, vascular ECs, and activated platelets[181,182]. Plasma S1P concentration varies from 200 nmol/L to 900 nmol/L in humans and rodents[183]. In this compartment, the majority of S1P is bound to serum proteins such as high density lipoprotein and albumin[184]. S1P is involved in many different cellular processes from proliferation to regulation of tight junctions, *etc*.[185,186]. S1P may act directly upon molecular targets in the cytosol or nucleus to modulate gene expression, but S1P can also act as an extracellular signaling molecule since specific S1P membrane transporters allow it to be extruded into the extracellular compartment[187].

Sphingosin-1-phosphate is the ligand of a family of 5 GPCR, denoted S1P1-5[188]. S1P1, S1P2, and S1P3 are ubiquitously expressed, whereas S1P4 and S1P5 exhibit more restricted distribution (lung, lymphoid system and brain). S1P1 receptors are exclusively coupled to $G_{i/o}$; they can activate Akt and Rac and down-regulate PKA by inducing a decrease in cAMP cytosolic concentration. S1P2 and S1P3 receptors are most efficiently coupled to $G_{12/13}$, leading to Rho activation, and to G_q, thereby activating the PLC/PKC/calcium signaling pathway[183].

Recently, the expression of SphK1 as well as S1PRs was demonstrated in whole kidney and in isolated renal cells. In the rat, S1P1-3 are more abundant in the medullary than in the cortical CD[189]. The cellular localization (*i.e.*, apical or basolateral) remains uncertain to date. Preliminary results from *in vitro* microperfusion of mouse CCDs indicate that S1P administration induces a functional response, suggesting that S1P receptors are expressed at the basolateral side of the CCD cells (unpublished observation by Morla L).

Intravenous and intrarenal infusions of S1P lead to renal vasoconstriction and cause natriuresis and diuresis despite a reduction in renal blood flow. Moreover, these effects have been shown to stem from both S1P1 activation and down-regulation of the AC/PKA pathway[190]. Interestingly, the effects of S1P1 activation on sodium and water excretion are additive to those of methylisobutylamiloride, furosemide, and HCTZ -which respectively inhibit apical Na^+ transporters in the proximal tubule, cTAL, and DCT/ CNT/CCD- but not to those of amiloride, a specific inhibitor of ENaC. This suggests that thenatriuretic and diuretic effects of S1P1 stem from PC. Finally it can also be noted that a S1P1 antagonist has anti-natriuretic properties[189].

The main characteristics of glomerulonephritis (proliferation of mesangial cells, stimulation of matrix production and inflammation) may involved the SphK1/S1P signaling system. Indeed, many studies have shown that S1P1, S1P2, and S1P3 are involved in inflammation and fibrosis in the glomerulus and proximal tubule[191-193]. However, so far, investigation of the specific pathophysiological contribution of each S1P receptor and SphK subtypes are not sufficiently selective and potent to yield clear-cut answers as to which of these should be targeted for treatment[194].

The recent study of Wilson *et al*[195] suggests that SphK1 is necessary for AngII-induced calcium signaling in isolated vascular smooth muscle cells and that store-operated calcium channels represent the exclusive intracellular target of SK1/S1P. Moreover, it shows that $SphK1^{-/-}$ mice do not develop hypertension following the infusion of angiotensin, suggesting its putative role in the AngII-induced hypertension[195].

Purinergic receptors (P2Y): Extracellular ATP and other nucleotides constitute local, intra-renal factors that regulate kidneys function. These nucleotides are secreted by all tubular cells on both apical and basolateral sides, either by exocytosis of nTP-filled vesicles or by specific nTP channels. ATP release occurs under mechanical stimulation. Cells express at their apical membrane a primary cilium that senses the flow rate. When luminal flow rises, the bending of the cilia is sufficient to induce ATP release at both poles of epithelial cells. ATP and other nucleotides can also be secreted under cell volume enhancement[196,197].

To date, seven P2X ($P2X_{1-7}$) and eight P2Y ($P2Y_{1,2,4,6,11-14}$) receptors have been identified. P2X are ligand-gated ion channels and P2Y are GPCRs. Since this review is primarily focused on GPCRs, we do not discuss here the role of P2X receptors in the distal nephron. Most P2Ys are coupled to G_q and their stimulation leads to the activation of PLCβ and intracellular calcium signaling. The $P2Y_{12-14}$ receptors may form a subfamily, as they appear to be coupled to $G_{i/o}$ and their stimulation triggers the inhibition of the AC/PKA pathway[198]. Many of these receptors subtypes are expressed along the nephron on both apical and basolateral cell membranes. Each nephron segment bears a specific combination of P2Y and P2X and these combinations vary from one species to another[199].

Reviews of recent studies of transgenic animals[198,200-202] indicate that $P2Y_2$ plays an important role in sodium and water handling by the kidney.

$P2Y_2$ receptors are expressed in cTAL[203], mDCT[204] and CD cells[205-207] and are generally found at apical and basolateral membranes, where their activation induces calcium signaling.

NKCC2 abundance and furosemide-induced natriu-

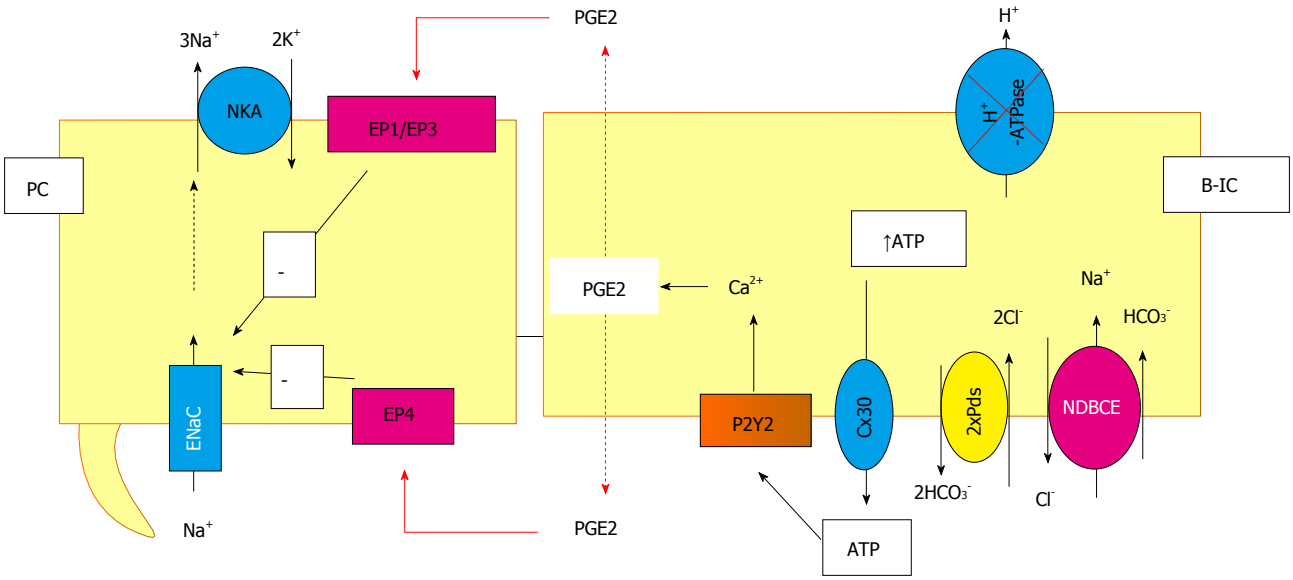


Figure 5 Cross-talk between principal and intercalated cells, example of the inhibition of the vacuolar proton pump. In this particular situation, the ATP is released by the B-intercalated in the lumen cell through connexion and then binds and activates the P2Y2 receptors. This activation leads to prostaglandin (PGE2) synthesis and release in both side of the cells. It binds to the EP receptors in principal cells, inducing, then, the inhibition of the Na^+ reabsorption. NDBCE: Na^+-driven bicarbonate/Cl- exchanger; PGE2: Prostaglandin; B-IC: Type B intercalated cells.

resis are both higher in $P2Y_2^{-/-}$ mice, which suggests that $P2Y_2$ receptors are an important modulator of sodium absorption in the cTAL; however their effects on sodium transport in the cTAL have not been assessed directly[199].

In the CNT/CCD of $P2Y_2^{-/-}$ mice, the open probability (Po) of ENaC is higher but its apical expression in PC is reduced. The activation of $P2Y_2$ by extracellular ATP should therefore inhibit ENaC activity. This effect is mediated in part by stimulation of PLC, which hydrolyzes PIP2 into IP3 and DAG. Since PIP2 binding to the β subunit of ENaC increases its open probability, reducing the amount of PIP2 in the apical membrane leads to a down-regulation of ENaC activity[201]. In addition, $P2Y_2$ activation raises cytosolic (Ca^{2+}) through IP3-mediated Ca^{2+} release from internal stores. ENaC is indirectly inhibited by intracellular Ca^{2+}, for instance through the Ca^{2+}-specific activation of Nedd4.2, an ubiquitin ligase that promote the endocytosis of ENaC[201]. In mDCT cells, a recent study showed that $P2Y_2$ receptor activation and calcium signaling causes a decrease in NCC expression, owing at least partly to the destabilization of NCC mRNAs[208].

Purines and $P2Y_2$ seem to be part of a negative feedback system that is activated to protect nephron cells from oxidative stress under high hormonal stimulation[209]. AVP, for instance, stimulates ATP release and $P2Y_2$ activation in the cTAL and CCD[210]. Moreover, studies of $P2Y_2^{-/-}$ mice have revealed the importance of this receptor in sodium homeostasis and BP maintenance, as these mice exhibit an increase in BP[211]. Interestingly, under aldosterone infusion and salt loading, the activity of ENaC and the salt-induced rise in BP are greater in $P2Y_2^{-/-}$ mice than in WT mice. Thus, $P2Y_2$ are important modulators of the action of aldosterone in the distal nephron, as they mediate the suppression of ENaC activity observed in WT mice under a high salt diet and mineralocorticoid infusion, a phenomenon known as the "aldosterone escape"[201].

Interestingly, Zhang *et al*[212] showed that the blunted aldosterone escape in $P2Y_2^{-/-}$ mice is associated with an impaired increase in PGE2 and NO urine excretion under high salt loading and aldosterone infusion. These results suggest that $P2Y_2$ are important activators of the cellular production of paracrine and autocrine messengers known to down-regulate the main sodium transporters in the nephron[213]. Gueutin *et al*[117] recently found that mouse CCDs are able to release PGE2 in a pathophysiological situation (Figure 5). Their study showed that ATP is released from B-type IC when H^+-ATPase is down-regulated, and that luminal ATP activates $P2Y_2$ receptors and calcium signaling in principal and IC. Interestingly, activation of the purinergic receptor induces the release of PGE2, which is known to inhibit sodium and water absorption in the distal nephron through the activation of EP1, EP3 and EP4 as described above. The effects of ATP through $P2Y_2$ activation or those of PGE2 through PE receptors on the Na^+/Cl^- electroneutral transport system in IC remain unknown[117].

CONCLUSION

This review recapitulates the "classical" and "novel" signaling pathways mediated by GPCRs that regulate Na^+ reabsorption in the distal nephron. The number of GPCRs that have been found to stimulate or inhibit the reabsorption of Na^+ in the distal nephron is strikingly large. However, the GPCRs discussed here may only be the tip of the iceberg. Indeed, by analyzing transcriptomic data, Pradervand *et al*[214] have identified the expression of 78 different GPCRs in the mouse DCT/CNT and CCD, many of which have not yet been

attributed a role in the kidney. Some of the most abundant GPCRs found by these investigators are known to be involved in the effects of chemokines, in the development of the CNS, in the immune system, or in the recognition of pheromones. The exploration of their role in the distal nephron and the potential discovery of their impact on Na^+ transport represent a wide-open field of new regulators of extracellular volume and BP.

In our opinion, the next important challenge is not only to discover new regulatory processes but also to understand how they are linked. As shown in this review, the activation of some GPCRs induces the stimulation of Na^+ reabsorption whereas others trigger the inhibition of Na^+ transport. The fine-tuning of renal Na^+ excretion clearly requires the simultaneous activation of different pathways (even with antagonistic effects) in the distal nephron. Moreover, depending on the physiological context (*e.g.*, the amplitude and duration of the stimulus), these different pathways are activated or inhibited to varying extents. Two scenarios can be imagined: (1) the global response involving the activation of many GPCRs is orchestrated by an initial conductor or a few conductors, such as the RAAS or CNS; and (2) all the systems controlled by GPCRs are assembled into a complex network consisting of positive and negative feedback loops acting in series. The existence of such a connected network would explain why several models characterized by a single GPCR deficiency (a given GPCR knockout mice, for instance) exhibit strong alterations in sodium handling, since the absence of one component would perturb the entire network.

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REVIEW

Dynamic interplay between adhesion surfaces in carcinomas: Cell-cell and cell-matrix crosstalk

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Abstract

Cell-cell and cell-matrix signaling and communication between adhesion sites involve mechanisms which are required for cellular functions during normal development and homeostasis; however these cellular functions and mechanisms are often deregulated in cancer. Aberrant signaling at cell-cell and cell-matrix adhesion sites often involves downstream mediators including Rho GTPases and tyrosine kinases. This review discusses these molecules as putative mediators of cellular crosstalk between cell-cell and cell-matrix adhesion sites, in addition to their attractiveness as therapeutic targets in cancer. Interestingly, inter-junctional crosstalk mechanisms are frequently typified by the way in which bacterial and viral pathogens opportunistically infect or intoxicate mammalian cells. This review therefore also discusses the concept of learning from pathogen-host interaction studies to better understand coordinated communication between cell-cell and cell-matrix adhesion sites, in addition to highlighting the potential therapeutic usefulness of exploiting pathogens or their products to tap into inter-junctional crosstalk. Taken together, we feel that increased knowledge around mechanisms of cell-cell and cell-matrix adhesion site crosstalk and consequently a greater understanding of their therapeutic targeting offers a unique opportunity to contribute to the emerging molecular revolution in cancer biology.

Key words: Cell-cell; Cell-matrix; Adhesion; Cancer; Crosstalk; Pathogens; Epithelial; Barrier function; Tight junction; Cell migration; Apical junctional complex; Adherens junction; Adhesion molecules; Extracellular matrix; Tyrosine kinases; GTPases; Rho

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Core tip: Deregulation of cell-cell and cell-matrix signaling makes well-established contributions to key elements of cancer initiation and progression. In this review we discuss mechanisms of crosstalk between these spatially-distinct adhesion sites in epithelial cells, with a view to understanding their therapeutic targeting in epithelial tumors (carcinomas). A particular focus is placed upon Rho GTPases and tyrosine kinases as mediators of inter-junctional crosstalk, in addition to the concept of opportunistic pathogenic infection as a paradigm for inter-junctional crosstalk. Overall, this review posits that a greater understanding of cell-cell and cell-matrix adhesion crosstalk will drive novel aspects of cancer drug discovery.

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INTRODUCTION

The semi-permeable plasma membrane acts as a barrier between interior and external environments of the cell. The plasma membrane forms specialised domains with distinct protein and lipid compositions crucial for many biological processes like cell adhesion, signaling, directed migration, cell division, cell fate determination and barrier function. These specialised domains contain concentration gradients of proteins differentiating the cell surface into basal, lateral and apical domains; facing respectively the extracellular matrix (ECM), adjoining cells and the outside environment (schematically represented in Figure 1).

Cell-cell interactions, functions and signaling

Cell-cell junctions form part of the lateral surface domain which segregates apical and basal plasma membrane constituents[1], and act as a physical barrier in regulating substance flow across the tissues. Throughout the developmental process of higher organisms, formation and remodeling of cell-cell junctions occurs repeatedly[2]. The main cell-cell anchorage systems are tight junctions (TJs), adherens junctions (AJs) and desmosomes, which are distinguished from one another structurally and based on molecular composition.

TJs

TJs are seen in various cell types like epithelial, vascular endothelial, mesothelial, schwann, oligodendrocytes and sertoli[3]. In mammalian cells TJs appear at the apical end of the lateral intercellular membrane, whereas in invertebrate-like insect epithelial cells their counterpart septate junctions are located in the basal domains. TJs also demarcate the boundary between the apical and basolateral membrane domains of a cell and serve as a semipermeable barrier to the transport of ions, solutes and water[3]. TJs are multi-protein complexes that can be subdivided into integral membrane and cytoplasmic plaque proteins. Junctional adhesion molecules (JAMs), occludin, claudins, tricellulin and marvelD3 are integral proteins that mediate cell-cell adhesion[4], while ZO-1, -2, -3 and cingulin are cytoplasmic proteins that connect membrane proteins to the cytoskeleton[5]. Occludin, tricellulin and marvelD3 contain a Marvel (MAL-related proteins for vesicle trafficking and membrane link) domain. Claudin, occludin, tricellulin and marvelD3 are tetra-spanning proteins with both N and C termini in the cytosol, and which possess two extracellular loops. JAMs belong to the immunoglobulin (Ig) superfamily and consist of two extracellular Ig domains, a single transmembrane domain and a C-terminal cytoplasmic domain containing a PDZ-interacting domain. Alterations in epithelial TJs have been reported to contribute to a variety of pathologies including inflammation and cancer, yet a complete mechanistic understanding of these phenomena remains elusive. For a more comprehensive review of this topic, the reader is directed to the following reviews[6,7].

AJs

AJs are complexes that form the initial link between neighbouring cells in a developing tissue barrier, and thereby make important contributions to embryogenesis and tissue homeostasis[8]. The core structural components of AJs are Nectin and Cadherin family cell adhesion molecules, which are linked to the actin cytoskeleton through their binding proteins afadin and catenins[9,10]. Cadherin extracellular domains form calcium-dependent homophilic interactions between neighboring cells[11], while their cytoplasmic domains interact with three distinct known catenins (α, β, γ)[10]. Nectins (1-4) are calcium-independent adhesion proteins with three IgG like loops in their extracellular domains and a cytoplasmic domain containing a C-terminal PDZ binding motif[10,12].

Desmosomes

Desmosomes are intercellular junctions that link intermediate filaments (IFs) to sites of intercellular adhesion, facilitating the formation of supracellular scaffolding that distributes mechanical forces throughout a tissue[13]. They are observed particularly in tissue types that experience mechanical stress, such as the intestinal mucosa, gallbladder, uterus and oviduct, liver, pancreas, stomach, salivary and thyroid glands and the epithelial cells of the nephron, but are most abundant in the skin and myocardium[14,15]. The components of desmosomes come from three gene families: Desmosomal cadherins (desmogleins and desmocollins), the armadillo family of nuclear and junctional proteins (plakoglobin and plakophilin 1-3) and finally the plakins (desmoplakin,

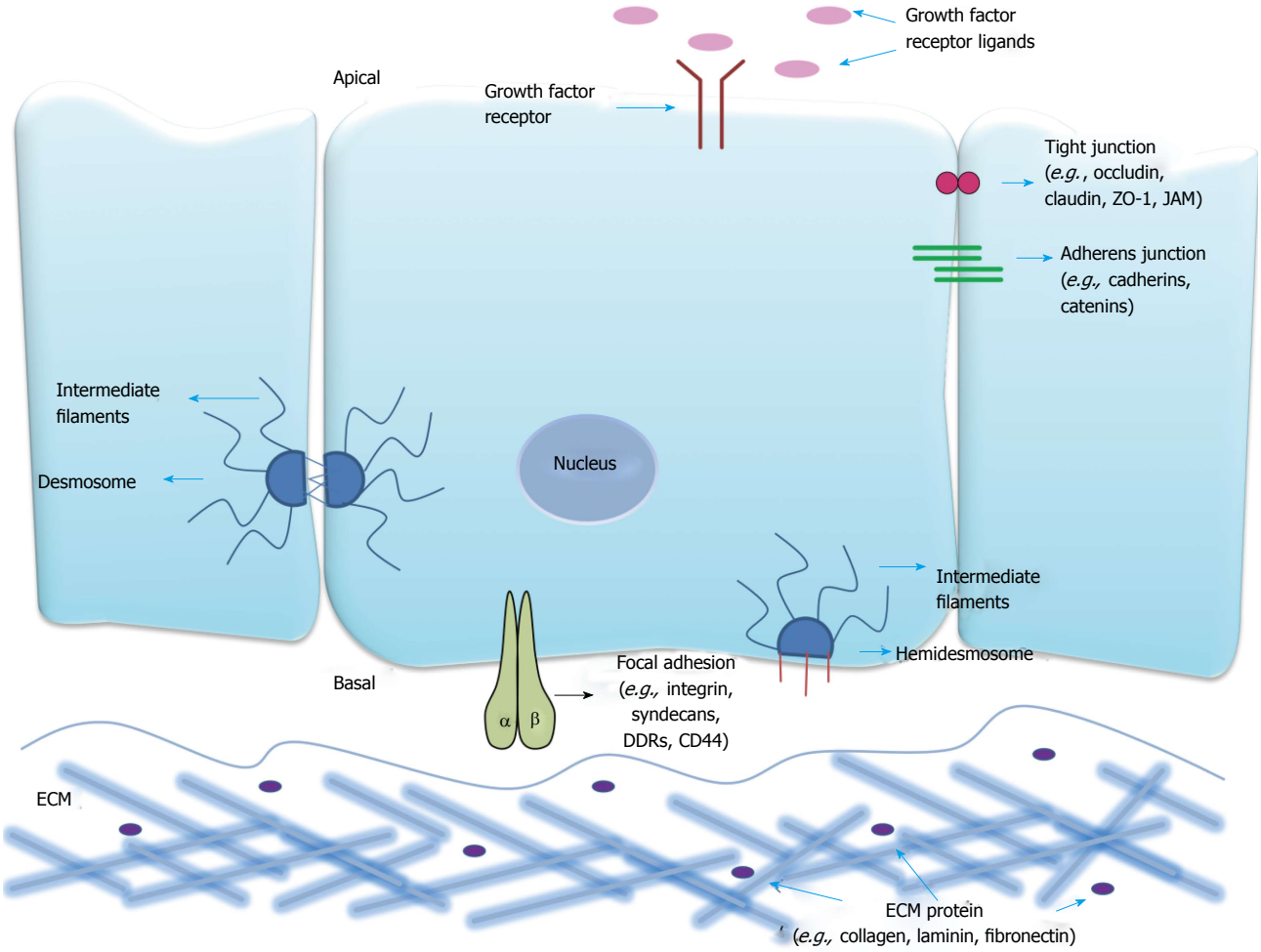


Figure 1 Epithelial cell-cell and cell-matrix junctions. Schematic drawing of key epithelial cell-cell and cell-matrix junctions, with representative constituent proteins listed as discussed in the manuscript text. ECM: Extracellular matrix; DDRs: Discoidin domain receptors; JAM: Junctional adhesion molecule.

plectin, envoplakin, periplakin)[16]. The cytoplasmic tails of desmosomal cadherins provide binding platforms for the armadillo family members. The plakin family members in turn link the stress-bearing IF cytoskeleton to specialized regions of the plasma membrane[13]. This interaction of IFs with desmosomes propagates the tensile strength imparted by the IF cytoskeleton across entire tissues, and is essential for tissue integrity[17].

Cell-matrix interactions, functions and signaling

Interactions between cells and the ECM are important in the overall architecture and maintenance of tissue integrity[18]. During development, these interactions play key roles in tissue morphogenesis, cellular polarisation and epithelial cell attachment[19,20]. Cellular interactions with the ECM rely upon interactions between ECM proteins, focal adhesion receptors and signaling molecules, including those of the cytoskeleton, to form multifaceted adhesion complexes. We refer interested readers to a recent review which discusses these complexes in further detail[21].

A plethora of cell-matrix proteins exist and are dynamically regulated depending on the tissue location, organisation, structure and components of the tissue microenvironment. This in turn dynamically regulates cellular responses[18]. Communication between cells and the ECM is mediated by transmembrane focal adhesion receptors including syndecans, discoidin domain receptors (DDRs), CD44 and integrins.

Syndecans are heparin sulfate proteoglycans that span the plasma membrane. Syndecans have been shown to play a role in multiple cellular processes including migration, differentiation and organisation of the ECM and cytoskeleton, features which are attributed to the presence of glycosaminoglycan chains in the extracellular domain[22].

DDRs are members of the receptor tyrosine kinase (RTK) family with collagen-specific ligands[23]. Collagen binds to DDR causing tyrosine phosphorylation and consequent activation of downstream signaling pathways. While the specific mechanisms of action of DDRs are still being elucidated, DDRs have been identified as important receptors in regulating cell polarity, adhesion, migration and invasion[24]. In addition, DDRs have been suggested as negative regulators of integrin signaling[25].

CD44 is a glycoprotein and member of the hyaluronate family of receptors. It mainly binds the ECM protein hyaluronan but can also bind other ECM proteins

including laminin, collagen and fibronectin[26]. CD44 is known to regulate cellular growth, differentiation, survival, migration and adhesion through different mechanisms including direct ligand binding, formation of co-receptors with RTKs and binding to ERM (ezrin, radixin and moesin) proteins thereby forming a link between the membrane and actin cytoskeleton[27].

Integrins are heterodimeric transmembrane receptors composed of α and β subunits that are considered the main matrix adhesion receptors mediating intracellular, cytoskeletal and ECM interactions[28]. Integrins can signal bidirectionally across the membrane, whereby intracellular signals can cause extracellular changes ("inside-out" signaling) and extracellular signaling can cause intracellular changes ("outside-in" signaling)[29]. Intracellular signaling *via* integrins is mediated through different signaling mediators including focal adhesion kinase (FAK), mitogen-activated protein kinases (MAPK) and members of the Rho-GTPase family[30]. Extracellular ligands for integrins include collagens, laminins or RGD-containing proteins[31]. Integrins regulate numerous important cellular features including migration, proliferation, survival and differentiation[32].

ECM remodelling is an important feature influencing cell to cell communication, a process which is regulated by degradation and modification of components of the ECM. Matrix metalloproteinases, ADAMTS (a disintegrin and metalloproteinase with thrombospondin motifs) and serine proteinases play a major role in this process to maintain homeostasis within the ECM[33].

CELL-CELL AND CELL-MATRIX CROSSTALK

Non-RTK signaling

Signaling crosstalk is an interaction between two or more independently-initiated signaling pathways, the outcomes of which include the amplification or attenuation of the signal. In the context of crosstalk between cell-cell and cell-matrix adhesive signaling, it is intriguing to note that both sites contain not only structural proteins but also signaling effectors which fit the bill as potential mediators of crosstalk. Notably, a number of non-RTKs are localized to cell-cell and cell matrix adhesions and act as key players regulating crosstalk in the context of adhesive networks[34-36]. Furthermore, tyrosine kinase signaling is one of the most frequently deregulated signaling pathways in solid tumors, and has been well established to contribute to the initiation and progression of tumors[37].

Non-RTK proteins in conjunction with cell-cell and cell-matrix adhesion are crucial effectors of a broad range of stimuli, and mediate activation of signal transduction events regulating cell survival, morphology and migration of malignant and non-malignant cells. One of the key molecules in this respect is the non-RTK Src, activation of which has dual functions in regulating formation of cell-cell and cell-matrix adhesions. Src is recruited and activated upon E-cadherin ligation and this provides a positive feedback loop that signals through Phosphoinositide-3 Kinase (PI-3 kinase) to promote the stability of cell-cell contacts[36]. However, constitutively active Src disrupts cell-cell contacts and alters cell morphology[38], and its presence at integrin-matrix adhesions leads to peripheral accumulation of activated myosin, leading to disruption of cell-cell junctions[39]. Recent studies have focused on Src-dependent regulation of E-cadherin and other tumor progression-related events such as epithelial to mesenchymal transition (EMT) with the development of metastasis. In pancreatic adenocarcinoma, c-Src (Src) is frequently activated and controls tumor progression[40,41]. The amount of E-cadherin protein in a cell plays an important role in tumor progression to invasive, metastatic carcinoma[41,42]. It was shown that Src downregulates E-cadherin and induces EMT, favoring metastasis in pancreatic adenocarcinoma[41]. In breast carcinomas, complete loss of E-cadherin may be an important step in the formation of lobular carcinoma *in situ*, as a precursor of invasive lobular breast cancer[43]. In colon cancer, Src-induced deregulation of E-cadherin has been shown to require integrin signaling[44]. Another study showed how the ECM environment controls tumor growth[45]. Specifically, in pancreatic cancer cell lines with high metastatic potential, E-cadherin expression levels were normal and cells possessed functional E-cadherin/catenin complexes. When the cells were cultured on type Ⅰ or Ⅲ collagen, E-cadherin gene expression was repressed and E-cadherin and catenin protein concentrations were reduced. In contrast, growth on fibronectin or collagen Ⅳ had no effect on the level of E-cadherin. Overexpression of activated c-Src in pancreatic cancer cells mimicked collagen-induced E-cadherin down-regulation and altered cell migration[45]. In non-transformed mammary epithelial cells PI-3 Kinase-Rac1-c-JNK signaling has been associated with collagen Ⅰ -induced cell scattering, up-regulation of N-Cadherin and consequent EMT[46]. It has been reported that other members of the Src kinase family mediate crosstalk between cell-cell and cell-matrix junctions, including the proto-oncogene c-Yes which is known to regulate epithelial tight junction formation by phosphorylation of a key tyrosine residue in occludin[47].

Further illustrating the potential importance of Src signaling in cell-cell to cell-matrix site crosstalk, the Src substrate FAK, itself a non-RTK, has also been shown to act as a downstream effector of integrin adhesion and signaling. By virtue of its binding to intracellular regions of β-integrin subunits, FAK plays a pivotal role as a signal integrator downstream of cell-ECM interactions and other receptor and non-RTKs[48]. Furthermore Src-FAK signaling promotes E-cadherin internalization during cancer progression, thus facilitating EMT and enhanced tumor cell motility[49]. In other cell types, as is the case with KM12C colon cancer cells that have retained E-cadherin, FAK can also have a negative influence on cadherin-mediated intercellular adhesion[44].

The non-RTKs Fes/Fer also regulate cell adhesion

and cytoskeletal reorganization through the modification of AJs[50]. Fer is involved in communication between cell-cell and cell-matrix junctions by shuttling between N-cadherin-containing AJs and β1-integrin focal adhesion (FA) complexes[51]; and can be activated by either cadherin or integrins[34,52,53]. Fer phosphorylates cadherin-associated cortactin and this process is involved in mediating intercellular adhesion strength[54]. In breast cancer Fer has been speculated to promote metastasis by regulating α6 and β1 integrin-dependent cell adhesion[55].

Given the fact that a number of non-RTKs mediate crosstalk between cell-cell and cell-matrix adhesion sites, any deregulation in the signaling network could have profound impacts on cell-cell and cell-matrix adhesion favoring malignancies.

RTK signaling

Several growth factor RTKs have also been reported to regulate cell-cell and cell-matrix adhesion both in cancer and normal cells. Among those, Ephrin receptors have been implicated in crosstalk with β-integrins in prostate cancer cells to strengthen adhesion to collagen type Ⅰ and preferentially metastasize to collagen Ⅰ -enriched bone tissue[56]. During breast cancer development, cancer cells metastasize to distant organs and interact with ECM and stroma to survive and remain dormant[57]. Another RTK which has been linked to adhesive crosstalk is the fibroblast growth factor (FGF) receptor. Specifically, estrogen-dependent breast cancer cells have been shown to be partially re-differentiated by FGF-2, which initiates upregulation of integrin α5β1 lost during malignant transformation[58]. Dual signaling by FGFR through PI3K and independently through integrin α5β1 induces activation of FAK and membrane localization and activation of the small GTPase RhoA and GRAF, a GTPase-regulator associated with FAK. This results in inactivation of RhoA, cortical rearrangement of F-actin and consequent switching of cancer cells to a dormant phenotype that promotes cancer cell survival[59]. In normal cells RhoA is essential for the assembly and function of epithelial cell-cell junctions whereas overexpression of RhoA in rat hepatoma cells induces an invasive phenotype[60]. Apart from this, FGFR signaling promotes cell adhesion in pancreatic cancer cells. N-CAM stimulates β1-integrin-mediated cell-matrix adhesion by activating FGFR signaling and could be a potential mechanism for preventing dissemination of tumor cells[61]. Activation of another growth factor receptor, that for EGF, prevents disruption of TJs in colon cancer cells. Specifically MAPK has been shown to interacts with occludin and mediates EGF-induced prevention of TJ disruption following hydrogen peroxide exposure[62]. Finally, the IGF1 receptor forms a ternary complex with E-cadherin and αv-integrins at cell-cell contacts in cultured colon cancer cells and human normal colonic mucosa. IGF1 binding to this receptor disrupts the complex and causes relocalisation of αv-integrins to focal contacts in conjunction with an increase in cell migration[63].

RTKs are known to play key roles in regulating several biological processes, and crosstalk with cell adhesion molecules is important in the context of normal development. However a large body of evidence from different laboratories suggests that such cooperation between cell-cell, cell-matrix and RTK signaling regulates cellular behaviors that are important for cancer progression.

Rho GTPases and signaling

Rho GTPases (guanosine triphosphatases) comprise a family of proteins within the Ras-like small G protein family, and include the molecules Rho, Rac, and Cdc42[64]. Rho GTPases play an important role in maintaining cell structure and polarity through their critical influence on dynamics of the actin cytoskeleton, thereby regulating cellular processes including cell adhesion and coordinating cell-cell and cell-matrix adhesion complexes[65]. Increased expression of Rho GTPases has been linked to many different cancer types including breast, lung, colorectal, testicular and prostate cancer[66-70]. Aberrant signaling of Rho GTPases has been attributed to promoting features of tumorigenesis including migration, invasion, cell survival, angiogenesis and metastasis[71]. As Rho GTPase molecules play an important role in regulating actin dynamics, cell adhesion and signaling processes, both at the cell-cell and cell-matrix level, it poses the question whether Rho GTPases mediate crosstalk between cell-cell specific and cell-matrix specific proteins and pathways, with particular reference to their involvement in cancer.

Rho GTPases are active when bound to GTP and inactive when bound to GDP, and a range of regulatory factors that dynamically influence the exchange kinetics of such reactions can themselves be signal control points or drug targets in cancer. These include the families of guanine nucleotide exchange factors which promote increased binding of GTP and therefore increase Rho GTPase activity[72]. In contrast, GTPase activating proteins inactivate GTP signaling by inducing GTP hydrolysis[72,73]. The switch between membranous active GTP-bound GTPases and cytosolic inactive GDP-bound GTPases is regulated by guanine dissociation inhibitors or GDI-like proteins[73].

Previous studies in our laboratory have shown a link in expression between the TJ protein JAM-A and the focal adhesion molecule β1 integrin in breast cancer tissues[74]. Further investigations to tease out the potential molecular crosstalk between JAM-A and β1 integrin identified the Rho GTPase Rap 1 as an important intermediary in controlling breast cancer cell migration[75]. Attenuation or knockdown of JAM-A in MCF7 breast cancer cells reduced the expression of β1 integrin along with αv and α5 integrins, resulting in reduced cell migration. The data presented in this study suggests a mechanism whereby activation of Rap1 by

JAM-A occurs indirectly though association of JAM-A with the downstream signaling molecules Afadin (AF-6) and PDZ-GEF2, the guanine exchange factor[75]. Notably this mechanism was previously identified in SKCO-15 colonic epithelial cells, whereby loss of JAM-A, Afadin or PDZ-GEF2 resulted in decreased Rap1 and β1 integrin expression in addition to decreased cellular migration[76].

Another mechanism through which Rho GTPases could potentially influence crosstalk between cell-cell and cell-matrix junctions is *via* coincident inputs from structural proteins located at both sites. One such example is the cytoskeletal protein vinculin, which is recruited to (and localises at) both cell-cell and cell-matrix junctions[77] with a higher affinity for cell-matrix than cell-cell adhesion sites[78]. At the cell-cell junction, vinculin stabilises E-cadherin through binding with β-catenin[79] and strengthens actin binding by binding with α-catenin[80]. At the cell-matrix junction vinculin plays an important role in mediating cell signaling between cytoskeletal and ECM proteins *via* its role in focal adhesion formation[81]. Activation of vinculin at the cell-matrix junction requires binding to several proteins including talin and paxillin[81,82] with interaction of active vinculin and paxillin being dependent on Rac1 activation[83]. Additionally, active vinculin is important with respect to cell-matrix adhesion *via* stabilising adhesion complexes and promoting integrin clustering[84]. Vinculin has also been implicated in facilitating membrane protrusion *via* extension of lamellipodia and cell spreading, following binding and recruitment of Actin-related protein (Arp) 2/3 to sites of integrin clustering[85]. Of interest, the formation of the vinculin/Arp 2/3 complex was found to be regulated by PIP2 (phosphatidylinositol-4,5-bisphosphate) and Rac1[85]. While research to date has mainly investigated the role of vinculin in the cell-matrix context, further investigation is warranted to investigate if Rac1 can directly regulate vinculin functionality at the cell-cell junction, thereby offering a potential common pathway through which vinculin can elicit its functions and mediate crosstalk between adhesion sites.

MAGI-1 (MAGUK with inverted domain 1) is a scaffolding protein containing a PDZ domain which has been shown to regulate structural and functional features at the cell-cell junction and to increase cell adhesion to matrix proteins[86,87]. Interestingly, MAGI-1 is required for vascular endothelial-cadherin-dependent Rap-1 activation, which, upon activation facilitates relocation of vinculin from the cell-ECM location to the cell-cell location thereby regulating AJ formation[88]. MAGI-1 is known to stabilise E-cadherin and β-catenin localisation at the AJ, which in turn increases actin stress fiber and focal adhesion formation. MAGI-1 has been implicated as a putative negative regulator of metastasis in a colon cancer model, whereby overexpression of MAGI-1 was shown to increase integrin-mediated cell adhesion to matrix proteins and outside-in signaling *via* FAK, ERK 1/2 and Akt, and to inhibit anchorage-independent growth, invasion and migration in addition to Wnt signaling[87]. This study proposes a mechanism whereby inhibition of cyclooxygenase-2, which is overexpressed in a number of cancers (including colon), upregulates MAGI-1 which in turn inhibits Wnt/β-catenin signaling by decreasing free β-catenin[87]. As such MAGI-1 identifies itself as an important regulator of cell-cell and cell-matrix functions and signaling.

Nectins have been identified as important molecules capable of co-operatively communicating between cell-cell and cell-matrix junctions *via* their role in integrin-cadherin crosstalk. Nectins are Ig-like cell adhesion molecules which regulate the formation of cell-cell junctions, including TJs and AJs[12]. Nectins have been shown to regulate cellular features including cell polarity, proliferation, movement and survival[89], the deregulation of which is implicated in cancer. Nectins can physically interact with the αvβ3 integrin at cell-cell adhesion sites, leading to activation of Src kinase which is required for the formation of cadherin-based AJs[90]. Src is also activated by nectins following nectin-nectin interactions at cell-cell contact sites, which induces activation of the GTPase Rap1, resulting in downstream activation of the other GTPases Cdc42 and Rac[90]. Cdc42 activation results in filopodia formation, while Rac activation leads to lamellipodia formation[91], important structures for cell migration and movement[92]. This identifies a potential mechanism whereby activation of Rap1 by nectin-based cell contact can influence actin dynamics at the cell-matrix interface, which in turn regulate functional behaviours associated with migration. Additionally, interactions between integrins and Rho GTPases can elicit pro-migratory functions, as integrins are known to activate the Rho GTPases Rac and Cdc42[93] with Cdc42 being shown to drive trans-endothelial migration of cancer cells by enhancing the expression of β1 integrin[94], an important process in metastasis. The versatility of Rho family GTPases in driving diverse signaling events clearly illustrates their potential to influence mammalian physiology and pathophysiology. However an intriguing aside is that mammalian Rho GTPase signaling is also commonly exploited for non-mammalian advantage, specifically in invasion or intoxication strategies of multiple bacterial pathogens. Interested readers are referred to an excellent recent review on this topic[95], while the next section of this manuscript will instead concentrate on the concept that pathogenic interactions with mammalian cells represent a paradigm of successful crosstalk between cell-cell and cell-matrix adhesion sites.

Pathogenic infection as a paradigm for inter-junctional crosstalk

By virtue of their strategic bridging location between the apical and basolateral membrane domains of epithelial cell surfaces, it has been well-established that TJ proteins are attractive targets for pathogens seeking to infect cells or to modify cellular functions towards a survival advantage. Among those TJ components known to be directly exploited as viral pathogen receptors or co-receptors are JAM-A (reovirus[96], rotavirus[97]) and

CAR (adenovirus 5, group B coxsackieviruses[98]), but many non-viral pathogens are also recognised to use TJ disruption as a broader primary mechanism to open up access to basolaterally-located attachment receptors (*e.g*., *Yersinia*[99]) or to orchestrate signaling events that drive disease [*e.g*., *Helicobacter pylori* (*H. pylori*)[100]]. An emerging literature is revealing that pathogens are masters of inducing crosstalk between epithelial cell-cell and cell-matrix adhesive interfaces to facilitate their own goals, of which various examples will be discussed herein.

In the case of viral infection of epithelial barriers, cell entry is a key requirement, and abundant examples illustrate cooperativity between cell-cell and cell-matrix sites to facilitate an attachment-invasion sequence. JAM-A has been described to act as an attachment receptor for the *Reoviridae* family member rotavirus *via* its VP4 spike protein[97], in conjunction with integrins acting as post-attachment receptors for the same non-enveloped virus[101]. Furthermore gene silencing screens have identified a potential role for not just JAM-A but also claudins-6, -9 and -14 in mediating rotaviral infection of cells[102]; which fits with the observation that a component of the VP4 spike (VP8) has sequence homology to the extracellular loops of claudin and can diminish TJ gate and fence function[103]. Compromising the paracellular barrier in this fashion would confer a distinct advantage upon the ability of the rotavirus to access basolaterally-located integrin co-receptors; which it could then bind *via* opportunistic expression of integrin-recognition tripeptide sequences in its outer capsid[104]. Accordingly, multiple viruses including the human cytomegalovirus[105] have been demonstrated to possess classic integrin binding sequences.

Another *Reoviridae* family member which has been shown to orchestrate crosstalk between cell-cell and cell-matrix sites to facilitate infection (and thereby survival) is the reovirus. Specifically, reovirus uses JAM-A as its cellular attachment receptor[96], whereupon β1-integrin is required for viral internalization *via* interactions with RGD/KGE sequences on the λ2 reovirus protein[106]. It is interesting to note that that the reovirus attachment protein sigma1 can, *via* a higher affinity interaction, substitute for one molecule of JAM-A in the homo-dimerisation event which characterises the adhesive function of JAM-A[107]. This could have implications for the integrity of the epithelial barrier which would, in turn, modify pathogen access to the normally restricted sub-epithelial compartment.

The final example of virus-induced adhesive crosstalk which will be discussed here is that of coxsackie and adenoviruses. A protein member of the Ig superfamily which is structurally related to JAM-A, namely coxsackie and adenovirus receptor (CAR), has been identified as the attachment receptor for both adenovirus-5 (Ad5) and group B coxsackieviruses[98]. However it has been demonstrated that post-attachment internalization of adenovirus depends upon interactions between virally-expressed RGD sequences and alpha-V integrins[108]. Accordingly, CAR overexpression can modify the activation status of integrins *via* activation of p44/42 MAP kinase, which concomitantly enhances cell-matrix adhesion and Ad5 infection[109]. Intriguingly, a feedback loop whereby Ad5 infection also promotes CAR dimerization and further MAPK-dependent viral infection[109] supports the assumption that infection dynamics are critically regulated by crosstalk between cell-cell and cell-matrix sites. Given that both CAR and integrin expression levels are sensitive to modulation by certain cytokines[110], it is likely that the specific environmental milieu of a tissue can influence not only adhesive crosstalk but consequently the likelihood of infection.

Mechanisms of infection differ somewhat in the context of bacteria, which, unlike viruses, are not entirely dependent on cellular invasion for their propagation. However a recurring theme is that bacteria often disrupt cell-cell junctional barriers to access basolaterally-located receptors, facilitating injection of bacterial proteins into host cells. One such example is *Yersinia pseudotuberculosis,* which has been shown to attach to a pool of β1-integrin that co-localizes with ZO-1 at TJ of renal epithelial cells[111]. The seemingly incongruous spatial overlap of a cell-cell and cell-matrix protein may, in this case, be explained by disruptions in F-actin and the redistribution of actin-affiliated cell-cell adhesion proteins following cellular penetration of the cytotoxic *Yersinia* outer membrane protein YopE[111]. Alternatively an environment of acute inflammation could promote access to basolaterally-located β1-integrin, as it has been demonstrated that colonic epithelial cells become sensitized to *Y. pseudotuberculosis* infection following the induction of micro-discontinuities in sites where neutrophils have trans-migrated across the epithelial barrier[99]. A pro-inflammatory cytokine balance could also perturb cell-cell adhesion and barrier function in a manner that permits pathogen access to basolateral receptors, as it has been demonstrated that IFNγ causes internalization of the tight junction protein occludin *via* macropinocytosis[112]. Interestingly, selective macropinocytosis of occludin has been illustrated downstream of Coxsackievirus B binding to CAR at TJ, with a block on viral entry in the absence of either occludin or a functional macropinocytotic pathway[113]. The nature of occludin as a common target for some bacterial and viral infections is further evidenced by a report that the *E. coli*-derived bacterial toxin cytotoxic necrotizing factor-1 (CNF-1) induces occludin and ZO-1 internalization *via* an uptake pathway involving caveolar lipid rafts[114]. In light of the crosstalk mediators alluded to in previous paragraphs, it is no coincidence that CNF-1 simultaneously activates Rho, Rac and Cdc42 in conjunction with impairment of epithelial barrier function and putative activation of focal adhesion assembly[114].

The final example of bacteria inducing inter-adhesion site crosstalk which will be dealt with in this review is that of *H. pylori*, the causative organism of peptic ulcer disease and itself a Group Ⅰ carcinogen. The *H.*

pylori effector protein CagA has been shown to disrupt TJ integrity by way of forming a complex with JAM-A and ZO-1[100]. Furthermore another gene in the *cag* pathogenicity island, CagL, reportedly binds to αV-β1-integrin *via* an RGD sequence, simultaneously activating FAK and Src activation whilst facilitating CagA entry into cells[115]. Taken together, such examples of cooperativity between cell-cell and cell-matrix adhesion sites make it intriguing to speculate that the crosstalk mechanisms which pathogens have evolved could be exploited for therapeutic advantage in not only infection but also cancer contexts.

THERAPEUTIC TARGETING OF PUTATIVE CROSSTALK MEDIATORS

Kinases as drug targets in cancer

Tyrosine kinase signaling is frequently deregulated in both solid and haematological tumors, and has been solidly linked to tumor initiation and progression. Both receptor and non-RTKs could mediate crosstalk signals between cell-cell and cell-matrix adhesion sites. In cancer cells RTKs are often constitutively active, and drug-based targeting of RTKs will only be successful if the targeted RTK is a major regulator of cancer cell survival. The most commonly deregulated RTKs include EGFR, HER2, MET, VEGFR, PDGFR, FGFR and IGF-1R[116]. Accordingly several monoclonal antibodies and small-molecule inhibitors have been developed to target these receptors. Since EGF receptor family members are frequently overexpressed in breast cancer patients[117,118], monoclonal antibodies like Trastuzumab (Herceptin), pertuzumab (Omitrag) and small-molecule inhibitors like lapatinib, gefitinib, erlotinib, AEE788 and CI-1033 have formed prototype targeted therapies[119]. For more detailed information on EGF receptor family members and their inhibitors, interested readers are directed to the following review article[120]. Further monoclonal antibody (pertuzumab) and small-molecule inhibitors (AEE788 and CI-1033) have been approved for clinical testing in various cancers like ovarian, prostate, and non-small cell lung cancer (NSCLC) whereas small molecule inhibitors (gefitinib and erlotinib) have been approved for treating NSCLC.

Another attractive option for targeted therapy in cancer has been the non-RTK Src, whose expression is known to be elevated in several tumors and has been implicated in disease progression, invasion and metastasis. Among the non-RTKs Src has been one of the most widely studied, encompassing its family members Lyn, Fyn, Lck, Hck, Fgr, Blk, Yrk, and Yes. To date several Src inhibitors such as dasatinib, SKI-606 (Bosutinib), and AZD0530 have been developed and are undergoing clinical trials in breast cancer patients[121-126]. Dasatinib is an effective inhibitor of several kinases including Bcr-abl, Src family kinases, c-KIT and PDGFR-β5[127], with promising preclinical results indicating its ability to inhibit the growth of breast luminal and basal tumors independently of ER or HER2 status[128,129]. Analogously, SKI-606 inhibits several Src family kinases and Bcr-Abl and suppresses migration and invasion of breast cancer cells[130]. AZD0530 (Saracatinib) is a more selective inhibitor of Src and Bcr-Abl in gastric and prostate cancer[131,132], and in breast cancer (when combined with estrogen blockers) has been shown to inhibit proliferation of cancer cells *in vitro* and *in vivo*[133].

Since both receptor and non-RTKs are frequently overexpressed in tumors and play key roles in signaling crosstalk, this section has highlighted the logic of targeting their signaling for downregulation as a mechanism of stalling cancer progression. However it must always be remembered that cancer is a complex disease resulting from deregulation of several signaling pathways, whether concurrently or sequentially, therefore long-term therapeutic strategies must always consider targeting multiple signaling pathways for the most effective outcome.

Rho GTPases as drug targets in cancer

Activation of Rho GTPases and their interactions with multiple different molecules can initiate many signaling pathways and downstream effects, including cancer-related behaviours such as migration, proliferation, cytoskeletal changes and apoptosis[134]. As such, Rho GTPases present themselves as attractive therapeutic targets, and many endeavors have been made to target these molecules in cancer, with promising preclinical results so far[134]. A Rac-specific small molecule inhibitor, NSC23766, has been found to inhibit features of invasion and anchorage-independent growth in human prostate cancer PC-3 cells[135]. Additionally, this compound was also found to inhibit breast cancer cell growth *via* cell-dependent mechanisms, either through cell cycle arrest or apoptosis[136]. Since then, several derivatives of this Rho GTPase inhibitor have been developed including AZA1 and AZA197.

A Cdc42 and Rac1 GTPase inhibitor, AZA1, a modified derivative of NSC23766, has been shown to inhibit migration and proliferation in addition to promoting apoptosis in prostate cancer cell lines *in vitro*[137]. This study also demonstrated increased survival and a reduction in tumor growth in a 22Rv1 androgen-independent xenograft model of prostate cancer[137]. In addition, another modified derivative of NSC23766 and a Cdc42 inhibitor, AZA197, has been shown to inhibit proliferation, migration and invasion in addition to exhibiting pro-apoptotic functionality in human colon cancer cell lines *in vitro*[138]. In this study, AZA197 also inhibited proliferation and promoted apoptosis while decreasing tumor growth and increasing survival in a human colon cancer xenograft mouse model[138].

Additionally, two Rac specific inhibitors, EHT 1864 and EHop-016, have shown anti-tumorigenic properties in breast cancer. EHT 1864, a Rac-specific inhibitor, was shown to inhibit estrogen-mediated breast cancer cell proliferation and to decrease growth

of tamoxifen-resistant breast cancer cells[139] thereby identifying potential subtypes of breast cancer which this Rho GTPase inhibitor may preferentially target. In another study, EHT 1864 was also shown to have anti-proliferative, anti-invasive and pro-apoptotic effects in breast cancer cells[140]. The small molecule Rac inhibitor, EHop-016, has also shown promising preclinical results in targeting Rac activity in breast cancer. In one study, treatment with EHop-016 resulted in reduced tumor growth, inhibition of metastasis and angiogenesis in addition to inhibiting proliferation and increasing apoptosis in MDA-MB-435 breast cancer cells[141].

Rho GTPases have been shown to play a role in cell signaling and functions critical to tumor formation and metastasis. Interestingly, Rho GTPases, as discussed in this review, have been shown to interact with and activate molecules present at both the cell-cell and/or cell-matrix locations and may provide mediators of crosstalk between these cellular locations. As demonstrated in different cancer models, inhibition of Rho GTPases suggests a novel and potentially beneficial approach in cancer treatment and in the prevention of metastasis. It is interesting to speculate that targeting specific Rho GTPases which function at both the cell-cell and cell-matrix junctions may indeed offer enhanced inhibitory effects and present potential advantages over single site-directed inhibitors.

Pathogenic mechanisms as drug targets in cancer

We have already illustrated that enzymes (such as kinases and GTPases) offer huge potential as cancer drug targets by virtue of their capacity to modify cell signaling, including that between cell-cell and cell-matrix junctions. However there is also an emerging literature on the concept that pathogens which bind to (or interfere with) cell-surface adhesion receptors could be used as tumor cytolytic agents, drug delivery systems or even as immunomodulators in various cancers. The simplest argument is in the use of pathogens or their derivatives as cytolytic agents. This has been well-evidenced by the interaction of *Clostridium perfringens* enterotoxin (CPE) with its cellular receptors, the tight junction proteins claudin-3 or -4, having a cytolytic effect on claudin-overexpressing breast cancer cells[142] and also brain metastases of breast primary tumors[143]. Therapeutic potential of using CPE has also been suggested for ovarian[144,145], endometrial[146] and pancreatic[147] tumors. Similarly, the JAM-A-binding pathogen reovirus has been mooted as a potential therapy for certain brain cancers, although the mechanisms of cytolysis may yet be JAM-independent[148]. Furthermore targeting the CAR (CAR; a TJ-enriched Ig superfamily member related to JAM-A) with conditionally-replicating adenoviruses has been discussed in melanoma treatment[149]. Cytolysis aside, using pathogens or modified derivatives to deliver transgenes[150] or cytotoxic molecules (akin to the "magic bullet" concept with monoclonal antibodies) is also an interesting concept. Although not without conceptual problems, some selectivity might be conferred by the careful selection of cancers in which the aforementioned pathogen receptors are known to be overexpressed. Finally the concept of immunomodulation *via* controlled pathogen attack of human cells also offers promise - from the very early observations of tumor regression in patients with certain *Streptococcal* infections (essentially a vaccination strategy involving the so-called Coley's toxin)[151] to the more recent report of differential sensitivity to infection of reoviruses expressing the co-stimulatory molecule B7-1[150]).

While a complete description of such phenomena is beyond the scope of this review, it is nonetheless intriguing to speculate that future developments in the arena of using pathogens or pathogenic derivatives to modulate cell-cell to cell-matrix adhesion site crosstalk (amongst other signaling mechanisms) may prove valuable in cancer therapeutics.

CONCLUSION

This review has endeavored to highlight pathways and signaling mechanisms of crosstalk between cell-cell and cell-matrix adhesion sites, with particular reference to the relevance of these concepts in cancer biology. Interestingly, successful crosstalk between spatially-distinct adhesion sites has been exemplified by the mechanisms which several pathogens use to invade or intoxicate epithelial barriers; and it is likely that much could be learned from such models in designing drugs to combat dysfunctional signaling crosstalk events associated with cancer progression. Some of the key putative mediators driving crosstalk between cell-cell and cell-matrix adhesion sites include Rho GTPases and kinases, hence these were concentrated upon in this review. Since Rho GTPases and kinases concurrently play a central role in tumorigenesis, we highlighted some current pharmacological strategies to target these molecules. We also alluded to the intriguing possibility of using modified bacterial or viral components as therapeutic tools to tap into crosstalk mechanisms which they have evolved mastery of. Taken together, we propose that rational strategies targeting specific regulators of cell-cell and cell-matrix adhesion may have far-reaching therapeutic potential in attenuating the signaling events associated with cancer initiation or progression.

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REVIEW

RNA-binding proteins related to stress response and differentiation in protozoa

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Abstract

RNA-binding proteins (RBPs) are key regulators of gene expression. There are several distinct families of RBPs and they are involved in the cellular response to environmental changes, cell differentiation and cell death. The RBPs can differentially combine with RNA molecules and form ribonucleoprotein (RNP) complexes, defining the function and fate of RNA molecules in the cell. RBPs display diverse domains that allow them to be categorized into distinct families. They play important roles in the cellular response to physiological stress, in cell differentiation, and, it is believed, in the cellular localization of certain mRNAs. In several protozoa, a physiological stress (nutritional, temperature or pH) triggers differentiation to a distinct developmental stage. Most of the RBPs characterized in protozoa arise from trypanosomatids. In these protozoa gene expression regulation is mostly post-transcriptional, which suggests that some RBPs might display regulatory functions distinct from those described for other eukaryotes. mRNA stability can be altered as a response to stress. Transcripts are sequestered to RNA granules that ultimately modulate their availability to the translation machinery, storage or degradation, depending on the associated proteins. These aggregates of mRNPs containing mRNAs that are not being translated colocalize in cytoplasmic foci, and their numbers and size vary according to cell conditions such as oxidative stress, nutritional status and treatment with drugs that inhibit translation.

Key words: Gene expression regulation; RNA-binding proteins; RNA-protein complexes; RNA granules; Protozoa; Stress and cell differentiation

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Core tip: RNA-binding proteins (RBPs) are numerous and widely distributed in nature. In addition to having different domains, these proteins are key modulators of gene expression and are involved in the cellular response to environmental changes, cell differentiation and cell death. In protozoa RBPs are crucial for the rapid gene expression remodeling that occurs in the course of cell differentiation or the stress response.

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INTRODUCTION

RNA-binding proteins (RBPs) are numerous and widely distributed in nature and are involved in gene expression control at all levels. There are several distinct families of RBPs. They are key modulators of gene expression and are involved in the cellular response to environmental changes, cell differentiation and cell death. The RBPs can differentially combine with RNA molecules and form ribonucleoprotein (RNP) complexes, thereby defining the function and fate of the RNA molecules within the cell.

RBPs and the RNA-binding domains

RBPs have multiple and diverse domains; many of them have a combinatorial set of RNA-binding domains (RBDs) that allow various modes of recognition to exert their function in the cell. The RBD recognizes and interacts with the RNA molecule based on sequence and/or structure. In addition to the RBD domain, many of the RBPs also have protein-protein domains that form complexes that are also subjected to intense post-translational modifications, such as methylation and phosphorylation, further increasing the complexity of the study and characterization of RBPs.

One of the most common domains found in RBPs is the RNA-recognition motif (RRM); it shows affinity with single-stranded RNA, DNA and proteins. Approximately 90 amino acid residues are involved in the recognition and binding of a target RNA sequence of 2 to 6 nucleotides. The proteins that contain this domain are involved in all pathways of the RNA cycle, from splicing to mRNA turnover. The RRM is usually present in multiple copies within a protein, and this multiplicity enhances the specificity of the ligand[1].

The zinc finger domain was first described as a DNA-binding domain (with the CCHH amino acid motif) and was subsequently described in proteins that interact with RNA (with the CCCH motif), such as Tis11 and Tristetraprolin (TTP)[2,3]. TTP is involved in transcript degradation through its interaction with the AU-rich elements in the 3'-untranslated regions (3'-UTRs) of some mRNAs, as described for tumor necrosis factor alpha and granulocyte-macrophage colony-stimulating factor mRNAs[4,5].

The Pumilio (PUF) protein takes part in regulating different processes in the cell, such as embryogenesis, development, and differentiation; however, the best-characterized function of PUF is as a post-transcriptional repressor[6,7]. The domain is characterized by a highly conserved C-terminal RBD composed of eight tandem repeats[6-10].

The hnRNP K-homology domain recognizes a specific sequence of single-stranded RNA and is found in both eukaryotes and prokaryotes. The domain is approximately 70 amino acids long, and the proteins are associated with a diversity of gene expression regulation mechanisms, such as splicing, transcriptional regulation, and translational control[6].

The Arg-Gly-Gly repeat (RGG) domain can interact with either RNAs or proteins, and this interaction can be based on the positive charge of the arginine amino acids[7]. Proteins containing RGG motifs are associated with several cellular processes, including transcription, splicing and mRNA export. One example of an RGG domain protein is the yeast Scd6 protein. It modulates translation by preventing the formation of the 48S pre-initiation complex by binding eIF4G through its RGG motif[8,9].

RBPs in protozoa

Most of the RBPs characterized in protozoa arise from trypanosomatids. Proteins such as TcDHH1[10] are involved in the formation of mRNA granules in *Trypanosoma cruzi* and *Trypanosoma brucei*, but it is believed that the function and composition of these granules can be different from those described for higher eukaryotes[11]. TcDHH1 binds to RNAs encoding stage-specific surface antigens from the infective trypomastigote forms. However, these mRNAs are also found at low levels in the non-infective epimastigote forms, leading to the hypothesis that TcDHH1 might be involved in the degradation of the targets in these forms[10,12].

Trypanosome gene expression regulation is mostly post-transcriptional, which suggests that some RBPs might display regulatory functions distinct from those described for other eukaryotes. One example is the poly-A binding proteins PABP1 and PABP2: Only PABP2 accumulates in the nucleus, indicating that PABP1 and PABP2 might be part of distinct mRNPs that associate with distinct populations of mRNAs[13]. Some proteins, such as TbDRBD3, TbPTB2, TbZPF3, TbPUF9, TbPUF1 and TbPUF2, can function as mRNA destabilization factors. However, the modulation mechanisms for most RBPs in trypanosomatids remain unknown[14].

In *T. cruzi*, the zinc finger protein TcZFP1 binds preferentially to C-rich regions[15]. However, TcZFP2 has a higher affinity for A-rich regions, and the mRNAs associated with this protein appear to be upregulated in metacyclic trypomastigotes, suggesting a possible role in parasite differentiation[16].

Some RBPs, such as TcPUF6[17] and TcUBP1[18], are involved in the downregulation of their target transcripts, while others, such as RBP19, can negatively regulate their own mRNAs[19]. Some RBPs, such as RBP42[20] and the Alba proteins[21], associate with the translation machinery. The RBP42 protein from *T. brucei* binds to the coding region of mRNAs of the insect form that encode metabolic proteins. RBP42 is an essential protein and associates with mRNAs bound to polysomes in the cytoplasm[20]. However, some RBPs, such as

TbRBP10, a cytoplasmic protein essential to the *T. brucei* bloodstream forms, appear to affect the expression of their targets indirectly through other proteins that form the mRNP complex[22].

TcRBP40 is found dispersed within the cytoplasm and is also concentrated in reservosomes, a storage organelle present in *T. cruzi* and some bat trypanosomes during a specific life cycle stage[23,24], suggesting that this organelle can also be involved in RNA processing[25]. There are also some RBPs with assigned nuclear functions, such as TbPTB2, which is involved in trans-splicing processing of some mRNAs[26]. Similarly, TbRRM1 is a nuclear RBP that associates with mRNAs from *T. brucei* and with the auxiliary splicing factor polypyrimidine tract-binding protein (PTB) 2, but not with components of the core spliceosome. A knockdown of TbRRM1 caused extensive alterations in mRNA abundance and regions enriched for the downregulated mRNAs were identified. In addition, in cells subjected to heat shock, TbRRM1 shifted from the nucleus to the cytoplasm, which led to the compaction of chromatin[27].

The *T. brucei* nucleolar RBPs PUF7 and PUF10 are involved in ribosomal RNA maturation and transcriptional control of procyclic stage-specific genes transcribed by RNA polymerase Ⅰ[28]. TcSUB2 is involved in the transcription/export pathway and is mainly localized to the nucleus[29].

In *Plasmodium*, DDX-6 class DEAD box RNA helicase (DOZI) is essential to zygote development because of its role in controlling mRNA stability[30,31]. In *P. falciparum*, both PfPuf1 (PFE0935c) and PfPuf2 (PFD0825c) are upregulated in gametocytes[32,33]. Microarray analysis detected increasing amounts of PfPuf1 and PfPuf2 mRNAs during gametocyte maturation[34]. Interestingly, the highest level of PfPuf2 expression was observed in sporozoites[35].

In *Toxoplasma gondii,* TgPuf1 displays different expression levels in tachyzoites and bradyzoites, indicating that it might function in regulating processes such as proliferation and/or differentiation that would enable the parasites to respond rapidly to changes in environmental conditions[36]. Another protein from *T. gondii* is the RGG single-strand-binding protein (TgSsossB), which interacts with the TgAlba proteins that are involved in translation regulation[37]. When the RGG domain was deleted from the protein, the mutant strain produced fewer plaques in stress conditions, a defect associated to a slow growth phenotype due to the exposure of extracellular parasites to stress. Moreover, the mutant lost its capacity to interact with the TgAlba complex[38].

RBPs and post-transcriptional control in stress

In unicellular and multicellular organisms, there is a very subtle balance between the cell's capacity to respond to stress and cell death. For the former, this balance needs to be even more strictly regulated, and there are many variables involved that take into account the strength and duration of the stimuli[39]. The stress response needs to be fast to allow cell survival, if we consider the cell' s time scale, at the moment the stress is triggered, the response starts immediately (within nanoseconds), and the cell metabolism adapts dynamically to the environment. Accordingly, there is modulation of gene expression, and trans-acting factors such as RBPs are key to this prompt response.

Post-transcriptional gene regulation occurs at different levels: Splicing, mRNA stability and translation regulation. With regard to splicing, it has been shown that when a cell is subjected to a low dose of ultraviolet radiation irradiation, the C-terminal domain of RNA polymerase Ⅱ is phosphorylated, and the splicing dynamics are altered. This alteration allows, *via* alternative splicing, the inclusion of exons with weaker splice sites[40,41]. One example of alternative splicing occurs with the MDM2 RNA during genotoxic stress. Under normal conditions, the MDM2 protein targets the p53 protein for degradation *via* ubiquitination; however, during stress, an exon is skipped in the MDM2 RNA, which ultimately results in the maintenance of p53 in the cell. This effect is only reversed upon restoration of the normal condition[42]. Splicing factors are also targets of regulation during stress. For example, hnRNP1 shuttles to the cytoplasm during osmotic stress, while the other splicing factors remain in the nucleus[43].

Extensive studies have been performed and highlight the importance of translational control during stress. For the cell, it is vital to control and arrest translation during stress, as 50% of the cell's energy is consumed during translation[44].

For most mRNAs, translation initiation occurs when the trimeric eIF4F complex binds to the transcript. This complex is formed by the protein eIF4E (the cap-binding protein), eIF4A (RNA helicase) and eIF4G (which forms the scaffold protein complex *via* its binding sites for eIF4E and eIF4A). The trimeric complex binds to the cap, followed by ribosome scanning from the RNA 5'-end to the first AUG site. Then, eukaryotic initiation factor 2 (eIF2) associates with tRNAimet, part of eIF2-GTP ternary complex that is required to bring the initiator tRNA to the ribosome[45,46]. When a stress signal is generated, two basic mechanisms are activated: The availability of eIF4E, which can be sequestered by eIF4EBPs (eIF4E-binding proteins), and the phosphorylation of eIF2α. Global translational arrest occurs, and only a few proteins are translated to help the cell overcome the stress. The most studied genes upregulated during stress are those encoding heat shock proteins (HSPs), whose transcription is stimulated by the heat shock factor that senses misfolded proteins in the cytoplasm and shuttles to the nucleus. HSPs are chaperones that help properly fold proteins[47]. Aside from HSPs, some transcripts that contain upstream open reading frames can be translated even under conditions of eIF2α phosphorylation[48,49]. The transcripts that contain internal ribosome entry sites (IRESs) on their 5'-UTRs can also bypass the inhibition of cap-dependent control (eIF4F). Interestingly, some

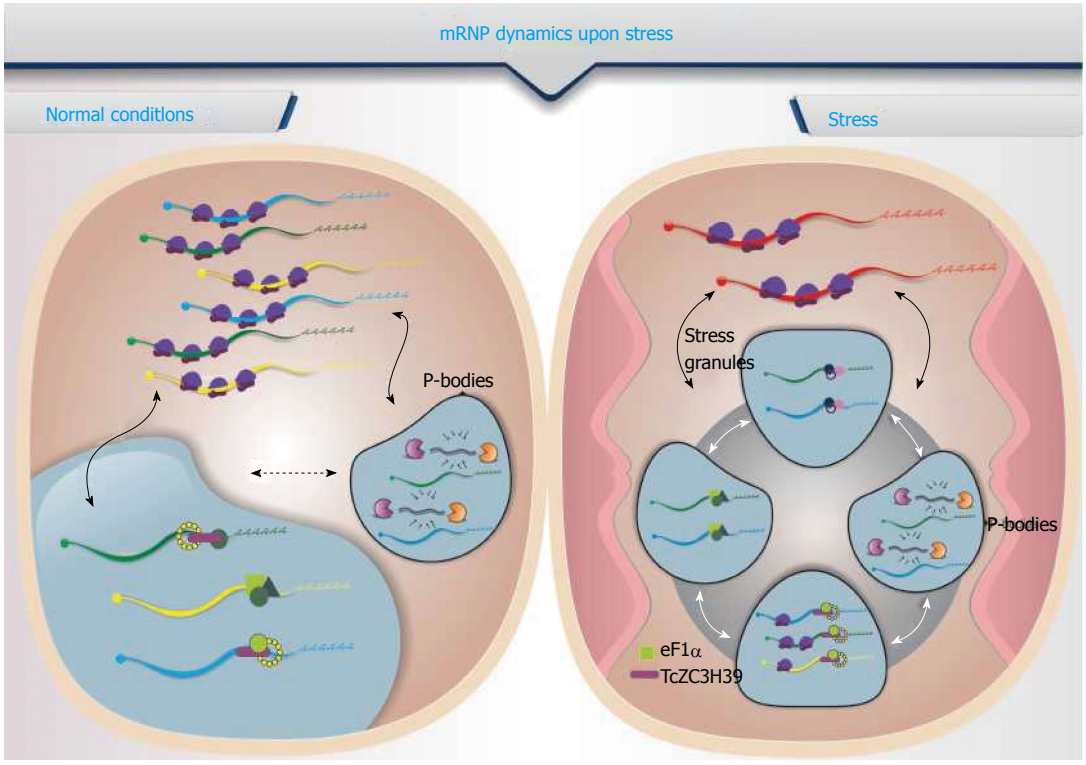


Figure 1 Messenger ribonucleoprotein compex dynamics during stress. Under normal conditions (left panel), several mRNAs exist in the cytoplasm and are normally translated. Some of these mRNAs can be degraded depending on the several translation rounds, and translation is repressed for some of them. Upon stress (right panel), most mRNAs leave the polysomes and are directed to either the degradation machinery or specific mRNP complexes (RNA granules), where they remain silent until normal conditions are restored. Few mRNAs are translated under conditions of stress. mRNP: Messenger ribonucleoprotein compex.

RBPs function as IRES-trans acting factors and interact with these IRES elements, resulting in the coordination of the subset of transcripts that will be translated[50]. For example, during stress, the PTB recognizes the IRES element in p53 transcripts and in other mRNAs associated with apoptosis and starvation and triggers their translation to respond to the stress conditions[51].

mRNA stability can be altered as a response to stress. Transcripts are sequestered to RNA granules that ultimately modulate their availability to the translation machinery, storage or degradation, depending on the associated proteins. Among the RNA granules, the processing bodies (P-bodies) are multiprotein aggregates of mRNPs containing mRNAs that are not being translated, associated with proteins involved in RNA degradation pathways and translation inhibition factors. These aggregates colocalize in cytoplasmic *foci*, and their numbers and size vary according to cell conditions such as oxidative stress, nutritional status and treatment with drugs that inhibit translation[52]. The proteins that have been identified as being involved in mRNA decapping [DCP1, DCP2, DHH1 and their activators, RCK/p54, pat1 (as part of the Lsm1-7 complex), the XRN1 exonuclease complex, and CCR4/POP2/NOT] are also found in P-bodies of mammalian and yeast cells[53-55]. Some proteins are essential for the formation of P-bodies, such as GW182 (which is involved in the route of degradation by micro RNA), RCK/p54 and Lsm4, as the absence of these proteins prevents the formation of these granules[54,56-58]. Some of the proteins characteristic of P-bodies are depicted.

There is a spatial separation between the mRNAs that are being translated from those that are associated with P-bodies. The mRNA translation status depends on the proteins associated with the mRNP complexes, which direct the mRNA molecules either to the polysomes for translation or to RNA granules for storage and/or degradation[55,59]. Observations indicate that several mRNAs present in the P-bodies undergo cap removal and are consequently prone to degradation[60,61]. However, the P-bodies may also act as mRNA storage sites, allowing mRNAs to return to the translation machinery. Experiments have shown that specific mRNAs accumulate in P-bodies in growing cells or cells under stress and that they emerge from the granules and migrate to the polysomes to be translated when normal conditions are restored[60,61].

Another type of mRNP granule found in eukaryotes is the stress granule. Unlike P-bodies, the stress granules are composed mainly of translation initiation factors, the 40 s ribosomal subunit and several RBPs[62]. Studies indicate that physical interaction occurs between the P-bodies and stress granules and that the mRNAs can switch between them[63] (Figure 1). The translation initiation complexes bound to mRNAs, which include eIF3, the eIF4F complex, eIF4B, the 40S ribosomal subunit and PABP-1, form the core of the stress granules and are considered markers of these

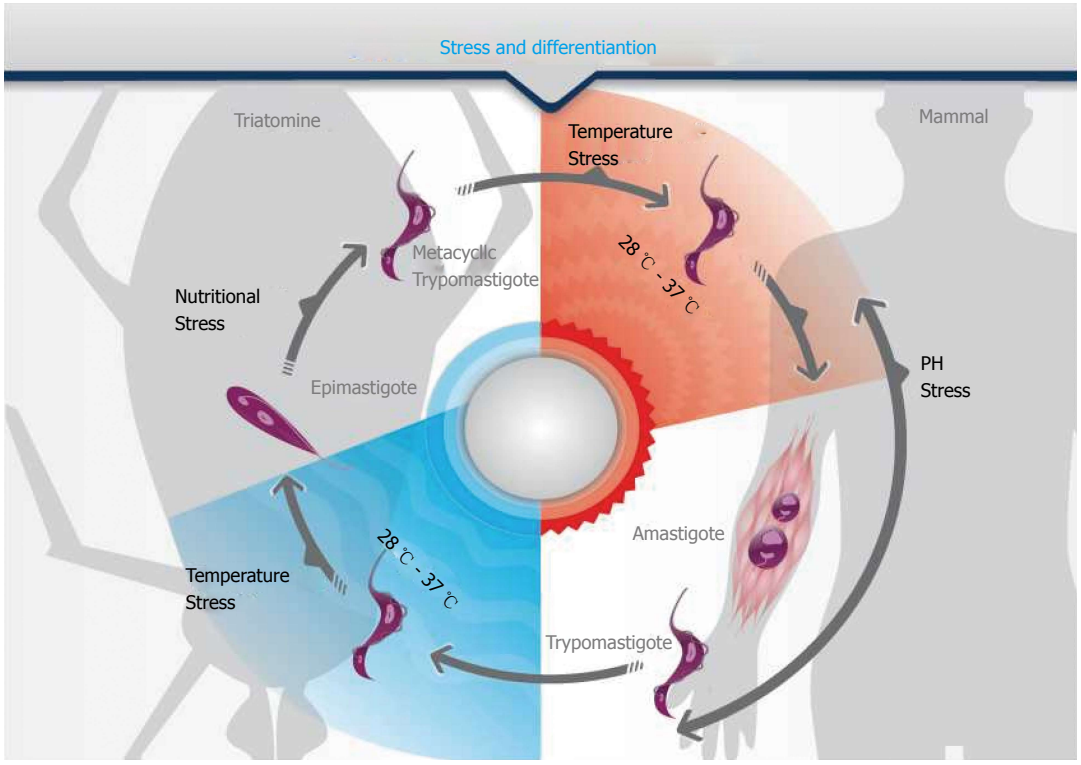


Figure 2 Physiological stresses and differentiation. The schematic representation shows the life cycle of *Trypanosoma cruzi*. This parasite alternates between two distinct intermediary hosts. The change to each host involves a temperature stress, either a heat shock (28 ℃ to 37 ℃) during the passage from the invertebrate to the vertebrate or a cold shock (37 ℃ to 28 ℃) when the parasite switches from the mammalian to the triatomine vector. Inside the hosts, nutritional and pH stresses are determinants for the differentiation from the non-replicative to the replicative forms (either epimastigotes in the insect or amastigotes in the mammal host) and from those to the infective trypomastigote forms.

structures[64]. Other proteins characteristic of stress granules may affect mRNA translation, such as T cell antigen internal-1 (TIA-1) and TIA-1-related, Fragile X mental retardation protein (FMRP) and Fragile X mental retardation-related protein-1, Argonaute, cytoplasmic polyadenylation element-binding protein, Pumilio and RNA-associated protein 55. Still others are involved in mRNA stability, such as RNA helicase RCK/p54, 5'-exonuclease 3' XRN1, proteins that bind to the ARE element, and Hur and TTP, which respectively stabilize and destabilize mRNA[65]. In mammalian cells, stress granules are formed as a result of phosphorylation of eIF2α by stress-activated kinases. The granules assemble when RBPs such as TIA-1, TTP, FMRP or G3BP bind to specific transcripts that are linked to the stalled pre-initiation 48S complex. After this initial nucleation stage, the polyA-binding protein (PABP) promotes the aggregation of these small granules by making them microscopically visible. Some proteins that do not have a canonical RBP domain can also associate with the granules through protein-protein interactions, which suggests that stress granules can interact with other signaling pathways in the cell[62,66].

Sheth *et al*[67] proposed an RNA cycle model describing the dynamics of mRNAs in the cytoplasm, from polysomes to P-bodies and stress granules. In this model, mRNAs are able to associate with polysomes being effectively translated into proteins; however, when a stress signal is triggered, mRNAs interact with components of the degradation/storage pathway, translation is stopped, and the mRNP that is formed is held in P-bodies. This mRNA can then be stored or degraded in the P-bodies or returned to the translation apparatus[55,66]. Stress granules represent a translation arrest site that increases in abundance as mRNAs are dissociated from polysomes; the association of mRNA with initiation factors may facilitate their re-entry for translation when the stress ceases[55]. The molecular mechanisms encompassed in the transition between polysomes, P-bodies and stress granules need to be clearly elucidated.

In several unicellular eukaryotes, stress is involved in triggering differentiation. This association is exemplified in Figure 2, which shows that nutritional, pH and temperature stresses are involved in the life cycle of the human pathogen *Trypanosoma cruzi*. Accordingly, it has been shown that formation of RNA granules increases during the nutritional stress preceding metacyclogenesis[10,12] (Figure 1). This process is dynamic because there is an important switch in the mRNAs sequestered to RNA granules in the course of the differentiation process[68].

RBPs in stress in protozoa

RBPs are crucial for the rapid gene expression remodeling that occurs in the course of cell differentiation or

the stress response. In some case, the changes in gene expression are accompanied by changes in mRNA localization within the cell. Accordingly, the localization of specific mRNAs is a meaningful tool to optimize protein synthesis and to maintain cell polarity[69]. Fluorescence *in situ* hybridization analysis using poly-T showed the increase of fluorescent granules during stress conditions in trypanosomatids, suggesting that the mRNAs were mobilized to form RNA granules. It was also observed that the cytoplasmic localization of specific transcripts changed during differentiation[70].

Proteomic analysis evidenced a global change in the protein composition of *T. cruzi* mRNPs isolated from polysomal and post-polysomal fractions from epimastigotes in exponential growth and epimastigotes under nutritional stress. More than 500 proteins were identified, and among them were several RBPs. Interestingly, a dynamic shift in protein composition was also observed in response to nutritional stress, especially for those proteins identified in the polysomal-free fraction of stressed parasites[71].

Some specific RBPs play important roles during the stress response in parasites. One example is TcZC3H39, a zinc finger protein from *T. cruzi* that exhibits a shift in the composition of the bound mRNA targets depending on the physiological conditions of the cell. Under stress conditions, the TcZC3H39-mRNP complex sequesters highly expressed mRNAs, thereby slowing translation activity. Accordingly, the number of bound mRNA targets was higher in stressed parasites than in non-stressed parasites. Under stress conditions, the TcZC3H39-mRNP complex was enriched in targets that are highly expressed under non-stress conditions, such as ribosomal and oxidative phosphorylation pathway proteins[68]. The protein content of TcZC3H39-mRNP consists of ribosomes, translation factors, RNA helicases and other RBPs, suggesting that this zinc finger protein might be part of complexes similar to stress granules, sequestering highly expressed mRNAs and the associated ribosomes, possibly slowing down translation in response to stress conditions[68].

The zinc finger protein ZC3H11 plays an important role during *T. brucei* heat shock. This protein is essential for the bloodstream form of the parasite and stabilizes HSP70 mRNA and other transcripts that encode chaperones necessary for proper protein folding or refolding during heat stress[72]. ZC3H11 interacts with the MKT1 and PBP1 proteins, forming a complex that stabilizes the associated transcripts[73].

TbDRBD3 is a cytoplasmic RRM protein from *T. brucei* that relocalizes to the nucleus and forms stress granules upon oxidative stress. It has been suggested that DRBD3 participates in the transport of target mRNAs that are remodeled in response to stress[74]. Accordingly, RNA-seq showed that the mRNAs associated with this protein encode ribosomal proteins, translation factors and enzymes, suggesting a role in protein synthesis in *T. brucei*[75].

TcSR62 is a nuclear RBP from *T. cruzi* that relocalizes to the nucleolus during actinomycin-D treatment[76]. Other RBPs, such as DRBD4 (PTB2 in *T. brucei*, a PTB homolog) and PABP, have also been observed to show this behavior during treatment with actinomycin-D or during severe heat shock[76]. It has been hypothesized that the nucleolus plays a role as a sensor or regulator of cell metabolism during the stress response[77,78].

T. brucei Alba 3 and 4 are found in stress granule-like structures during nutritional deprivation. They partially co-migrate with polysomes and also associate with the helicase DHH1, translation initiation factors and PABP1, indicating a role in translation modulation[21].

The heat shock response in *T. brucei* involves a reduction of the polysomes and a corresponding increase in the number of RNA granules, such as P-bodies and stress granules. The P-bodies from *T. brucei* are composed of XRNA (mRNA decay machinery), Scd6 and DHH1. These RNA granules could act as sites for RNA storage or degradation during stress and differentiation[79].

In *T. cruzi*, the DHH1 protein is also localized in cytoplasmic foci that resemble P-bodies and/or stress granules. The protein interacts with PABP, and its localization changes in response to nutritional stress; the granules increase in number and also become bigger, likely due to the shift of mRNAs from the polysomes to the RNA granules[12].

It is important to mention that proteins lacking canonical RBDs but having other functions in the cell can also associate with RNAs and become structural components of RNP complexes[80]. The moonlighting theory proposes that some multifunctional enzymes normally associated with metabolic pathways can also play a role in regulating gene expression in prokaryotes and eukaryotes. This "moonlighting" role was previously described for glyceraldehyde-3-phosphate dehydrogenase, which can be found within the nuclei of mammalian cells acting in transcription, DNA repair, RNA binding and transport, and telomere binding[73,81,82]. Other examples are inosine monophosphate dehydrogenase[83], acetyl-CoA carboxylase[84], lipin-1[85], aminoacyl-tRNA synthetase[86], Llv5p, which is required for the synthesis of branched chain amino acids[87], phosphofructokinase[88] and the mitochondrial enzyme Arg5,6, that participates in the transcription of target genes in addition to its role in arginine biosynthesis[89].

Another example of a moonlighting protein in *T. cruzi* is EF-1α, which associates with a specific subset of mRNAs during stress conditions. This protein can associate with large protein complexes independent of the translation machinery. A specific subset of mRNAs associates with EF-1α-mRNPs in unstressed or stressed epimastigotes. Some mRNAs are common to both physiological conditions, whereas others are specific to a given physiological condition. Gene ontology analysis identified enrichment of gene sets involved in single-organism metabolic processes, amino acid metabolic processes, ATP and metal ion binding, glycolysis, glutamine metabolic processes, and cobalt and iron ion

binding[90].

RBPs and eukaryotic cell differentiation

In parasites, several RBPs involved in differentiation have been described. The RRM-containing domain proteins RBP10 and RBP6 from *T. brucei* are implicated in life-cycle differentiation[22,91]. Accordingly, RBP10 knockdown led to a great alteration in the mRNA levels of transcripts normally found in high levels in the bloodstream forms[22]. The CCCH zinc finger proteins ZFP1, ZFP2 and ZFP3 have also been described as having roles in differentiation in trypanosomes[92,93]. Knockdown of ZFP1 disturbed the bloodstream differentiation by repositioning the kinetoplast in *T. brucei*[94]. A similar result was obtained with the RNAi knockdown for ZFP2: The parasite was unable to differentiate[92]. ZFP3 overexpression enhanced the differentiation levels to procyclic forms, and it is believed that these three proteins are part of the same differentiation pathway[93,95].

CONCLUSION

Despite evidence of the role for RBPs in gene expression regulation, particularly in protozoa as a means to respond promptly to environmental changes, the detailed mechanisms remain to be clearly elucidated. We anticipate that the complexity of these mechanisms should increase, as we do not yet know how different combinations of RBPs in a given complex affect the fate of the bound mRNAs. Furthermore, the modulation of the interactions within a complex could also be affected by post-translational modifications of the RBPs, creating an RBP code analogous to the histone code.

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REVIEW

Understanding the molecular mechanisms of cancer prevention by dietary phytochemicals: From experimental models to clinical trials

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Abstract

Chemoprevention is one of the cancer prevention approaches wherein natural/synthetic agent(s) are prescribed with the aim to delay or disrupt multiple pathways and processes involved at multiple steps, *i.e.*, initiation, promotion, and progression of cancer. Amongst environmental chemopreventive compounds, diet/beverage-derived components are under evaluation, because of their long history of exposure to humans, high tolerability, low toxicity, and reported biological activities. This compilation briefly covers and compares the available evidence on chemopreventive efficacy and probable mechanism of chemoprevention by selected dietary phytochemicals (capsaicin, curcumin, diallyl sulphide, genistein, green/black tea polyphenols, indoles, lycopene, phenethyl isocyanate, resveratrol, retinoids and tocopherols) in experimental systems and clinical trials. All the dietary phytochemicals covered in this review have demonstrated chemopreventive efficacy against spontaneous or carcinogen-induced experimental tumors and/or associated biomarkers and processes in rodents at several organ sites. The observed anti-initiating, anti-promoting and anti-progression activity of dietary phytochemicals in carcinogen-induced experimental models involve phytochemical-mediated redox changes, modulation of enzymes and signaling kinases resulting to effects on multiple genes and cell signaling pathways. Results from clinical trials using these compounds have not shown them to be chemopreventive. This may be due to our: (1) inability to reproduce the exposure conditions, *i.e.*, levels, complexity, other host and lifestyle factors; and (2) lack of understanding about the mechanisms of action and agent-mediated toxicity in several organs and physiological processes in the host. Current research efforts in addressing the issues of exposure conditions, bioavailability, toxicity and the mode of action of dietary phytochemicals may help address the reason for observed mismatch that may ultimately lead to identification of new chemopreventive agents for protection against broad spectrum of exposures.

Key words: Cancer prevention; Dietary phytochemicals;

Chemoprevention; Experimental efficacy; Mechanisms of action; Clinical trials; Bioavailability; Toxicity

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Core tip: Review compares the available evidence on the chemopreventive efficacy and probable mechanisms of chemoprevention by selected dietary phytochemicals in experimental systems and clinical trials. All the dietary phytochemicals covered have demonstrated chemopreventive efficacy against carcinogen-induced tumors in rodents at several organ sites. Mechanism of observed chemopreventive action(s) involve phytochemical-mediated redox changes, modulation of enzymes and signaling kinases leading to effects on multiple genes and cell signaling pathways. Clinical trials with these compounds have not demonstrated their chemopreventive efficacy. Probable reasons for observed mismatch between the two systems and areas of current research efforts and recommendations have been presented.

Maru GB, Hudlikar RR, Kumar G, Gandhi K, Mahimkar MB. Understanding the molecular mechanisms of cancer prevention by dietary phytochemicals: From experimental models to clinical trials. *World J Biol Chem* 2016; 7(1): 88-99 Available from: URL: http://www.wjgnet.com/1949-8454/full/v7/i1/88.htm DOI: http://dx.doi.org/10.4331/wjbc.v7.i1.88

INTRODUCTION

Cancer is a disease characterized by out-of-control cell growth leading to spread of abnormal cells to other body parts by local invasion and/or distant metastasis. It is one of the major and growing public health problem, currently accounting for over 12% deaths globally. With steady rise in life expectancy globally, it is estimated that the number of deaths due to cancer may double in next 50 years and new cases of cancer cases may rise to 15 million by 2020[1]. Majority of human cancers are caused by environmental and life-style factors. The etiology of all cancers is associated with inherited genetic aberrations (5%-10%) and acquired genetic abnormality (90%-95%) caused by exogenous and/or endogenous environmental agents[2].

Number of epidemiological studies have successfully shown that exposures to certain environmental chemicals/mixtures (exogenous-societal, occupational, lifestyle, industrial, agricultural, medicinal, *etc*., endogenous), physical agents (UV/solar, ionizing radiations, heat) and biological agents (certain viruses - EBV, HBV, HCV, HPV, HIV1, bacteria - *H. pylori*, parasites-liver fluke, schistosoma) increase the cancer risk at specific organs[3,4]. Experimental studies in animals, tissues/cells (*in-vitro)* have also provided supportive evidence for organ specific increased risk of cancer. Several of these associations have been established to be causative, *e.g.*, use of tobacco and oral cancer, while for several other human cancers such as breast, prostate, esophagus, *etc*. though associated factors have been documented, their causal association is yet to be determined.

Dose and duration of exposure to environmental carcinogens and their complex interactions with genetic and/or acquired host factors are some of the critical determinants, for the development of cancer[2]. Response to carcinogen exposure may further be modulated by host (physical activity, obesity) and life style (diet, tobacco, alcohol) factors leading to disease or no-disease.

CARCINOGENESIS: MULTIFACTORIAL AND MULTISTEP PROCESS

Carcinogenesis refers to environmental carcinogen [chemical/physical/biological agent(s)] -induced etiological pathways and processes that lead to cancer. It is a complex, multi-factorial, multi-step, multi-path and in humans a multi-years process comprised of at least three steps *viz*. initiation, promotion and progression[5] (Figure 1). Exposure of normal cells in tissues to carcinogenic agent results in genomic DNA damage (critical genes) and its fixation through a cycle of DNA replication leads to initiation and that is irreversible step. In the promotion process (reversible), clonal expansion of initiated cells occurs due to promotory stimuli resulting in formation of an actively proliferating, pre-malignant tumor cell population. While in progression (irreversible process) additional genetic changes lead to a new clone of tumor cells with increased proliferative, invasive, and metastatic potential.

Exposure to environmental carcinogens results in a series of genetic mutations and such alterations in at least two classes of genes, *i.e*., proto-oncogenes and/or tumor suppressor genes have been associated with tumor development. Activation of proto-oncogenes by qualitative or quantitative genetic changes results in enhancement of proliferative signals. Alternatively environmental carcinogen-mediated loss or inactivation of tumor suppressor genes (normal genes) also leads tumor development[6,7]. Genetic mutations, genomic instability, and series of epigenetic events, such as chronic inflammation are known to play role in transformation of normal to malignant cells. All transformed cells exhibit certain common characteristics such as sustained proliferation signals, evasion of growth suppressors, resistance to cell death, replicative immortality, and ability to induce angiogenesis and activation of invasion and metastasis[8].

WHY CANCER PREVENTION?

In spite of significant advancement in understanding the molecular mechanism of cancer development, very limited success has been achieved in early detection

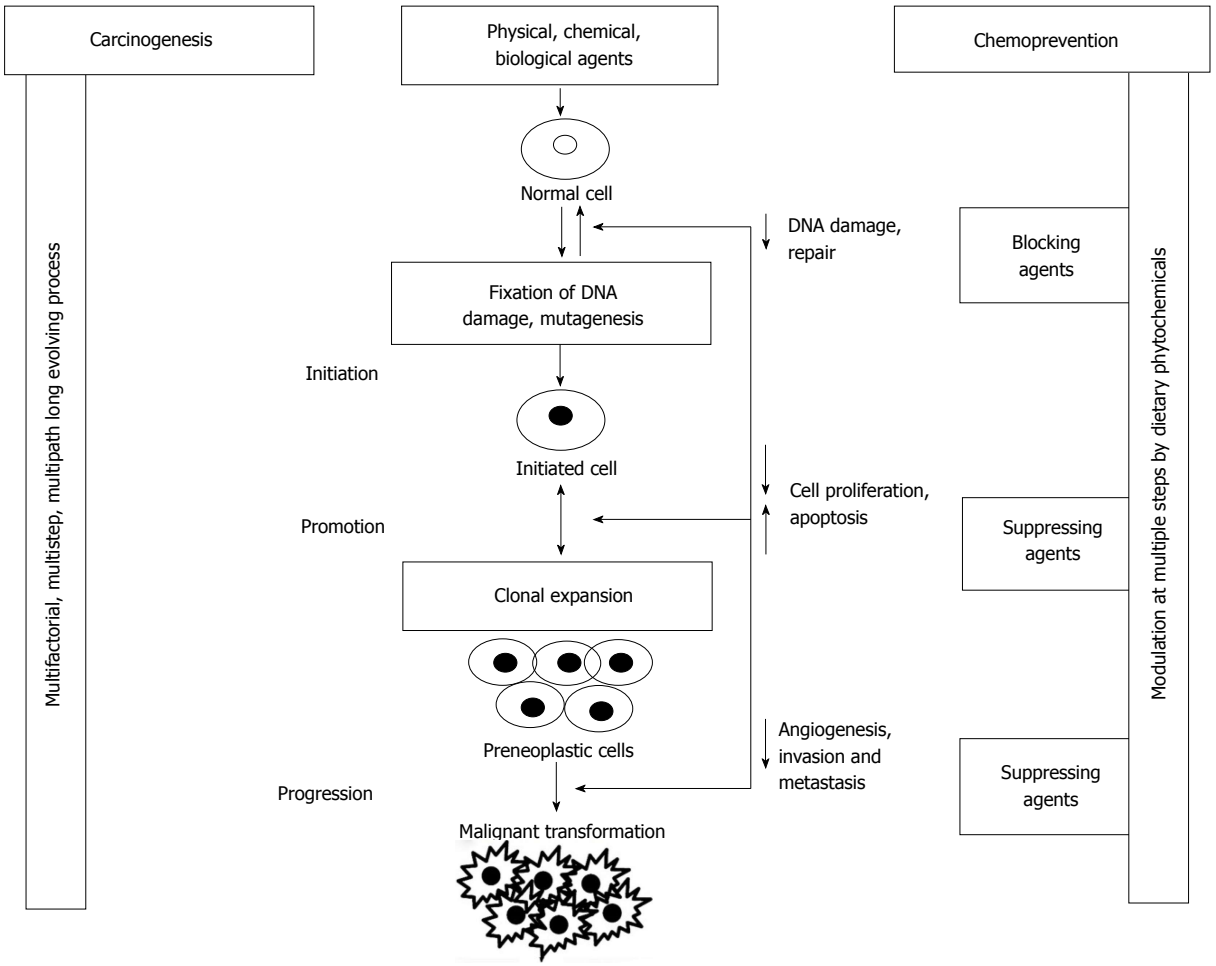


Figure 1 Schematic diagram showing multi-factorial, multi-step process of environmental agent(s)-mediated carcinogenesis and step(s) which are modulated by chemopreventive dietary phytochemical(s).

and treatment for most of the cancers. For patients with metastatic cancer, even the most advanced treatment strategies have not been successful in saving their lives while patients with less advanced cancer, treatment results in high morbidity and significant social and economic burden. Moreover, development of chemo/radio-resistance and recurrence of tumors are major challenges in the fight against cancer.

Considering limitations in early detection and successful treatment of cancer, preventive interventions have attracted increasing attention and significant research efforts. Several cancer prevention approaches and interventions have demonstrated potential for success in epidemiological and clinical trials across the globe[9,10] (Table 1).

CANCER PREVENTION AND APPROACHES

Preventing cancers by avoiding or minimizing exposures, especially occupational and societal (habit-related) exposures to known environmental carcinogens is likely to be most practical and cost effective strategy[3,11]. However these approaches of elimination of carcinogen from the environment and/or minimizing the exposure to carcinogens have not achieved desired success due to lifestyle choices and modern developments. Additional cost effective and practical approaches in cancer prevention interventions include pursuing lifestyle or dietary changes, chemopreventive interventions or prophylactic resection of high-risk organs in certain germ-line mutation carriers[9,10,12]. One of these approaches with enormous potential is chemoprevention which can be defined as the use of natural/synthetic/biological agents to suppress or prevent either the initial phases of carcinogenesis (blocking agents) or delay the progression of premalignant cells to invasive disease(suppressing agents)[12-14]. Improved understanding of the molecular basis of carcinogenesis has further enhanced the interest and potential of preventive interventions, *i.e.*: (1) Process of carcinogenesis involving set of genetic and epigenetic changes has been shown to be modulated by several environmental agents and changes in lifestyle and host factors; and (2) Preventive strategies have achieved good measure of success in other environmental diseases such as infectious diseases (vaccines) and cardiovascular events (treating risk factors with statins, lifestyle changes). Chemoprevention of skin,

Table 1 Successful cancer prevention approaches

Cancer prevention approach	Measures undertaken	Demonstration in epidemiological studies/clinical trials	Ref.
Avoid/minimize exposure to known carcinogens/risk factors	Quit cigarettes smoking	Epidemiological study followed up for 40 yr on male British doctors demonstrated decrease in risk	[39-41]
	Decreasing proportion of young smokers	Decrease in mortality due to lung cancer among younger male cohort	
	Reduction in number of cigarettes/day or duration of smoking and time since stopping smoking	Epidemiological study - decrease in the rate of lung cancer	[42]
	Ban the production and reduce the usage of carcinogenic aromatic amines	Reduction in bladder cancer among the dye workers	[43]
	Improvement in food preservation techniques	Significant reduction in incidence and mortality due to gastric cancers	[44]
Vaccination	HPV vaccines Gardasil (HPV 6,11,16,18) Cervarix (HPV 16,18)	Have shown 95%-100% effectiveness in preventing the cervical cancer precursor lesions and in preventing cervical adenocarcinoma *in situ*. Protection conferred was highly variable in those with prior HPV infection	[45-47]
	HBV vaccines Engerix-B (HBV-DNA) Recombivax (HBV surface antigen)	Rate of HCC decreased in children (6-14 yr)	[48]
Surgical intervention	Prophylactic resection of high risk organs-bilateral mastectomy and oophorectomy in BRCA1/ BRCA2 mutations carriers	Decrease in breast cancer risk and breast and ovarian cancer risk	[49-51]
	Colostomy in FAP patients	Decrease in colorectal cancer risk in patients with APC mutations	[52,53]
Chemoprevention			
Non melanoma skin cancer	Topical application of 5-FU, Immiquimod, Diclofenac, PDT with delta-aminolevulinic acid, Ingenolmebutate	Partial or complete clearance of actinic keratosis leading to decrease in cancer	[54-64]
Skin melanoma	Daily or discretionary sunscreen (SPF15+) for 4 yr	Reduction in invasive melanoma in a community based trial	[22]
Breast cancer	Administration of selective estrogen receptor modulating agent(s) (Tamoxifen, Reloxifen)/ aromatase inhibitors (Exemestane)	Trials have demonstrated efficacy of these agents in breast cancer prevention, reduction in recurrence and mortality	[25,65,66]
Prostate cancer	Administration of androgen receptor blockers (Finasteride, Dutasteride)	Reduction in prevalence and risk in clinical trials	[67-70]
Colorectal polyps Adenomas and cancer	Administration of NSAIDs- Aspirin, Celecoxib, Rofecoxib, Sulindac, DFMO	NSAIDs-mediated reduction in colorectal adenomas Significant increase in time to first colorectal cancer occurrence	[24,27,51,71-75]

HPV: Human papillomavirus; HBV: Hepatitis B virus; HCC: Hepatocellular carcinoma; BRCA: Breast cancer; 5-FU: 5-Fluorouracil; PDT: Photodynamic therapy; NSAIDs: Non-steroidal anti-inflammatory drugs; APC: Adenomatous polyposis coli; DFMO: Difluoromethylornithine.

breast, prostate, cervical, and colon cancer by various prevention agents has further stimulated the interest in the research area[10,12,15] (Table 1).

The aim of cancer chemoprevention is to delay or disrupt cancer causation associated pathways and multiple steps of carcinogenesis *viz*. initiation, promotion, and progression. Cancer preventive strategies can be classified into primary, secondary, and tertiary prevention. Primary cancer prevention is meant for general population and those at increased risk of cancer development. Secondary prevention is employed in patients with premalignant lesions that may progress to cancer. Tertiary cancer prevention is employed in preventing cancer recurrence (local invasion and/or distant metastasis) or second primary tumor among those who already have developed the disease.

This review article is targeted to briefly elaborate and compare the available evidence and understand the mechanism of chemoprevention by dietary phytochemicals in experimental and clinical investigations without presenting details on the experimental models, methods, and clinical trials.

DIETARY PHYTOCHEMICALS AS CHEMOPREVENTIVE AGENTS: EXPERIMENTAL STUDIES

Amongst environmental chemopreventive compounds, diet/beverage-derived agents due to their long history of exposure to humans, high tolerability, low toxicity, and reported biological activities are fast becoming lucrative targets for chemoprevention. Large number of pure compounds and extracts from dietary components has been evaluated in various experimental models for testing the chemopreventive efficacy of dietary

Table 2 Chemopreventive efficacy of dietary phytochemicals in experimental studies

Compounds	Chemopreventive agent administered (before + during + after carcinogen treatment) route-range of dose	Prevention of carcinogen induced tumors			Pathways modulated		No. of studies reporting protection	Selected ref.
		Target organs	Tumor multiplicity/ tumor incidence (#)	Latency period	Cell proliferation/ apoptosis	Detoxication pathways		
Tocopherols	Diet: 0.3%, 100-4000 mg/kg	Lung, Vagina, Prostate, Colon, Mammary gland	↓(3)/NR	NR	↓(3)/↑(2)	↑(2)	8	[76,77]
Indoles	Diet: 0.25%, 500- 4000 ppm, 0.014 mmol/g, 112 μmol/g, Gavage 50-250 mg/kg BW	Endometrium, Liver, Mammary gland, Lung	↓(5)/↓(2)	↑	↓(4)/↑(3)	↑(3)	14	[31]
Genistein	s.c.: 100-400 mg/mL (100 μL), Gavage: 1-500 mg/kg BW Diet: 100-300 mg/kg	Mammary gland, Prostate, Seminal vesicles, Buccal pouch, Uterous, Colon, Lung	↓(5)/↓(2)	NR	↓(5)/↑(3)	↑(3)	20	[78]
Curcumin	Diet: 0.05%-2%, 500-2000 ppm, Topical: 1-100, 3000 nmol	Skin, Digestive system, Intestine[1], Mammary gland, Liver, Kidney, Cheek pouch, Esophagus	↓(14)/↓(8)	NR	↓(12)/↑(8)	↑(9)	23	[79-82]
Dially sulphide	i.g.: 150-200 mg/kg BW, Topical: 0.5%-1% (100 μL)	Colon, Kidney, Forestomach, Urinary bladder, Thymus, Lung, Esophagus, Buccal pouch, Skin, Liver, Colon, Kidney	↓(6)/↓(2)	NR	↓(5)/↑(3)	↑(2)	17	[83,84]
Resveratrol	Diet: 60-200 mg/kg	Urinary bladder, Skin, Mammary gland[1], Buccal pouch, Tounge, Prostate, Lung, Salivary gland	↓(5)/↓(1)	↑	↓(7)/↑(6)	↑(4)	24	[85-88]
Retinoids	Topical (Oral): 0.5-1.0 mmol/kg BW Diet: 150-200 mg/kg	Urinary bladder, Skin, Mammary glands[1], Buccal puch, Tounge, Prostate, Lung, Salivary gland	↓(6)/↓(1)	↑	-/-	NR	16	[89-91]
Capsaicin	Diet: 1% capsaicinoids (64.5% capsaicin), Topical (Oral): 10-102 mg/kg BW	Lung, Skin, Tongue, Colon	↓(4)/NR	-	↓(4)/↑(3)	NR	10	[92,93]
Phenethy isothiocyanate	Diet: 5-25 μmol/g, 0.01%-0.1%	Lung, Esophagus, Urinary bladder, Colon	↓(3)/↓(1)	NR	-/-	NR	9	[94-96]
Lycopene	Drinking water: 17 ppm Diet: 10-300 mg/kg Gavage: 15-20 mg/kg BW	Prostate, Lung, Colon, Mammary gland[1], Buccal pouch, Liver	↓(5)/↓(2)	↑	↓(4)/↑(5)	↑(5)	18	[97]
Green, Black tea polyphenols	Drinking water: 1%-5%, 200-1000 ppm Diet: 0.05%, Topical: 1-200 mg/animal	Liver, Skin, Mammary gland, Lung, Buccal pouch, Colon, Esophagus, Prostate gland	↓(15)/↓(6)	↑	↓(9)/↑(10)	↑(8)	24	[98-104]

[1]Spontaneous tumors. NR: Not reported; #: No. of studies reported.

phytochemicals[16,17]. Model system have been employed based on their ability to rapidly answer, demonstrate expression of multi stage carcinogenesis, tissue/cell specificity, hormone responsiveness, invasiveness, modulation of tumor growth, histological types, and particular relevance to most common human cancers.

Chemopreventive activity of an agent is generally investigated employing carcinogens and/or spontaneously induced tumor and/or appropriate pathways or biomarkers in experimental animals/cell culture assays. In experimental models chemopreventive activity is ascertained based on observed increase in latency period and/or decrease in incidence and/or multiplicity of tumors or by modulation of disease/process associated biomarkers. Alternatively, development of pre-malignant lesions such as hyperplasia formation has been studied instead of carcinogenesis to study initial development[18]. In animal models regression of tumor or tumor xenografts and metastasis has also been reported[19-21].

There are various dietary phytochemicals of plant origin (various plant parts-leaves, vegetables, fruits, seeds, *etc.*) such as capsaicin, curcumin, diallyl sulphide, genistein, green/black tea polyphenols, indoles, lycopene, phenethyl isothiocyanate, resveratrol, retinoids and tocopherols which are most frequently studied. These dietary phytochemicals have been demonstrated to modulate incidence and/or multiplicity and/or latency period of carcinogen/spontaneously-induced tumors at various organ sites in experimental rodent models (Table 2). Surrogate markers like cell proliferation and apoptosis and various detoxication pathways (xenobiotic enzyme inductions/inhibition, lipid peroxidation, *etc.*) have also been successfully used for demonstrating protective action in *in vivo* system. Overall, the mechanisms observed in the dietary phytochemical-mediated inhibition are diverse (Figure 2). The observed anti-initiating and anti-promoting activity of dietary phytochemicals in experimental models involve modulation of redox status, direct interaction with

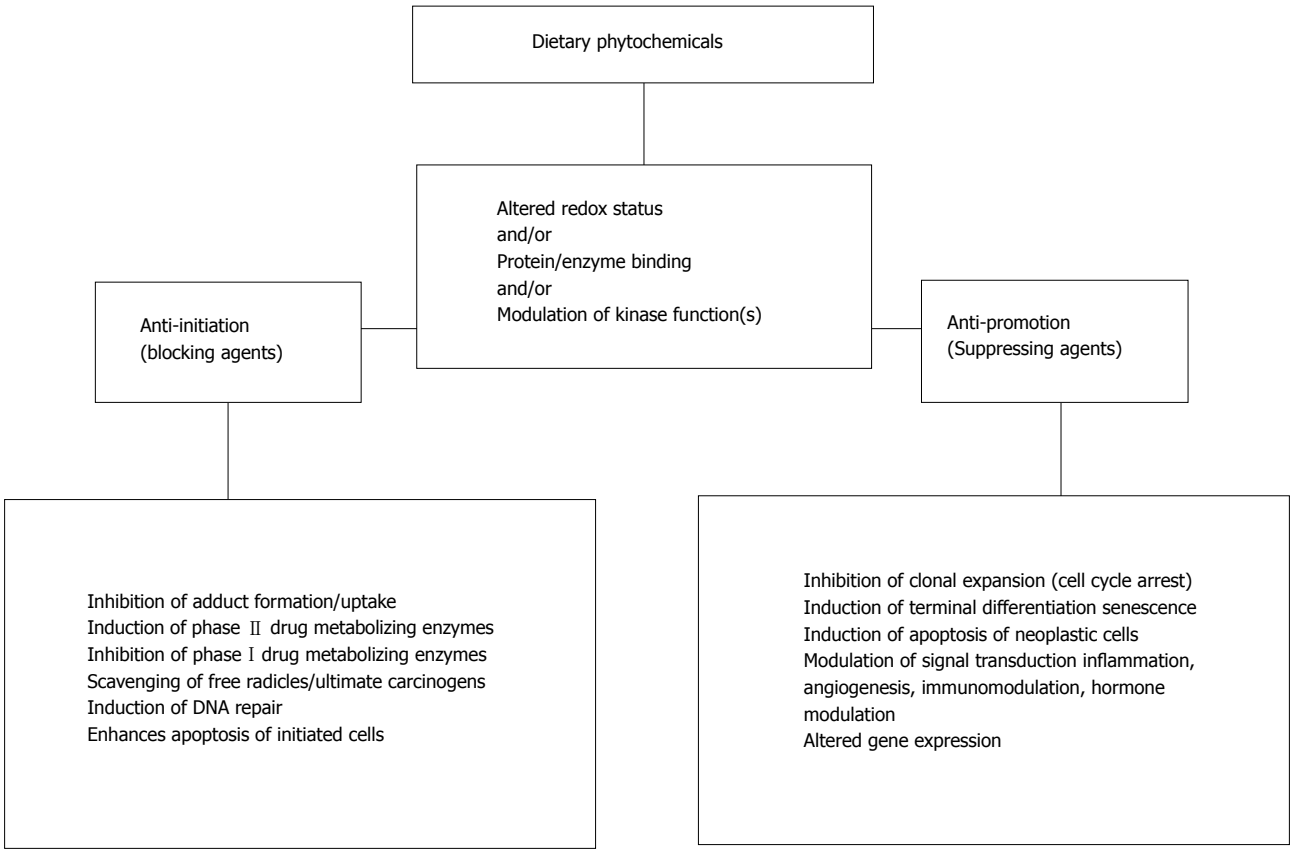


Figure 2 Mechanism(s) of chemopreventive action(s) (*in vivo*) of dietary phytochemicals observed in experimental systems. For details see[16,17,21].

proteins and modulation of signaling kinases leading to effects on genes and cell signaling pathways at multiple levels (Figure 2).

Following findings are noted after comparing and analyzing the outcome of all available experimental studies (Table 2) evaluating the chemopreventive efficacy of dietary phytochemicals: (1) Effects are moderate and generally decrease in multiplicity, burden of tumor, and/or moderate increase in latency period have been observed without major change in incidence of tumor; (2) Relatively long and repeated exposures (prior, during, and post-carcinogen) to dietary phytochemicals have generally been needed for observing protective effects; (3) Most of the dietary phytochemicals have been demonstrated to be effective against several classes of environmental carcinogens at multiple organ sites; (4) Bioavailability of dietary phytochemicals and their metabolites have not been reported from most of the experimental studies that demonstrated their chemopreventive efficacy; and (5) In most of these studies doses of chemopreventive agent(s) administered or effective doses appear to be much higher than normal dietary exposures received in human, although chemopreventive doses have not been reported to be toxic.

CLINICAL TRIALS

Comprehensive evaluation of preclinical efficacy and safety of dietary phytochemicals, the promising compounds has been evaluated in appropriate clinical trials. A brief summary of the outcome from these trials has been presented (Table 3) and following points may be summarized: (1) In most of the trials chemopreventive agents were administered to patients with cancer or high risk individuals, *i.e*., after occurrence of damage or disease; (2) Though there are several clinical trials conducted using dietary phytochemicals, results from only approximately 20% trials have been reported. Other trials have either not been completed or their results are not reported due to issues of toxicity, bioavailability and other unknown reasons under the conditions employed for the trials; (3) Bioavailability issues have been reported for curcumin, genistein, resveratrol, lycopene and green tea; (4) Toxicity issues have been reported in clinical trials conducted with tocopherols and retinoids; and (5) Very few trials with curcumin and green tea have shown beneficial effects as judged by modulation of biomarkers and symptoms.

If direction of results from clinical trials evaluating the chemopreventive potential of dietary phytochemicals is compared with that of their chemopreventive effects in experimental models, it is clear that efficacy observed in experimental studies has not been observed in clinical trials. To understand the differences between experimental studies and clinical trials that may have contributed in the observed mismatch in the direction of outcome, the probable major differences have been

Table 3 Evaluation of dietary phytochemicals in clinical trials

Compounds	No. of clinical trials		Chemopreventive agent administered		Target organs	Efficacy	Bioavailability/ toxicity issues	Ref.
	Conducted	Results reported	High risk individuals/ patients[1]	Route-dose range				
Curcumin	52	3	-/+	Oral: 0.036-8 g daily	Multiple organ sites	+(2)/-(1)	+(1)/-(3)	[105-107]
Genistein	29	7	-/+	Oral: 160-600 mg/d	Breast, urothelial bladder, Mammary gland	NR	+(1)/-(1)	[108,109]
Indoles	4	0	-/+	Oral: 2 serving (½ cup/serving) daily	Prostate, breast, blood	NR	NR/NR	NR
Tocopherols	28	9	+/+	Oral: 50-500 mg/d, 400-1000 IU/d	Prostate, lung	NR	NR/+(2)	[110]
Dially Sulfide	0	0	-/-	NR	NR	NR	NR/NR	NR
Resveratrol	5	2	-/+	Oral: 20-80 mg/d	Colorectal, colon	+(2)	+(1)/-(2)	[32,111]
Retinoids	102	17	+/+	Oral: 1-80 mg/d	Lung, blood, prostate, kidney, skin, blood, head and neck, liver	+(3)/-(7)	NR/+(2)	[112,113]
Capsaicin	2	0	-/+	Oral: 112 mg twice daily for 6 mo, one capsaicin lozenge 4 times daily	Prostate, head and neck	NR	NR/NR	NR
PEITC	9	1	-/+	Oral: Broccoli (300 g) soup four times daily for 5 d, Broccoli seed extract (250 mg), 40 mg PEITC capsules 4 times a day	Lung, oral	-(1)	NR/-(1)	NR
Lycopene	26	24	-/+	Oral: 15-90 mg/d	Prostate	+(1)/-(1)	+(2)/NR	[114,115]
Green tea	85	6	-/+	Oral: 400-2000 mg twice a day, (daily dose is equivalent of 9 cup-of-green tea per day (0.9 g/d GTE, 0.6 g/d EGCG)	Blood, colorectal, prostate, pancreas	+(3)/-3	+(3)/-(3)	[116-118]

[1]Study cohort is exclusively of cancer patients. Source: https://clinicaltrials.gov. +: Present; -: Not observed; NR: Not reported; PEITC: Phenethyl isothiocyanate.

listed below: (1) Experimental systems are generally simple and homogenous in terms of genetic, host and lifestyle factors and conditions are controlled, while in clinical trials additional variables of host and lifestyle factors, exposure complexity and metabolic competence makes the system more complex; (2) Exposure to potential chemopreventive agent in experimental systems is generally before, during and/or after carcinogen treatment when cells/tissues/processes are generally normal while in clinical trials considered in this review administration of chemopreventive agent is after damage/disease, *i.e.*, high-risk individuals/cancer patients; and (3) Chemopreventive efficacy of dietary phytochemicals in experimental systems has been generally at doses higher than their normal dietary exposure to human while bioavailability and toxicity issues have probably affected the outcome in clinical trials.

To understand the role or contribution of listed factors (differences between two systems), a comparison of similarities and differences in the properties and biological effects of dietary phytochemicals with approved chemopreventive agents has been made (Table 4).

Considering the chemopreventive efficacy of approved chemopreventive agents both in experimental studies as well as clinical trials[22-29], it appears that differences in host and lifestyle factors and the time of exposure to chemopreventive agent, *i.e.*, before and after damage/disease, may not be the major determinants in the direction of outcome between the two systems. Based on these comparisons and reports in literature on the properties and bioavailability of dietary phytochemicals in clinical trials, bioavailability, metabolic processing and toxicity related differences appear to be the major and important factors.

CURRENT STATUS AND RECOMMENDATIONS

Several clinical trials have found that administration of dietary phytochemicals resulted in low undetectable levels in blood[30-32], which probably curtailed its progress from laboratory to the clinic. Search for potential factors contributing to low bioavailability suggest: (1) low aqueous solubility (polarity, poor dissolution rate) of compounds; (2) poor absorption (lipophilic nature); and (3) extensive metabolic conversion to conjugates/ metabolites. Number of approaches are currently being evaluated for enhancing the bioavailability such as use of nano-dietary phytochemicals (particles)/ dietary phytochemicals in nano-carriers, incorporation of polymers, proteins/amino acids, phospholipids and/ or solid lipids, use of liposomes for delivery of dietary phytochemicals or using phytochemicals coated with essential natural oil and use of synthetic analogues[33]. With continuing efforts to enhance the bioavailability of

Table 4 Properties and biological effects of dietary phytochemicals and approved chemopreventive agents

Dietary phytochemicals	Approved chemopreventive agents
Occurrence	
Natural compounds of plant origin	Majority are synthetic compounds while some are biological and/or natural agents
Long History of exposure to humans through food	Human exposure as prescribed drug/vaccine
Easily available and relatively cheaper	Available on prescription and relatively expensive
Properties	
Anti-oxidant/polyphenolic in nature, sparingly soluble in water	Widely differing properties and characteristics
Chemopreventive efficacy	
Established in experimental models, yet to prove efficacy in clinical trials	Established both in experimental models and clinical trials
Effects-weak to moderate	Effects-moderate to strong
Successful against different classes of carcinogens and at multiple organ sites	May not be successful against different classes of carcinogens and at multiple organ sites
Mechanism(s)	
Modulators of redox status and kinase functions, inducer of phase Ⅱ enzymes	Mechanisms are diverse and may not be related to redox status and/or kinase functions, may not be modulating phase Ⅱ enzymes
Modulate multiple pathways/targets	Modulation of specific targets/pathways
Specificity	
Relatively less specific/non-specific (Pleotropic effects)	Relatively specific to defined agent/exposure and/or molecular pathways
Toxicity	
Depends on dose and duration of exposure Non-toxic at the doses present in food	Depends on dose and duration of exposure

dietary phytochemicals using various approaches, we also need to understand if there is linear relationship between bioavailability and bioefficacy and identify the factors that are responsible for observed toxicity of these compounds in clinical trials but not in experimental studies.

Considering the limitations of currently available/employed animal models in identification of environmental chemopreventive agents, existing models need to be improved and new experimental models need to be developed. Efforts to achieve/incorporate exposure complexity similar to those in humans and replication of human host and/or lifestyle factors in animal models if successful may help in addressing the current limitations. Before embarking on clinical trials, experimentally successful chemopreventive agent(s) need to be thoroughly investigated for the mechanism(s) of observed chemopreventive actions and/or toxicity. Similarities and differences in circulating levels of chemopreventive agent(s) and metabolic products attained in experimental *vs* real life also need to be understood. Since process of carcinogenesis involves complex interplay between various factors it is suggested to study and understand the role of diet (animal *vs* plant sources), calorie content and physical activity on the dietary phytochemical(s) (single or mixture)-mediated effects in experimental systems and in humans. This suggestion is based on the available experimental and epidemiological observations on modulatory effects of calorie content[34,35], diets rich in fruits and vegetables[36] and moderate physical activity[37,38]. Experimental and clinical observations based on mechanistic studies, gene-environment interactions and complementary nutritional studies may help in identifying environmental chemopreventive agents. However, cancer being multi-factorial, multi-step complex evolving process, establishing or understanding the protective effect of one such factor or class of agent alone may not address the problem satisfactorily. Identifying novel prevention approaches and developing number of dietary chemopreventive agents differing in mechanism of their chemoprotectory action may help in slowing down and delaying /prevention of environmental cancers.

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REVIEW

Recent advances in production, purification and applications of phycobiliproteins

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Abstract

An obligatory sunlight requirement for photosynthesis has exposed cyanobacteria to different quantity and quality of light. Cyanobacteria can exhibit efficient photosynthesis over broad region (450 to 650 nm) of solar spectrum with the help of brilliantly coloured pigment proteins called phycobiliproteins (PBPs). Besides light-harvesting, PBPs are found to involve in several life sustaining phenomena including photoprotection in cyanobacteria. The unique spectral features (like strong absorbance and fluorescence), proteineous nature and, some imperative properties like hepato-protective, anti-oxidants, anti-inflammatory and anti-aging activity of PBPs enable their use in food, cosmetics, pharmaceutical and biomedical industries. PBPs have been also noted to show beneficial effect in therapeutics of some disease like Alzheimer and cancer. Such large range of applications increases the demand of PBPs in commodity market. Therefore, the large-scale and coast effective production of PBPs is the real need of time. To fulfil this need, many researchers have been working to find the potential producer of PBPs for the production and purification of PBPs. Results of these efforts have caused the inventions of some novel techniques like mixotrophic and heterotrophic strategies for production and aqueous two phase separation for purification purpose. Overall, the present review summarises the recent findings and identifies gaps in the field of production, purification and applications of this biological and economically important proteins.

Key words: Phycobiliproteins; Nutraceutical; Fluorescence; Pharmaceutical; Cyanobacteria

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Core tip: The present review compiles the overall research that has been done so far in the field of production, purification and application of phycobiliptoteins (PBPs). The comparative accounts for different methods for PBPs production, purification have been discussed. The potential applications of PBPs in various industries including food, cosmetics and pharmaceutical have been debated rationally. Remaining questions and research gaps in respected field have been highlighted as conclusion.

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INTRODUCTION

Cyanobacteria are considered as an earliest group of a prokaryotic organism, performing an oxygenic photosynthesis and thus accepted as an ultimate source of atmospheric oxygen[1]. They are widely distributed over aquatic and terrestrial environments including extreme habitats such as hot springs, deserts and polar region[2]. Exposure to various environmental stresses like ultraviolet radiation[3], high temperature, high/low light irradiation and many others have forced cyanobacteria towards gradual evolution to maintain the cell viability[4,5]. Especially, an obligate sunlight requirement for photosynthesis have exposed cyanobacteria to the wide range of solar radiation; which in turn, positively pressurized them to synthesize a number of photon absorbing pigment molecules[6-9]. Cyanobacteria exhibit efficient photosynthesis over the wavelength range 450 to 655 nm by absorbing light energy through a family of brilliantly colored pigment proteins called phycobiliproteins (PBPs)[10]. They stacks together in sequential fashion to form a giant mound - phycobilisome (PBS), which is stabilized and tied up by colourless linker proteins (LPs) on the outer surface of photosynthetic lamellae[11].

PBS

Principally, PBS is built up of various functional PBPs plus a few LPs in a manner to efficiently absorb and funnel photo-energy toward photosystems (Figure 1). PBPs, representing almost 80% of PBS mass[12], are pigment proteins consists of protein scaffold with covalently attached chromophore (a linear tetrapyrrole chain) (Figure 2)[13]. De Marsac and Cohen-Bazire[12] (1977) first time demonstrated the presence of 15%-20% LPs in PBS on SDS-PAGE and was suggested to have role in assembly and stabilization of PBS. Moreover, they were also found to have assisting role in optimization of absorbance characteristics and energy transfer of the PBPs to favor a unidirectional energy flow from the peripheral PBPs towards the reaction centres *via* PBS core[14]. Besides light-harvesting action, PBS also plays an important role in photo-protection mechanisms under high-irradiances[15]. Moreover, PBS may also be utilized as nutrient source under nitrogen and phosphorus starvation conditions[16].

PHYCOBILIPROTEINS

PBP is the family of brilliantly colored water-soluble pigment proteins. PBPs are classified based on their spectral characteristics. Phycoerythrin (PE, $\lambda_{A\ max}$ = 540-570 nm; $\lambda_{F\ max}$ = 575-590 nm), phycocyanin (PC, $\lambda_{A\ max}$= 610-620 nm; $\lambda_{F\ max}$: 645-653 nm) and allophycocyanin (APC, $\lambda_{A\ max}$ = 650-655 nm; $\lambda_{F\ max}$ = 657-660 nm) are the majorly found PBPs (Figure 3)[17-19]. The bright color, non-toxic protein nature and antioxidant virtues of PBPs open up the doorway for their potential application in various industries. To fulfil the increased demand of PBPs in the market, many scientists have been trying to improve the production and purification of PBPs.

PRODUCTION OF PBPs

Photoautotrophic production

Generally, PBPs are produced photo-autotrophically by two ways: By growing the red algae and cyanobacteria at large scale in open man-maid ponds or in closed bioreactor under the natural sunlight. *Spirulina platensis* is majorly used host for the PBPs production because it can grow in open ponds without being out-competed by contaminating organisms and even in alkaline conditions (pH 10.5)[20,21]. Approximately, > 3000 tons of *S. plentesis* cell mass are produced worldwide predominantly at tropical and subtropical locations around the Pacific Ocean[22,23]. In open pond, the poor availability of light below the surface level is the major problem that hampers the cell mass production. Enclosed photobioreactor (Figure 4) is used as the better option for PBPs production at large scale, which facilitates the equal light provision, CO_2 distribution, temperature control over the outdoor culturing.

Mixotrophic production

Provision of any carbon source enhances the cell mass concentrations as compared to photoautotrophic cultures[24,25]. Vonshak *et al*[24] (2000) have been observed that the maximal photosynthetic rate and the light saturation value of mixotrophically growing culture are higher than that in autotrophic condition. Feeding of glucose to *S. plentesis* was found to increase the biomass production up to 10 g/L[26]. The enhanced tolerance against high light stress was noticed in mixotrophic condition as compared to autotrophic condition[24]. Mixotrophic production is carried out in the presence of some carbon source along with the sunlight in specially designed bioreactors, which helps the photoautotrophs to compete against other contaminating microbes. *S. plentesis* was shown increased PC production in indoor mixotrophic conditions than that in outdoor photoautotrophic cultures[26,27].

Heterotrophic production

Cyanobacteria and red algae living in extreme habitat acclimatized themselves to utilize the carbon source from their surrounding rather than being photo-autotrophic. To produce the PBPs from this extremophiles, the

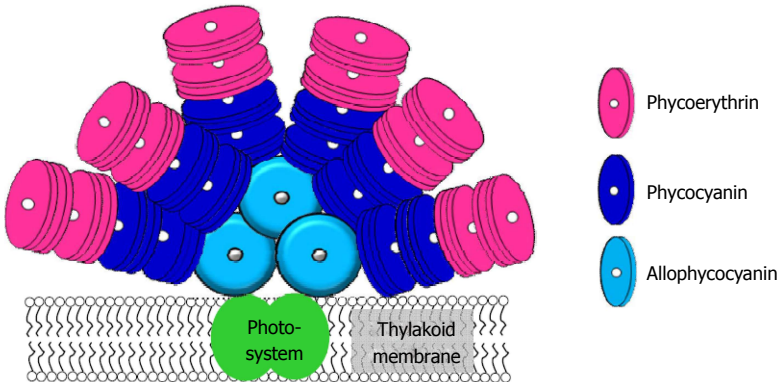


Figure 1 Schematic diagram of phycobilisome situated on the thylakoid membrane.

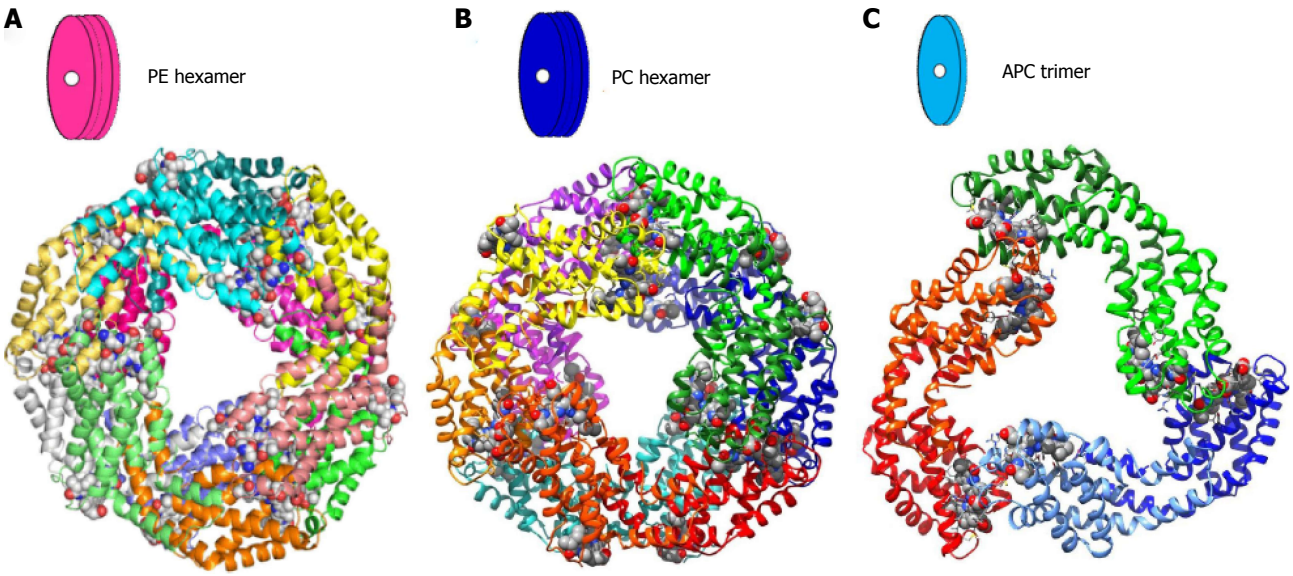


Figure 2 The phycobiliptotein is mainly made up of hexameric and trimeric disks of phycobiliptoteins. Schematic diagram and ribbon model of the structure of A: Phycoerythrin-hexamer; B: Phycocyanin-hexamer; C: Allophycocyanin-trimer. The apoprotein and chromophores are shown by ribbon and ball-stick model, respectively. PBPs: Phycobiliptoteins; PE: Phycoerythrin; PC: Phycocyanin; APC: Allophycocyanin.

heterotrophic strategies are being employed. *G. sulphuraria,* generally inhabiting in hot and acidic niche, is widely used as host for the heterotrophic production of PC[28]. The acidic pH and high temperature of culture decreases the risk of contamination and thus, permits the heterotrophic culture to be an axenic even for long time. The properties of heterotrophic PC resemble those of photoautotrophic origin.

Recombinant production

Although being very difficult, recombinant production is only alternative for the heterotrophic synthesis of PBPs. The co-expression of the numerous gene (for apoprotein synthesis, chromophore synthesis and chromophore attachment) is required for the production of recombinant PBPs. Recently, Overkamp *et al*[29] (2014) have demonstrated the recombinant PBPs production using the eukaryotic PBP lyses. Cherdkiatikul *et al*[30] (2014) cloned and overexpressed the *S. plentesis* allophycocyanin and phycocyanin apo-proteins in *E. coli BL21* through pETDuet-1 vector. Several other reports have also demonstrated the recombinant production of PBPs apoproteins (alpha- and beta-subunits)[31,32], however, the recombinant production of holoproteins is poorly reported[33,34].

PURIFICATION OF PBPs

Due to owing diverse applications in various fields, it becomes essential to develop an easy and cost-effective method for PBPs purification from cyanobacteria to meet its increasing demand. Researchers have made many efforts to purify PBPs with maximum purity and yield. Bermejo Román *et al*[35] (2002) described a fast two-step chromatographic method for the purification of PE from *Porphyridium cruentum, i.e.*, expended-bed adsorption chromatography using streamline DEAE followed by conventional ion-exchange chromatography (DEAE-cellulose). The same authors also demonstrated the baseline separation of α-, β- and γ-subunits of PE by a reverse-phase HPLC gradient semi-preparative method. Rossano *et al*[36] (2003) extracted and purified R-PE

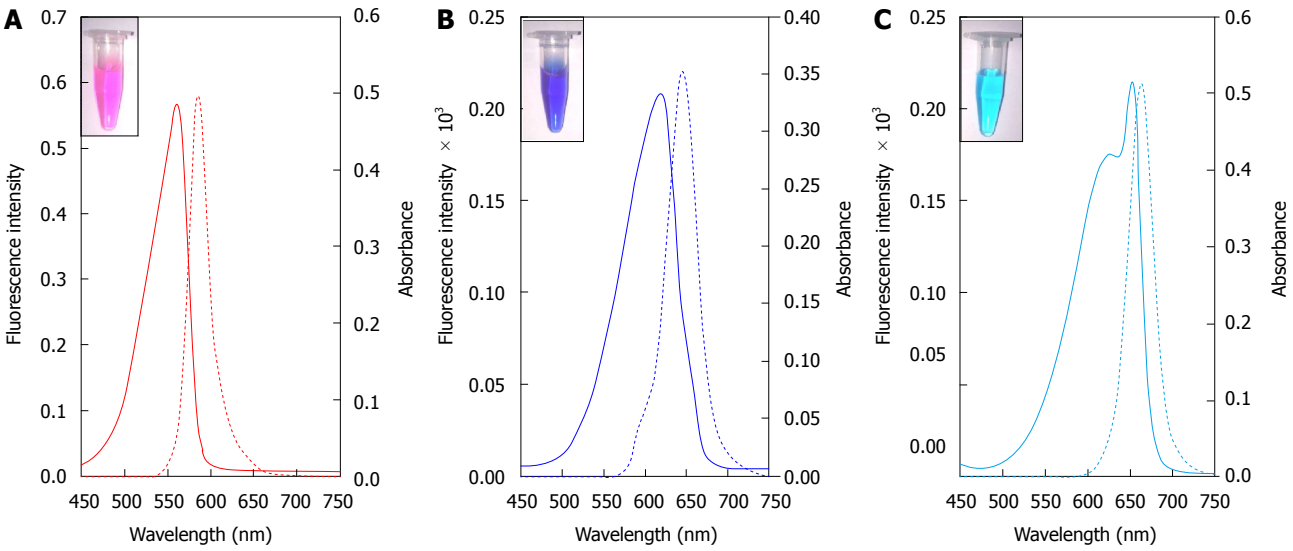


Figure 3 UV-visible absorbance (solid line) and fluorescence emission (dotted line) spectra, and appearance (inset) of A: Phycoerythrin, B: Phycocyanin and C: Allophycocyanin.

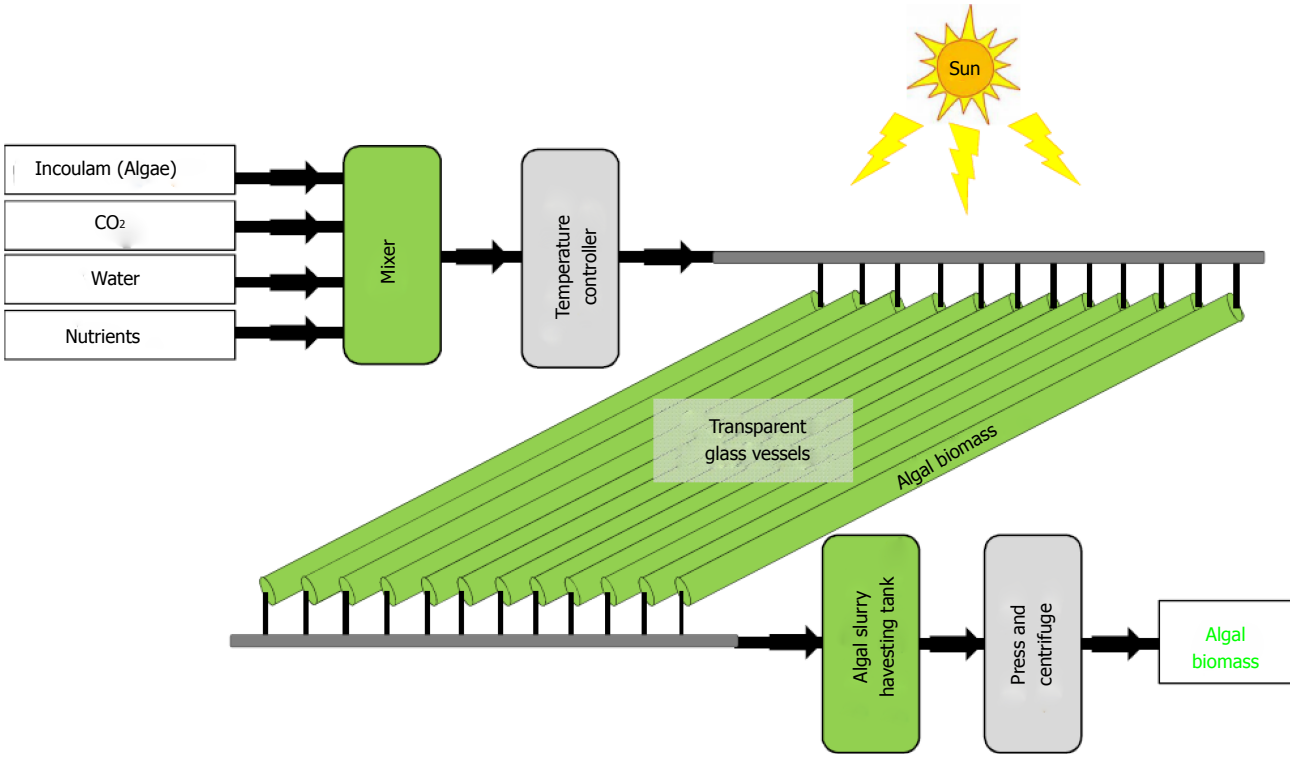


Figure 4 Photo-bioreactor: The graphical representation of the structure and operation of widely used sun light based enclosed photo-bioreactor for the large-scale production of algal biomass.

from Mediterranean red algae *Corallina elongata*, using hydroxyapatite column. Isailovic *et al*[37] (2004) isolated and characterized R-PE subunits and its enzymatic digests. Their results showed efficient absorption and fluorescence of the R-PE subunits and digested peptides, originating from the incorporation of PEB and PUB chromophore groups in them. Tripathi *et al*[38] (2007) extracted and purified an unusual PE from a terrestrial and desiccation-tolerant cyanobacterium *Lyngbya arboricola*. PE was purified using acetone precipitation, gel filtration and ion-exchange chromatography resulting in PE with high purity ratio (> 5). Soni *et al*[39,40] (2006, 2008) purified and characterized PC by using ammonium sulphate precipitation coupled with ion-exchange and hydrophobic interaction chromatography, respectively. Zhang *et al*[41] (1999) purified PC and APC using ion-exchange chromatography, however they used pH gradient to elute proteins rather than slat gradient. Patil and Raghavarao[42] (2007) separated PC and APC using aqueous two phase separation techniques. Recently,

Table 1 List of various procedures employed for phycobiliptoteins purification from cyanobacteria and red algae

PBPs	Ref.	Species	Purification procedure	Purity	Yield (%)
PC	Boussiba *et al*[70]	*S. platensis*	Ammonum sulphate precipitation; Hydroxyapatite chromatography; Ion exchange chromatography	4.15	-
APC	Reuter *et al*[71]	*Rhodella violace*	Gradient centrifugation; Hydroxylapatite chromatography; Preparative "native" PAGE	-	-
PC	Abalde *et al*[72]	*Synechochoccus* sp.	Hydrophobic interaction chromatography; Ion exchange chromatography	4.85	-
PC	Zhang *et al*[41]	*S. platensis*	Ammonum sulphate fractionation; Ion exchange chromatography; Gel filtration chromatography	5.06	-
PE	Galland-Irmouli *et al*[73]	*Palmaria palmate*	Preparative "native" PAGE	3.2	-
PC	Rito-Palomares *et al*[74]	*S. maxima*	Two-phase aqueous extraction; Ultrafiltration; Ammonum sulphate precipitation	3.8	29.5
PC	Minkova *et al*[75]	*S. fusiformis*	Rivanol treatment; Ammonum sulphate precipitation; Gel filtration chromatography; Ammonum sulphate precipitation	4.3	45.7
PC	Soni *et al*[39]	*O. quadripunctulata*	Ammonum sulphate precipitation; Gel filtration chromatography; Anion exchange chromatography	3.31	44.2
PC	Benedetti *et al*[76]	*A. flos-aquae*	Ammonum sulphate precipitation; Hydroxyapatite (electrostatic interaction) chromatography	4.78	-
PC	Patil *et al*[77]	*S. platensis*	Chitosan adsorption; Two-phase aqueous extraction	5.1	66.0
PC	Patil *et al*[77]	*S. platensis*	Chitosan adsorption; Two-phase aqueous extraction; Ion exchange chromatography	6.69	-
PC	Niu *et al*[78]	*S. platensis*	Expanded bed adsorption chromatography; Ion exchange chromatography	3.64	8.7
PC	Patil *et al*[42]	*S. platensis*	Repeated two-phase aqueous extraction; Ultrafiltration	4.05	85.0
PC	Soni *et al*[40]	*P. fragile*	Ammonum sulphate fractionation; Hydrophobic interaction chromatography	4.52	62.0
PC and APC	Yan *et al*[79]	*S. platensis*	Ammonum sulphate precipitation; Ion-exchange chromatography	5.59 and 5.19	67.04 and 80.0
PE	Sun *et al*[80]	*Heterosiphonia japonica*	Ammonum sulphate precipitation; Gel filtration chromatography; Ion exchange chromatography	4.89	-
APC	Parmar *et al*[81]	*Geitlerinema* sp. A28DM	"Ethodin" precipitation; Gel filtration chromatography	3.2	66.0
PE	Niu *et al*[82]	*Porphyra yezoensis*	Expanded bed absorption; Ion-exchange chromatography	> 4	-
APC	Su *et al*[83]	*S. platensis*	Ammonum sulphate precipitation; Hydroxyapatite chromatography; Ion-exchange chromatography	5	43.0
PE	Parmar *et al*[84]	*Phormidium* sp. A27DM, *Lyngbya* sp. A09DM, *Halomicronema* sp. A32DM	Ammonum sulphate precipitation; Gel filtration chromatography	> 3.5	> 60.0
PE	Mishra *et al*[85]	*Pseudanabaena* sp.	Ammonum sulphate precipitation; Gel filtration chromatography; Ion exchange chromatography	6.86	47.0
PC	Johnson *et al*[86]	*Nostoc* sp.	Ion exchange chromatography; Two-phase aqueous extraction	3.55	-
PE, PC and APC	Sonani *et al*[43]	*Lyngbya* sp. A09DM	Triton X-100 mediated ammonium sulphate precipitation; Ion exchange chromatography; Gel filtration chromatography	6.75, 5.53 and 5.43	76.16, 60.23 and 71.91
PE	Johnson *et al*[86]	*Nostoc* sp.	Ion exchange chromatography; Two-phase aqueous extraction	-	-

PBPs: Phycobiliproteins; PC: Phycoerythrin; APC: Allophycocyanin; PE: Phycoerythrin; PAGE: Polyacrylamide gel electrophoresis.

Sonani *et al*[43] (2014a) and Rastogi *et al*[44] (2015c, 2015d) have developed a protocol, which facilitates the purification of all three major PBPs from marine isolate *Lyngbya* sp.. A09DM by Triton X-100 mediated ammonium sulphate precipitation. A novel microwave assisted extraction and pre-purification of PBPs from thick exopolysaccharide cell wall containing *Porphyridium purpureum* was demonstrated[45]. Cuellar-Bermudez *et al*[46] (2015) nicely reviewed various strategies and their successes in purification of PBP from algae. Several protocols employed so far for the purification of PBPs have been depicted in Table 1.

APPLICATIONS OF PBPS

Due to inherent bright color, non-toxic protein nature, easy availability and potential free radical scavenging capacity, the pigment PBPs are being employed in food, cosmetics, pharmaceutical industries and in several other biomedical researches (Figure 5).

Colorant in foods

PBPs are widely used as a natural protein dye in various food and cosmetic industry. PC isolated from *S. platensis* is widely used as a natural pigment in food such as chewing gum, dairy products and jellies[47]. Although, having lower comparative stability in the presence of heat and light, PC is considered more versatile due to its health-beneficial effects than that of gardenia and indigo in jelly gum and coated soft candies[48]. PBPs are also used in many other food products such as fermented milk products, ice creams, soft drinks, desserts, sweet cake decoration, milk shakes, maintaining the color for at least 1 mo at room temperature. Experiments of

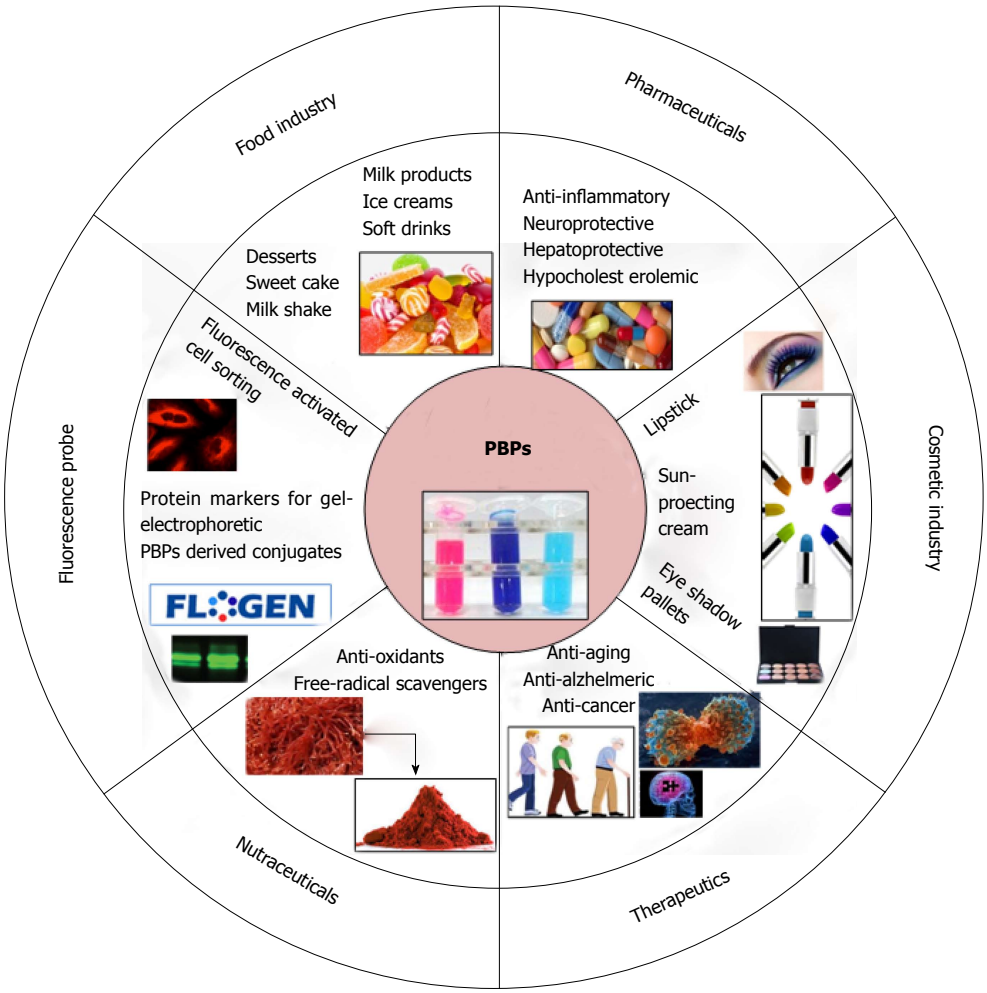


Figure 5 Application of phycobiliproteins in food, cosmetics, pharmaceutical and biomedical industries. PBPs: Phycobiliptoteins.

Jespersen *et al*[48] (2005) regarding the stability PC as colorants, stated that the shade of blue color produced from the red microalga *Phorphyridium aerugineum* does not change with pH (between 5 to 9 pH), temperature (up to 60 ℃) and in the presence of light. However, it is susceptible towards high temperature (> 60 ℃) and within the highly acidic and alkaline pHs. In addition to its colouring properties, PE possesses a yellow fluorescence. Transparent lollipops made from sugar solution, dry sugar-drop candies for cake decoration (that fluoresce under UV light), and soft drinks and fluorescence alcoholic beverages were prepared and tested as a results of enormous efforts to exploit this spectral characteristic[49].

Pharmaceutical and neutraceutical

PBPs isolated from various cyanobacterial species have been reported to show a variety of pharmacological and health beneficial effects including antioxidant, anticancerous, neuroprotective, anti-inflammatory, hepatoprotective and hypocholesterolemic. A number of physiological abnormalities have been averted in various experimental animals by PBPs administration[50-54]. When PC was evaluated as an antioxidant *in vitro*, it was found to scavenge alkoxyl, hydroxyl and peroxyl radicals, and inhibits microsomal lipid peroxidation induced by Fe^{+2} – ascorbic acid or the free radicals initiators 2, 2′-Azobis (2-amidinopropane) dihydrochloride (AAPH)[55-58]. PC reduces the levels of tumor necrosis factor in the blood serum of mice-treated with endotoxin and it showed neuroprotective effects in the rat cerebellar granule cell cultures[59]. PC has been reported to inhibit the cell proliferation of human leukemia K562 cells in a dose- and time-dependent manner[57]. APC, at nontoxic concentration to the host cells (0.045 ± 0.012 μmol/L), was found to inhibit enterovirus 71- induced cytopathic effects, viral plaque formation, and apoptosis[60]. Bei *et al*[61] (2002) found the role of PE in improving the selectivity of photodynamic therapy treatment of mouse tumor cells S180 and human liver carcinoma cells SMC 7721. Sonani *et al*[43,62] (2014a, 2014b) has recently reported the anti-oxidant based anti-aging activity and anti-Alzheimeric potential of PE (isolated from *Lyngbya* sp. A09DM) in wild type and transgenic *Caenorhabditis elegans* (*C. elegans*) (Figure 6). Singh *et al*[63] (2014) have recently given the putative therapy of Alzheimer's disease (AD) by *in silico* molecular docking between the structures of PC and β-secretase (molecule assumed to

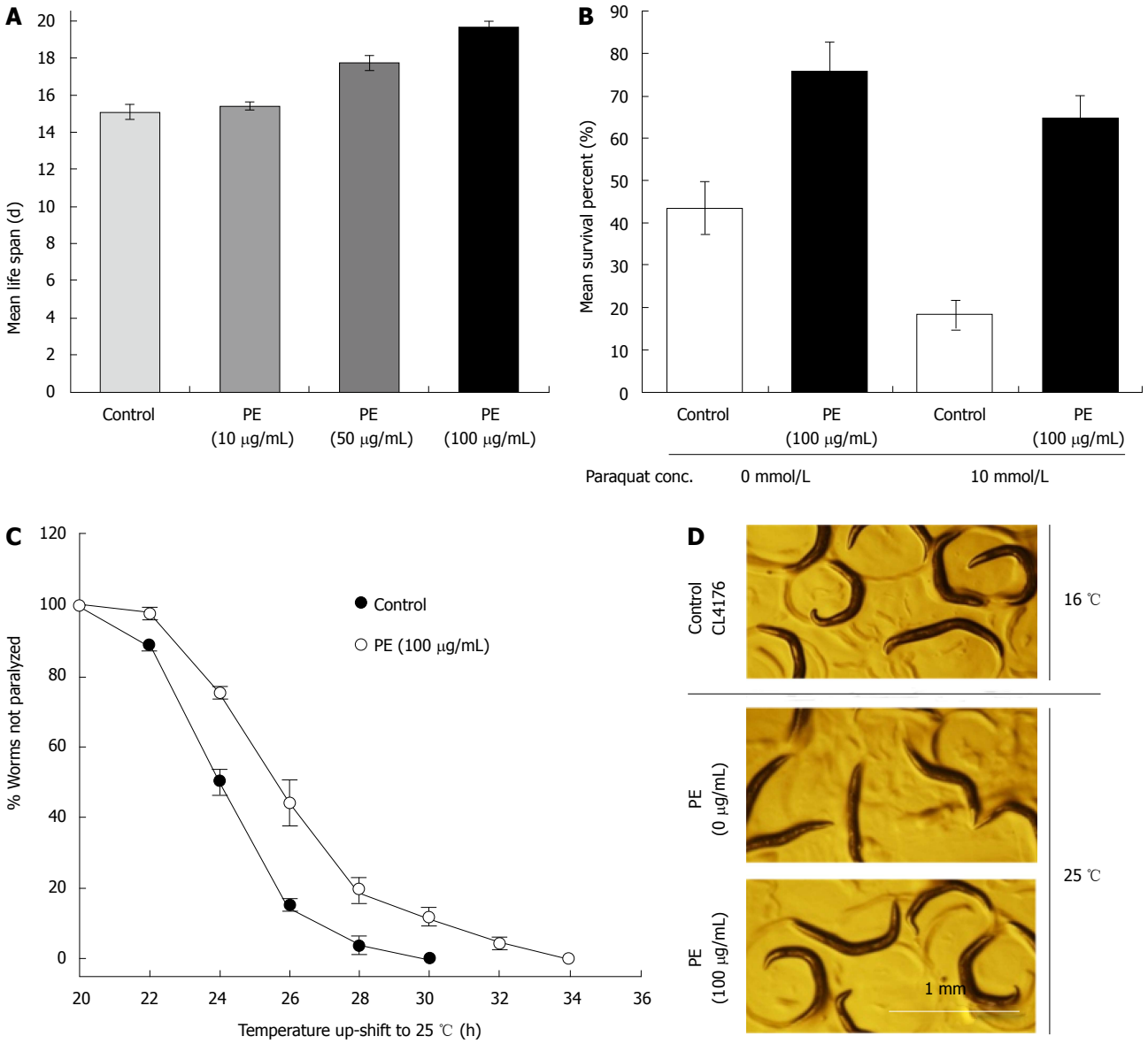


Figure 6 Phycoerythrin-feeding increases mean survival percentage of *Caenorhabditis elegans*. A: Phycoerythrin-feeding increases the mean life span of wild type N2 *C. elegans* in dose-dependent manner[43]; B: Phycoerythrin-feeding increases mean survival percentage of *C. elegans* in under normal and oxidative (paraquat) stressed conditions[43]; C: PE-treatment moderates the heat-induced Alzheimer associated paralysis in the muscles of *CL4176 C. elegans*[63]; D: Representative images of *CL4176* animal grown at 16 ℃, at 25 ℃ and at 25 ℃ with phycoerythrin. Phycoerythrin effectively moderates the Alzheimer associated phenotype in *CL4176 C. elegans*[63]. PE: Phycoerythrin; *C. elegans*: *Caenorhabditis elegans*.

be possible drug target for AD) (Figure 6C). However, the exact mode of actions of PBPs in various diseases is still unclear and further research is needed to elucidate the action point of PBPs in various metabolism pathways.

Fluorescence probe

High molar extinction coefficient of PBPs is attributable to trimeric and hexameric packing as well as higher number of chromophore attached to them in comparison with monomers[64,65]. Due to their unique fluorescent properties, PBPs get applications in flow cytometry, fluorescent immunoassays and fluorescence microscopy for diagnostics and biomedical research[66]. Moreover, they can be used as protein markers for electrophoretic techniques[67]. Synthesis of conjugates of PBPs with molecules having biological specificity, like immunoglobulins, protein A, biotin and avidin, were reported and showed that PBP conjugates are excellent reagents for two color fluorescence analysis of single cells using fluorescence activated cell sorter[66,68]. A series of low-molecular weight PBPs (derived from *Cryptomonad*) are evaluated for their utility for flow-cytometry labelling[69].

CONCLUSION

During last two decade, the field of PBPs research has made the significant progress. New purification methodology allows obtaining the ultra-pure PBPs at

large scale. Some new applications of PBPs in pharmaceuticals and nutraceuticals create more demand of PBPs in the commodity-markets. To meet such increasing demand of PBPs, it is very necessary to develop the new strategies for mixotrophic and heterotrophic production. A protein engineering or recombinant strategy also seems to be promising options to enhance the spectral properties and stability PBPs. Furthermore, more extensive research in the field of PBPs-application is still needed to elucidate the complete mode of action of PBPs during its anti-oxidants, anti-proliferative, anti-aging and neuro-protective effects using various model organisms.

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REVIEW

Longevity of animals under reactive oxygen species stress and disease susceptibility due to global warming

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Abstract

The world is projected to experience an approximate doubling of atmospheric CO_2 concentration in the next decades. Rise in atmospheric CO_2 level as one of the most important reasons is expected to contribute to raise the mean global temperature 1.4 ℃-5.8 ℃ by that time. A survey from 128 countries speculates that global warming is primarily due to increase in atmospheric CO_2 level that is produced mainly by anthropogenic activities. Exposure of animals to high environmental temperatures is mostly accompanied by unwanted acceleration of certain biochemical pathways in their cells. One of such examples is augmentation in generation of reactive oxygen species (ROS) and subsequent increase in oxidation of lipids, proteins and nucleic acids by ROS. Increase in oxidation of biomolecules leads to a state called as oxidative stress (OS). Finally, the increase in OS

condition induces abnormality in physiology of animals under elevated temperature. Exposure of animals to rise in habitat temperature is found to boost the metabolism of animals and a very strong and positive correlation exists between metabolism and levels of ROS and OS. Continuous induction of OS is negatively correlated with survivability and longevity and positively correlated with ageing in animals. Thus, it can be predicted that continuous exposure of animals to acute or gradual rise in habitat temperature due to global warming may induce OS, reduced survivability and longevity in animals in general and poikilotherms in particular. A positive correlation between metabolism and temperature in general and altered O_2 consumption at elevated temperature in particular could also increase the risk of experiencing OS in homeotherms. Effects of global warming on longevity of animals through increased risk of protein misfolding and disease susceptibility due to OS as the cause or effects or both also cannot be ignored. Therefore, understanding the physiological impacts of global warming in relation to longevity of animals will become very crucial challenge to biologists of the present millennium.

Key words: Reactive oxygen species; Redox regulation; Oxidative stress; Survivability; Climate change; Disease susceptibility; Global warming; Longevity; Thermal stress

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Core tip: Oxidative damages, generated by reactive oxygen species induce aging in cells *via* several senescence markers. Thermal stress under global warming can elevate oxidative damages with alleviated redox capacity in animals. Protein misfolding may also occur in animals under such conditions. Elevated temperature may also make the animals susceptible to diseases that are aggravated under thermal stress. Oxidative stress (OS) and disease susceptibility may push the animals to age faster under thermal stress. We propose a perspective by drawing relationships among rise in habitat temperature, OS, protein misfolding, disease susceptibility, aging and longevity in both poikilotherms and homeotherms.

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INTRODUCTION

Prediction of the impacts of climate change on metabolism of organisms is one of the major concerns for the contemporary eco-physiologist. Rise in environmental temperature due to multiple ecological changes is presumed to be one of the main factors altering the physiology of the inhabiting organisms. Poikilotherms can be considered as one of the major targets to be in trouble under the condition of rise in temperature of their natural habitat. These organisms do not have physiological mechanism(s) to regulate their body heat in relation to changing environmental temperature. As a result, their energy metabolism is considerably affected by changing environmental temperature[1]. On the other hand, physiological disorders noticed in organisms due to thermal sensitivity in relation to global warming or acute rise in environmental temperature are not only restricted to poikilotherms but also can affect the physiology of homeotherms and plants as well[2]. For example, heat waves generated as a consequence of global warming may have devastating impacts on plants leading to severe dehydration and drying their foliage parts[3,4]. However, this article only focuses on the influences of altered temperature due to climate changes especially on cellular active oxygen species metabolism in animals. Because, altered temperature influences physiology and that may govern their survivability and longevity. Emphasis has been paid to draw a positive correlation between oxidative stress (OS) and metabolism of animals. For example, folate metabolism has long been recognized as an important nucleic acid synthesis mechanism for proliferating cells but now it has been discovered that folate metabolism also generates reducing power through producing NADPH. Impaired folate metabolism increases cell sensitivity to OS[5]. Damaging toxicological role of surplus level of reactive oxygen species (ROS) on lipids, proteins, carbohydrates and nucleic acids as a function of thermal stress *via* multiple cellular processes are discussed in this article. Mitochondria act as the main hub for ROS and OS metabolism[6]. So, emphasis has been paid on the role and distribution of mitochondria in cells of animals on aging as a function of geographical distribution. Finally, correlations have been drawn among OS, metabolism, protein misfolding, disease susceptibility, ageing and longevity in animals. In spite of homeotherms having different adaptive capacity against thermal stress in comparison to poikilotherms, it has been argued, why both are susceptible to toxic effects of ROS and will experience OS under rise in habitat temperature.

ACTIVE OXYGEN SPECIES METABOLISM

ROS are toxic to cells and therefore, studied under toxicology because they non-specifically damage all the cellular biomolecules. ROS or active oxygen species are usually used as ecotoxic biomarkers in various climatic studies[6]. Metabolism of active oxygen species includes the pathways where O_2 performs a central role directly or indirectly to regulate the biochemical processes related to oxidation of nutrient molecules to produce energy. As a result of the above process, different ROS are produced as by-product due to incomplete reduction

of O_2. Surplus ROS are highly toxic to cells[6]. If the generated ROS in cells are not neutralized immediately, they can damage the macromolecules present in their vicinity in the cells. This ROS induced cellular damage is now well established and is called as OS. Generation of OS in cells is considered as one of the markers of toxicity and is correlated with several senescence related processes. Physiology of OS comprises of oxygen (O_2) respiration by mitochondria, leakage of O_2 to produce ROS and oxidation of tissues by ROS, responses of both enzymatic and non-enzymatic redox regulatory molecules against the produced ROS and oxidative phosphorylation and generation of ATP molecules in mitochondria *etc*. The state of OS in eukaryotes occurs either due to over production of cellular ROS or due to the diminished redox regulatory capacity to scavenge the (over) produced ROS. Both the above conditions also can arrive in cells to generate OS. As stated above, the generated ROS if not neutralized immediately, can oxidize lipids, protein and DNA present in the cells which leads to OS. ROS, such as superoxide radical ($O_2^{\bullet -}$), hydroxyl radical and hydrogen peroxide (H_2O_2) are generated mainly due to incomplete reduction of O_2 during cellular respiration. They are non-specifically highly reactive in nature and can oxidize lipids, proteins and nucleic acids to lipid peroxides, protein carbonyls and respective nucleic adducts, few of the important oxidised products of macromolecules, respectively[6]. As a result of oxidation of the above biomolecules, reduced efficiency of enzymatic and other functions of proteins, loss of membrane fluidity, unwanted reduced or alleviation in gene expression, complete or partial arrest in several anabolic processes occur in cells. It is noteworthy that ROS (for example H_2O_2) that are also reported to be useful as at lower concentrations, they mediate several cellular signal transduction processes[7,8]. However, under abnormal conditions due to external (mainly environmental) or internal (cellular) factors including thermal stress, maintenance of the nominal amount of ROS to regulate signal transduction processes is not ensured[7].

Aerobes are equipped with both enzymatic as well as non-enzymatic antioxidant defenses to counteract the over production of ROS. Superoxide dismutase (SOD), the first enzyme of enzymatic antioxidant defense, dismutates the toxic superoxide radicals to H_2O_2 and molecular oxygen[9]. H_2O_2 (another toxic oxidant) is further neutralized by two cellular enzymes, catalase (CAT) and glutathione peroxidase (GPx)[10]. CAT breaks down H_2O_2 to H_2O[11] and O_2 while GPx reduces H_2O_2 and organic hydroperoxides to H_2O and other non-reactive metabolites at the cost of oxidation of a reduced glutathione (GSH) molecule. The oxidized glutathione is reduced back to GSH by the enzyme glutathione reductase with the help of the reduced nicotinamide adenine dinucleotide phosphate. Peroxiredoxins are a group of ubiquitous antioxidant enzymes that regulate the levels of cytokine-induced peroxides[12]. The oxidized form of peroxiredoxins is non-catalytic in nature. To self recharge after reducing H_2O_2, these enzymes require thioredoxin[13]. They require electrons from the reduced thioredoxin to restore their enzymatic catalytic function[14]. Thioredoxins are a class of small ubiquitous redox proteins which also play a key role in removing ROS[15,16]. Their redox functions are mainly due to the reduction of other proteins by cysteine thiol-disulfide exchange[17,18]. Due to the above redox regulatory function of thioredoxins, their roles in reducing OS and decrease in aging have been established in thioredoxin over expressed mice models[19,20]. Glutaredoxins are a group of small redox enzymes which confer their antioxidant activity by reducing dehydroascorbate, peroxiredoxins and methionine sulfoxide reductase[17,21-23]. Non enzymatic antioxidant defence system comprises of small molecules such as ascorbic acid, vitamin E and GSH which directly scavenge ROS[6]. Under thermal stress, the levels of redox regulatory molecules are found to be alleviated or insufficiently enhanced which fails to combat OS in organisms[24] (Figure 1).

OS, temperature and aging

A hypothesis describing one of the important causes of aging postulates that the senescence-associated loss of functional capacity is due to the accumulation of toxic ROS and its consequences as molecular oxidative damage[25]. Tissue repair and regeneration is an important topic in aging. The link between aging and inflammation is highlighted in many studies[26]. Chronic, progressive low-grade inflammation can accelerate aging *via* ROS-mediated exacerbation of telomere dysfunction and, cell senescence, thereby indicating that aging, inflammation, necrosis factor Kb, DNA damage and cellular senescence are closely linked[26]. Aging is characterized by a progressive decline in the efficiency of physiological function and by the increase in susceptibility to disease and finally to death. Publications from different research groups indicate that ageing has a strong positive correlation with OS in animals[27,28]. The "free radical theory of aging" is one of the most plausible and acceptable explanations for the mechanistic basis of aging which postulates that aging and its related diseases are the consequence of free radical-induced damage to cellular macromolecules and the inability to counterbalance the produced high level of ROS by endogenous anti-oxidant defenses[29]. This leads to alteration of the nature of membrane fluidity due to lipid oxidation, the reduced enzymatic and other functions of proteins due to oxidation of proteins and alternation of gene expression due to DNA damage. The origin of this explanation has a foundation in the "rate of living theory" and longevity of an organism is thus supposed to be influenced by its rate of cellular active oxygen species metabolism especially OS status. In this context, mitochondrial rate of ROS production is more important. Indeed, the mitochondrial rate of free radical production seems to have a much stronger correlation

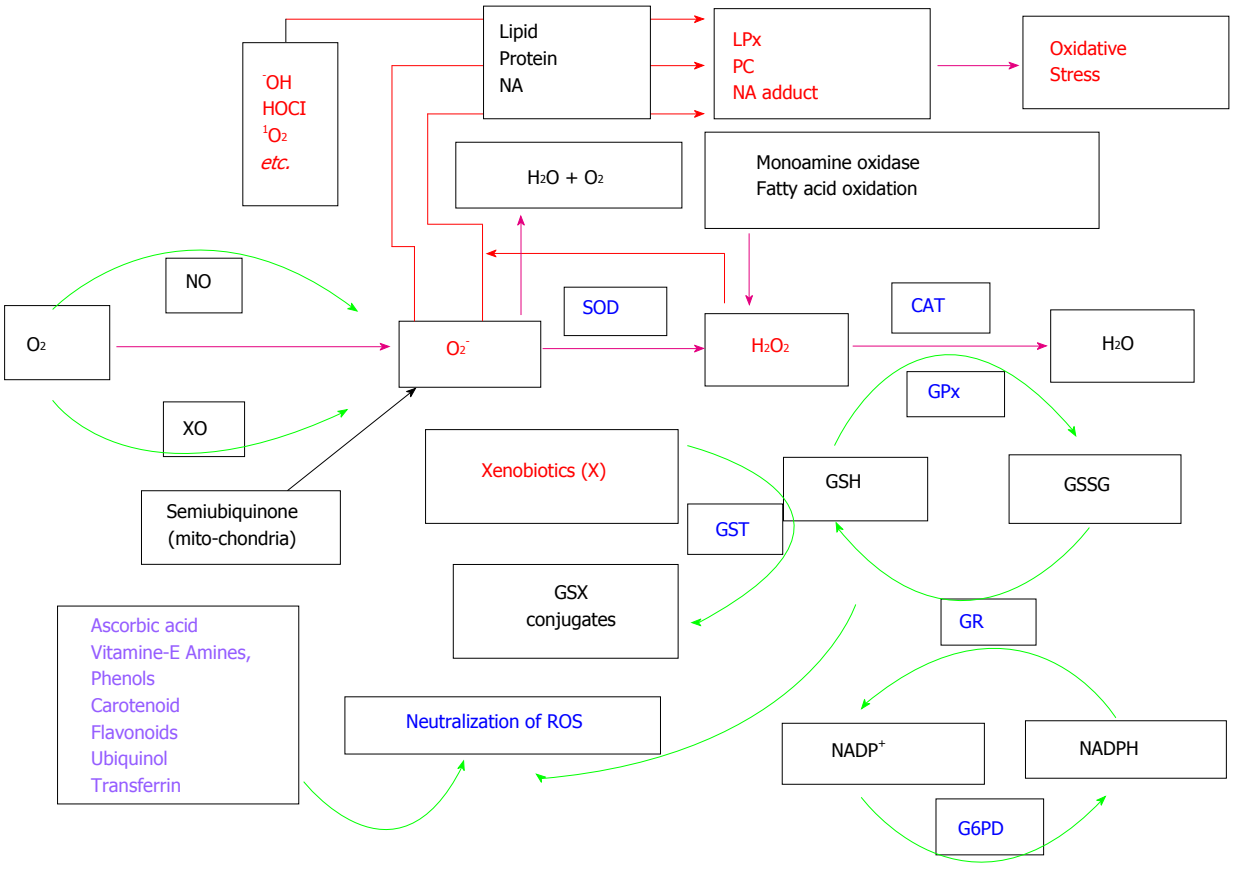


Figure 1 Pathways of active oxygen species metabolism. OS physiology starts with O_2 consumption in animals. O_2 is incompletely reduced to super oxide radicals (O_2^-) *via* NO or XO or in mitochondria due incomplete reduction. O_2^- is dismutated to H_2O_2 by enzyme SOD. H_2O_2 is then scavenged by either CAT, or glutathione peroxidase with the help of the reduced glutathione (GSH, which form oxidised glutathione, *i.e.*, GSSG). GSSG is recycled to GSH by the enzyme GR with the help of NADPH (which is converted into NADP+). NADP+ is reduced back to NADPH by the enzyme G6PD. GST is also responsible to remove xenobiotics (which are responsible to produce ROS) from cells with the help of GSH. Peroxiredoxins and thioredoxins system and glutaredoxins are responsible for scavenging H_2O_2 and reduction of other proteins (not shown in Figure). Redox regulatory non-enzymatic molecules such as ascorbic acid, flavonoids and phenols can also remove ROS such as OH, HOCL, O_2, O_2^- and H_2O_2. With insufficient antioxidant defence, more ROS accumulation in cells occurs and it leads to oxidation of biomolecules such as proteins, lipids and nucleic acids to form PC, LPx and NA adducts, respectively. Formation of LPx, PC and NA leads to a disorder condition called as oxidative stress. OS: Oxidative stress; NO: Nitric oxidase; XO: Xanthine oxidase; SOD: Superoxide dismutase; CAT: Catalase; GR: Glutathione reductase; G6PD: Glucose-6-phosphate dehydrogenase; GST: Glutathione-S-transferase; PC: Protein carbonyls; LPx: Lipid peroxides; NA: Nucleic acid.

with maximum longevity in animals[30,31].

In the modern aging theory, the "rate of living" is defined by metabolism rather than the loss of vital substance, although, advances in aging research continue to challenge this theory. The fiction in modern "rate of living" theory is whether metabolic rate or metabolic stability is the determinant of longevity is still under debate. In a review article, López-Otín *et al*[32] summarized the cellular and molecular hallmarks of aging, and proposed nine candidate hallmarks such as genomic instability, telomere attrition, epigenetic alterations, loss of proteostasis, deregulated nutrient sensing, mitochondrial dysfunction, cellular senescence, stem cell exhaustion and altered intercellular communication. Many of the above factors are directly or indirectly found to be the "cause" or "effects" of OS and ageing in animals[6]. Recently, Missios *et al*[33] reported the first experimental evidence that telomere dysfunction enhances the requirement of glucose substitution for the maintenance of energy homeostasis and IGF-1/mTOR-dependent mitochondrial biogenesis in aging tissues.

Climatic factors have significant effects on the active oxygen species metabolism and also on the life cycle of animals[10,34,35]. Especially, temperature has a very strong and positive correlation with cellular levels of both ROS and OS[6]. This particular phenomenon is also found to be true at mitochondrial level in certain ectotherms[36-39]. The main reasons for high cellular ROS and OS are attributed to malfunction of the mitochondrial complex enzymes especially complex Ⅰ and Ⅲ or over expression of certain complex enzymes with uncoupling between electron transfer or leakage in electron transport chain and oxidative phosphorylation[40]. The later case may appear due to disturbance in chemi-osmotic balance between inter membrane space and matrix of mitochondria. It leads to a less efficient oxidative phosphorylation state/system which results in reduced rate of ATP generation. In aquatic ecosystems where temperature is elevated sharply, the availability of environmental O_2 is also depleted due to its inverse correlation with temperature. Less environmental O_2 results in decrease in O_2 uptake and the final availability

of O_2 to mitochondria also becomes less. Availability of less O_2 in mitochondria may also leads to less ATP generation as O_2 is one of the key molecules in the final step reaction of oxidative phosphorylation where, it is reduced to form water molecule[41]. Less cellular ATP level leads to metabolic depression and in extreme cases it ends in with collapse of cellular metabolism and finally death of animals[39]. A positive correlation is also observed between temperature and production of ROS in mammalian mitochondria. Rise in temperature, therefore, may lead to an undesired state with unfavorable conditions such as cellular OS due to high level of ROS, less ATP level and metabolic depression. Insufficient expression of redox regulatory molecules under energy deficient condition may also attribute to the increase in ROS level and OS in animals[29]. The levels of the above state can be augmented under thermal stress. High ROS level under thermal stress condition also may lead to chromosomal aberrations. In totality, the above reasons altogether may direct to hinder the normal physiology of animals and it may negatively influence their survivability under the state of thermal stress. In extreme cases, animals may encounter permanent metabolic depression. Therefore, it can be predicted that continuous exposure to gradual or sudden rise in habitat temperature may elevate the toxic active oxygen species anabolism and can thus adversely influence survivability and finally the longevity of the inhabiting animals.

Although, not many experimental records are available directly on correlation between rising temperature and longevity in animals, Auad *et al*[42] observed that rise in temperature in combination with elevated CO_2 level decrease the duration of nymphal stadia, the longevity and reproductive success of the aphid *Sipha flava* (*S. flava*). Based on their results, they predicted that populations of the aphid *S. flava* may significantly decrease under future climatic conditions when the concentration of atmospheric CO_2 and temperature are projected to increase. Similarly, several authors also have pointed that increase in habitat temperature above 20 ℃-24 ℃ (which is the optimum temperature required for the growth of aphids) can directly decrease or negatively influence the fertility, reproduction, development, life expectancy, survival and abundance of aphids[43,44]. Although, effects of temperature on the life cycle and above mentioned related processes were studied in aphids with an objective to control their population for the purpose of pest management, negative influence of rise in temperature on the life cycle of insects in general and aphids in particular cannot be ignored. This is because, both increase in CO_2 level and rise in temperature may affect the life cycle of herbivorous insects indirectly by influencing host plant physiology and phytochemistry[45]. As a result, it affects the food quality and food intake rate of the herbivorous insects[45]. From the above discussion, it seems that focus has been given by researchers to investigate particularly how insects respond to climatic changes[46-48]. Global climatic changes may alter ecosystem functioning and species interactions by promoting a shift in the geographical range of herbivores[49], by facilitating the spread new adventives and invasive species[50], and by altering the natural climatic control of herbivorous species[49]. As a result, due to a shift of the normal life processes of herbivores, it can affect the life of carnivores by influencing the prey-predator relationship[51,52]. Occurrence of the above multiple processes as the consequences of climatic changes are not only restricted to terrestrial ecosystems but also can affect the aquatic ecosystems. For example, in aquatic environment especially in coastal marine bodies, climatic changes also negatively modulate the active oxygen species metabolism as well as the other physiological processes such as reproduction, excretion and respiration of the inhabiting ectotherms[3,36,38,39,53].

The effects of climate changes on population dynamics of animals are of critical concern[54,55]. The enhancement in global warming is primarily due to increase in atmospheric CO_2 level produced from anthropogenic activities and secondarily due to other reasons such as chlorofluorocarbons, halons, methyl chloroform, methyl bromide and carbontetrachloride[56-58]. Irrespective of the sources, rise in CO_2 level along with environmental temperature may influence the longevity of both poikilotherms and homeotherms. However, the poikilotherms are predicted to be more venerable to the above state under rise in mean global temperature due to lack of ability to regulate their body temperature[3,59]. Irrespective of the capacity of thermo regulation in animals, the nature of food habit also can be an indirect factor that may influence the longevity of animals under the rise in environmental thermal stress. This can be correlated to the alterations in biochemistry and physiology in plant tissues under altered climate-controlled conditions. As a consequence, it can affect food quality for herbivores and may modulate the performance of herbivorous animals in general and insect species in particular[60,61]. Although, elevated CO_2 levels tend to increase the rate of photosynthesis and plant biomass[62,63], it reduces host plant quality for herbivores because of increase in foliar carbon and secondary organic compounds[60,63] and decrease in availability of nitrogen[64,65]. Finally, this could indirectly affect all the herbivores in general and arboreal herbivores in particular due to food quality and their host-plant dependency.

Mitochondria, the main hub for ROS production

Mitochondria of cells that act as the hub for ATP production are the main centers for generation of ROS or active oxygen species. ROS are produced in mitochondria as a result of incomplete reduction of O_2 due to leakage of electrons at the complex enzyme centers of inner mitochondrial membrane[66]. Especially, leakage of electrons at complexes Ⅰ and Ⅲ reduces O_2 for formation of ROS such as O_2^-, H_2O_2 and ^-HO (Figure 2). In the matrix of mitochondria, a successful electron transport

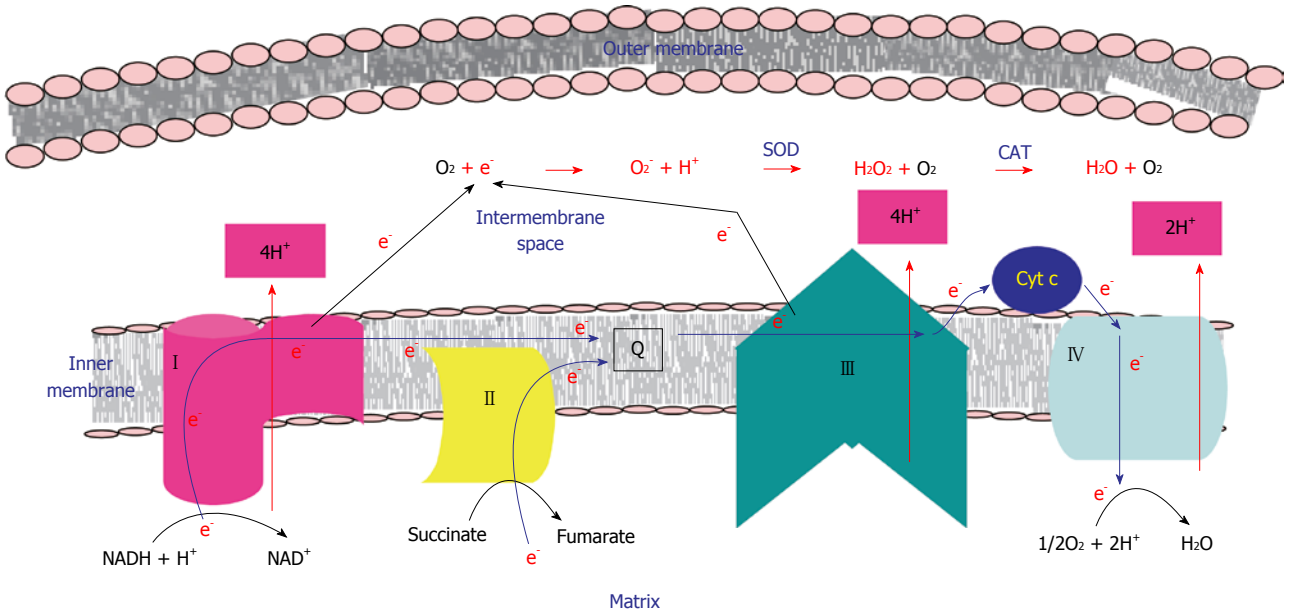


Figure 2 Production of reactive oxygen species in mitochondria. ETC enzymes such as Ⅰ (complex Ⅰ), Ⅱ (complex Ⅱ), Ⅲ (complex Ⅲ) and Ⅳ (complex Ⅳ) are shown to be located in the inner membrane of mitochondria. During electron transport in ETC, e^- *via* complex Ⅰ (from NADH) and via complex Ⅱ (from $FADH_2$) are passed to complex Ⅲ through Q and then subsequently passed to complex Ⅳ. In the sequence, they are finally delivered to the reaction in which O_2 is reduced to H_2O. In coupled with the above process, protons are pumped to intermembrane space *via* complexes Ⅰ, Ⅲ and Ⅳ. This forms a chemiosmotic gradient to avail free energy for synthesis of ATP molecules *via* complex Ⅴ enzyme (not shown in the figure). However, due to leakage of electrons at complex Ⅰ and complex Ⅲ enzymes during electron transport, O_2 molecules are incompletely reduced to form ROS such as O_2^- anion radical, H_2O_2 and OH radical (formed due to the interaction between O_2^- and H_2O_2). Enzymes such as SOD and CAT catalyzes the reactions at the respective steps as shown in the figure. It leads to production of subsequent other ROS. Blue thick arrows indicate direction of flow of electrons in ETC. Red arrows indicate pumping of protons from matrix to intermembrane space. Black arrows indicate leakage of electrons to intermembrane space[40]. ETC: Electron transport chain; e^-: Electrons; Q: Ubiquinol; O_2^-: Superoxide; H_2O_2: Hydrogen peroxide; OH: Hydrogen; SOD: Superoxide dismutase; CAT: Catalase.

from several universal electron acceptors to O_2 molecule leads to the development a chemiosmotic gradient across the inter membrane space and matrix[67]. The process is accompanied by H^+ pumping into the inter membrane space *via* complex Ⅰ, Ⅲ and Ⅳ enzymes and then its back movement into the matrix *via* complex Ⅴ enzyme (ATPase) along its concentration gradient. The free energy available in the last step is coupled with ATP synthesis by the F_0F_1-ATP synthase complex and the process is referred as oxidative phosphorylation[68]. Under several pathophysiological disorder conditions, more electrons leak particularly at complex Ⅰ and Ⅲ enzymes which then reduce O_2 to O_2^-[69,70]. Then the second and third reduction of O_2^- produces the other two ROS namely the hydroxyl radical ($^-$OH) and H_2O_2. It indicates that the process of oxidative phosphorylation includes both, the energy producing machinery culminating with ATP production, and generation of ROS in the mitochondria. The whole machinery of electron transport chain and oxidative phosphorylation works as a function of O_2 uptake by mitochondria. Therefore, measurement of the rate of mitochondrial respiration with oxidative phosphorylation and function of electron transport chain are the important indices for studying the mechanism of ROS generation, OS induction and the energy status of the organism[71,72]. Analysis of OS indices, levels of ROS and redox regulatory parameters has immense importance in several core evolutionary concepts of animal biology such as in life history trade-offs, sexual selection and senescence in free ranging organisms[73]. Therefore, much importance has given to cell senescence and aging in animals in relation to the toxic role of active oxygen species anabolism in mitochondria[6].

Mitochondrial distribution and aging

Global warming is likely to uncouple and alter the phase relationships existing between temperature and photoperiod and it may have significant consequences on animal's reproduction and longevity. For example, it can affect life cycle of organisms that develop gametes during the winter and spawn in the spring in temperate northern latitudes[3]. Therefore, it is predicted that environmental factors related to climate change especially rise in temperature is more likely to have significant impacts on fecundity and spawning success in animals[3]. Ageing and longevity in animals are correlated with each other and the active oxygen species production may act as a link between the above two. Therefore, fecundity and ability of mitochondria in animals in relation to their colder and warmer habitat must have a major role in relation to the current topic. Because mitochondria act as the hub for the production toxic active oxygen species[6,36]. In this context, studies in both laboratory and field intervention indicate that not only temperature acts as a major factor under climate

change by contributing to the production of toxic active oxygen species in animals but also other environmental factors such as salinity and O_2 tension can contribute to influence it[36,74-76]. Mitochondrial function and production of ROS in the intertidal mud clam *Mya arenaria* collected from the colder parts of the globe is found to be positively correlated with temperature[53]. Similarly, the production of active oxygen species in aquatic organisms can vary as a function of the climate change especially temperature[39,77]. As per the prevailing climatic changes, the fecundity, function, efficiency, cellular distribution of mitochondria can vary in the inhabiting organisms[78-80]. For example, rise in temperature can alter the above functions of mitochondria[81]. Even a very acute rise in habitat temperature can influence the respiration, mitochondrial efficiency and thereby, increases the oxidative injury in hepatic tissues of broiler chickens[82]. It is reported that telomere dysfunction enhances the requirement of glucose substitution for the maintenance of energy homeostasis and IGF-1/mTOR-dependent mitochondrial biogenesis in aging tissues. Based on the above facts in relation to mitochondria, the geographic distribution of organisms through invasiveness to other regional of suitable temperature has been also proposed[83]. So, invasiveness of animals to a comparatively colder or warmer habitat on the basis of mitochondrial thermal adaptation may be correlated to an evolutionary objective in animals to live longer in the best suited habitat. Therefore, the geographic distribution and longevity in animals ranging from bivalves to birds based on their oxidative metabolism are closely correlated with each other[31].

Although climate change and human longevity are very interesting issues, it is pity that there are very few commentaries found in literature. It is speculated that the longevity of animals living in colder climates should be higher than those live in warmer climates. A very relevant logical discussion is made on this particular issue at "longevity-and-antiaging-secrets.com"[84]. It is noticed that vegetation in colder regions lives longer, for example, mosses in tundra climate can live up to 150-200 years which is too shorter in warmer regions. The regions are mainly attributed to high fecundity of mitochondria with low cellular metabolic rate. So, it can also be predicted that the more the number of mitochondria, the higher the longevity in animals including human being can be expected. For example, the northern people live longer and people exposed to hunger and hot climates during generations have shorter life period. The amount of sunlight people get in colder climates (which affects vitamin-D levels) and also seasonal disorders can be the reasons to affect human longevity. The fact is explained as the increase in aging of the skin (which is also a part of ageing and related to vitamin-D level) of people who live in sunnier climates. The skin of people in hot climates certainly seems to age faster than that of people who live in colder climates[84]. Although the determining factor is exposure to sun light rather than temperature, both have always a positive correlation with each other. It can be concluded that with multiple reasons, people living in colder countries have higher life expectancy than the people living in warmer countries[85]. It is observed that in polar countries the life expectancy is > 60 years while it is < 40 years in courtiers present in equatorial lines[84].

GLOBAL WARMING, HUMAN HEALTH AND AGING

Climate and weather are important abiotic components of complex ecosystems. The dynamic balance between the biotic and abiotic components of ecosystems is often disturbed if changes occur in climate or weather[86]. Instability in any ecosystem due to changes in its abiotic components especially temperature can increase the risk of changes in pathogen prevalence, altered pathogen transmission profiles, and increase in host susceptibility[59]. These instabilities can have dramatic effects on human's health, livestock, wildlife and marine systems[87,88]. The World Health Organization (WHO) warned in its 2007 report that infectious diseases are emerging at a rate that has not been seen before. Since the 1970s, more than 40 infectious diseases have been discovered, including SARS, MERS, Ebola, avian flu, and swine flu. It was predicted that the ranges of infectious diseases and vectors will be changing in altitude, along with shifts in plant and animal communities and the retreat of alpine glaciers. Additionally, extreme weather events especially gradual increase in global temperature creates conditions conducive to "clusters" of insect-, rodent- and water-borne diseases[89]. Accelerating climate change carries profound threats for public health and society also[89]. The key point of presenting such information is that susceptibility of an animal to disease is always has negative correlation with its longevity and a positive correlation with OS. Therefore, attribution of climate change including global warming to longevity of animals including human through susceptibility to different diseases may not be ignored.

Effects of global warming on human body

A very nice relationship is drawn by Goldsmith *et al*[58] among global warming, exposure of human to ultraviolet radiation (UV) (due to depletion of ozone layer) and longevity through disease (such as cancer and increased risk of health disorder processes including immunosuppression) susceptibility. Rise in CO_2 level may not be the single factor responsible for global warming. Several chemicals such as chlorofluorocarbons, halons, methyl chloroform, methyl bromide and carbontetrachloride are also responsible for global warming and they act as ozone-depleting substances too. The ozone layer absorbs most of the UV radiation of sun and protects plants and animals from exposure to such radiation. Fortunately, where a high probability is there for damage of human DNA upon exposure to UV radiation, ozone layer strongly absorbs it. When chlorofluorocarbons

and halons are released into the atmosphere, they rise slowly to reach the stratosphere. Once they reach there, under the influence of the sun's ultraviolet light, chlorine is released and reacts with ozone. As a consequence, depletion of the ozone layer starts. Finally, it ends with hole(s) in ozone layer. When UV and visible radiation from sun strike the skin through such holes in ozone layer, part is remitted, part is absorbed by chromophores in various layers of skin, and part is transmitted inward to successive layers of cells until the energy of the incident beam is dissipated. Two major physical processes namely absorption and scattering with varying wavelengths limit the penetration of UV and visible radiations into skin. UV wavelength less than 320 nm are readily absorbed by proteins, DNA and other components of epidermal cells and UV radiation rays are energetically enough powerful to break the bonds of DNA molecules, and thereby damage cells by the process of mutation, unwanted gene expression, *etc*. These damaged DNA molecules are not repaired, and can replicate, leading to dangerous forms of skin cancer (basal, squamous, and melanoma). Prolonged human exposure to solar UV radiation may result in acute and chronic health effects on the skin, eye and especially on immune system[90]. Acute complaints like heat strokes, itchy plaques, actinic prurigo, papular lesions, urticaria, photoallergy *etc*. may occur in human. The chronic effects of UV exposure can be much more serious, even life threatening and include premature aging of the skin, suppression of the immune system, damage to the eyes, and finally skin cancer[90]. In the most serious cases, skin cancer and cataracts can occur commonly due to chronic inflammation. According to a report from WHO, a growing body as evidence suggests that environmental levels of UV radiation may suppress cell-mediated immunity and thereby enhance the risk of infectious diseases and limit the efficacy of vaccinations[91]. UV radiation induced immunosuppression and photocarcinogenesis are two important phenomena that can affect human longevity. It is important to note that the immunosuppressive properties of UV radiation refer exclusively to the adaptive immune response. T-cell mediated immune reactions are suppressed on UV exposure but not host defence reaction against bacterial attacks[58]. Under these conditions, the highest risk of skin cancers that appear to accompany is exposure to UV radiation from sun through the holes in ozone layer (that are created indirectly due to global warming). Therefore, direct impact of UV radiation induced immunosuppression on life expectancy is anticipated. The fact is partially supported by a study over 300000 people born in Maine. The people who were born in a peak solar year lived 1.5 years less than those born in non-peak years[91].

Global warming and vector born diseases

ROS produced by phagocytes are critical for host defense against infection[92]. The negative regulator of ROS production, which is localized to the endoplasmic reticulum of phagocytes, has been discovered[93]. On the other hand, climate change and its effects on emerging infectious diseases were elegantly predicted by Epstein[89] in 2001. Especially infection rate of diseases those are insect vector born can be aggravated in comparatively warming habitats. For example, diseases carried by mosquito vectors are particularly sensitive to meteorological conditions. Excessive heat kills mosquitoes, but within their tolerance and survivable range, warmer temperatures increase both their reproduction and biting activity[94] and the rate at which pathogens mature within them. McArthur[95] had determined in 1972 that at 20 ℃, *Plasmodium falciparum,* a malaria causing protozoa, takes 26 d incubation time but just at rise in 5 ℃ higher temperature, *i.e.,* at 25 ℃, they develop in 13 d. So, warmer temperatures could mean longer and more frequent waves of mosquito infestations. A very small change in the Earth's temperature has a big impact on the above process[96]. The longevity of *Anopheles* mosquitoes, the carriers of malaria is only few weeks. Therefore, increase in temperature, permits the parasites to mature in less time in the body of mosquito to transfer the infection[95]. Due to the temperature thresholds in *Anopheles* mosquito, the transmission of falciparum malaria occurs where temperature exceed 16 ℃[97]. The mosquito *Aedes aegypti,* who acts as vector for both yellow and dengue fever, is restricted by the 10℃ winter isotherm. Temperature less than that can slow their life cycle and under freezing temperatures, eggs, larvae and adults of *Aedes* are killed. Expanding tropical conditions can also elevate the transmission rate of the disease. Warm nights and warm winters favour insect survival. Therefore, rise in temperature can be positively correlated with the risk of disease susceptibility in human and it can affect the longevity[87-89,98].

Temperature, protein misfolding, diseases and longevity

One of the vital elements required for maintenance of the structural architecture of cells is protein. At least 30000 different proteins with a different role are identified in humans[99]. The main functions of proteins in all organisms are to make cellular building blocks and to operate cellular functions. To fulfill specific function(s), each protein has to maintain its own unique sequence and shape in native conformation. Protein folding is the process by which the newly synthesized protein molecule folds into its unique, three-dimensional native structure[100]. The sequence of protein synthesis in relation to folding may be described as the synthesis of primary amino acid chains (peptides) which joined together to form secondary α-helix and the β-sheet and random coils of peptides. It can further fold to have the tertiary structure refers to the distribution of both α-helices and β-sheets and random coils in the protein, wherein, these elements are folded into a compact conformation stabilized by hydrogen bonds or

ionic interactions[101]. These are referred as monomeric proteins. The monomeric proteins after formed in three-dimensional structure can become multimeric form and the conformation is called as quaternary structure of proteins in which the different polypeptide chains are connected. The required native conformations of proteins in cells are achieved by post translational modifications including folding[101].

Several molecular and cellular mechanisms are associated with protein folding. One of such examples is the involvement of chaperons[102]. On the other hand, synthesis of incorrectly shaped (misfolded) proteins occurs in cells due to many factors. One of such factor is exposure to environmental changes[99]. More specifically the degree of misfolding can increase due to exposure of animals to increase in temperature, high or low pH, agitation, elevated glucose, or oxidative damages. For examples, misfolding or denaturation of streptokinase[103] and β-lactoglobulin[104] at increased temperature and SOD[105], LDL, ApoC-Ⅱ[106,107] due to oxidative damages are reported. The rate of misfolding is accelerated by the altered amino acid composition in proteins due to mostly mutations. Protein misfolding and the subsequent aggregation are associated with various, often highly debilitating, diseases for which no sufficient cure is available yet. Although, many recent efforts have been made to develop treatments for diseases associated with protein misfolding, not much success has been achieved. Diseases that are associated with protein misfolding represent a group of disorders that have protein aggregation and plaque formation in common[108]. Amyloidosis is a group of such protein misfolding diseases, in which protein aggregates accumulate either systemically or locally in certain organs or tissues. Other neurodegenerative diseases namely Alzheimer's disease (Amyloid-β, tau), Parkinson's disease (α-synuclein), Huntington's disease (Huntington), familial British dementia (Abri), Spongiform encephalopathies (Prion), hereditary cerebral hemorrhage with amyloidosis (cystatin C), amyotrophic lateral sclerosis (CuZn SOD) and other diseases such as diabetes mellitus (IAPP, amylin), atherosclerosis (modified lactate dehydrogenase) and sickle cell anemia (hemoglobin) are found to be the leading protein disorder related diseases[99]. These protein misfolding diseases share similar pathological aspects including hypertension, OS, or hyperglycemia, and all of these can result in protein misfolding[101]. All the above diseases or pathways are found to be influenced by climatic changes in animals. It is now clear that both temperature and OS cause protein misfolding and associated diseases. Particularly, protein misfolding is highly temperature dependent[108,109] and under rise in environmental temperature, the risk of disease susceptibility is also predicted to influence ageing and longevity in animals *via* protein misfolding. Therefore, rise in environmental temperature can raise OS in one hand and increases the risk of protein misfolding and disease susceptibility on the other hand in animals including humans. As a result it can influence the longevity in animals.

ADAPTATION OF ANIMALS AND OS UNDER HIGH HABITAT TEMPERATURE

Adaptation mechanism and OS in animals

Many animals including vertebrates maintain various long or short term adaptation strategies to cope with fluctuation in environmental temperature. Hibernation and aestivation are two such examples during which animals cope with fall or rise in environmental temperature, respectively. Aestivation is a state of dormancy or seasoning noticed in animals and is characterized by inactivity and metabolic depression in response to high temperatures and arid conditions[110]. It takes place during times of hot and dry season of the summer months. Fossil records show that the act of aestivating may be several hundred million years old. The depression of metabolic rate during aestivation causes a reduction in macromolecule synthesis and degradation. To stabilize the macromolecules, aestivators usually enhance antioxidant defenses and elevate chaperone proteins. At such time period, metabolic depression related responses such as tissue specific large glycogen reserves, anaerobic glycolysis, attempt for reduced ATP generation and energy utilization with specific biochemical and transductional adjustments are few of the strategies adapted by animals to avoid environmental insults especially hyperthermia states[20,111-115]. Increase in redox status or low O_2 uptake are also two other strategies adapted by animals to keep the cellular ROS level low with reduced OS level[39,41,116]. This is one of the widely used strategies adapted across all forms of hypometabolism. These physiological and biochemical concerns appear to be the core elements of hypometabolism throughout the animal kingdom[117]. For example, the above phenomenon is observed in insects such as lady beetles (*Coccinellidae*)[118] and mosquitoes[119], in Australian land snail *Rhagada tescorum*[120], in amphibians such as *Broomistega putterilli*[121] and California red-legged frog and in water-holding frog[122], in African lungfish[123,124], in mammals[125] such as Malagasy fat-tailed dwarf lemur[126] and East African Hedgehogs[127].

From the above discussion, it is expected that under high temperature in summer season, the aestivating animals due to adaptation especially metabolic depression (here it is an adaptation) and in non-aestivating animals due to long term adaptation to temperature[128], rise in OS is not expected[6]. And, the phenomena must be true in natural populations. However, reports on natural populations of several groups of animals including ectotherms (sensitive to temperature) indicate that they experience OS under rise in temperature in summer season[129-154] (Table 1). Their redox regulatory capacity *via* different antioxidant molecules became insufficient to neutralize the over produced toxic ROS and it induces cellular OS under rise in temperature condition (Table

Table 1 Oxidative stress and redox status in natural population of various animals under high habitat temperature

Name of the animal	Modulator	Tissue/organ/cell fractions	ROS	OS	AOE	Ref.
Mytilus spp. (bivalve) and Macoma balthica (bivalve)	High temperature in summer	Whole animal	NA	NA	NA	[129]
Anodonta anatina freshwater duck mussel	High temperature in summer	Whole animal	NA	NA	NA	[130]
Perna viridis (green-lipped mussel)	Low temperature Winter	Digestive gland	H_2O_2↓	TBARS↓	SOD↓, GPx	[131]
-do-	-do-	Gills	H_2O_2↓	TBARS↓	SOD↓, GPx	[131]
-do-	-do-	Mantle	NA	TBARS↓	SOD↓, CAT↓	[131]
Crassostrea rhizophorae (mangrove oyster)	High temperature in summer	Gill	NA	Lipdperoxide	CAT	[132]
Viviparus acerosus (river snail)	High temperature in summer	Whole body	NA	NA	SOD, CAT↓, GPx↓	[133]
Ancylus Fluviatilis (lipmet)	High temperature in summer	Whole body	NA	NA	NA	[134]
Nacella (Patinigera) magellanica (limpet)	High temperature in summer	Whole body (for O_2 consumption), Digestive gland (biochemical studies)	NA	NA	SOD↓, CAT↑, GPx↑	[135]
Gammarus roeseli (freshwater gammarid crustacean)	High temperature in summer	Tissue pool	NA	MDA↑	CAT, GPx↑	[136]
Scylla serrata (mud crab)	High temperature in summer with high salinity	Hepatopancreas	H_2O_2↑	TBAR↑, PC↑	SOD↓, CAT↑, GPx↓	[34]
-do-	-do-	Muscle	H_2O_2↑	TBARS↑, PC↑	SOD↓, CAT↑, GPx↓	[34]
-do-	-do-	Gill	H_2O_2↑	TBARS↑, PC↑	SOD↓, CAT↑, GPx↓	[34]
Geophagus brasiliensis (cichlid fish acará)	Comparatively high temperature in spring	Liver	NA	TBARS↑, GSSG↑	SOD↓, CAT	[137]
Gasterosteus aculeatus L. (three-spined stickleback fish)	High temperature in summer with reproductive activity	Liver	NA	TBARS↑	GPx↓	[138]
Lepomis macrochirus (bluegill fish)	High temperature in summer	Whole animal	NA	NA	NA	[139]
Solea senegalensis (Senegal sole fish)	High temperature with heavy metal load	Liver	NA	TBARS↑	CAT↓, GPx↓	[140]
Barbus barbus L. (barbell fish)	High temperature in summer	Liver	NA	NA	SOD↓, CAT↑	[141]
-do-	-do-	Muscle	NA	NA	SOD↓, CAT↑	[141]
Trachemys scripta elegans (red-eared sliders Turtle)	High temperature in summer	Whole animal	NA	NA	NA	[142]
Caiman yacare (crocodile)	High temperature in summer	Liver, Kidney, Lung	NA	GSSG↑	NA	[143]
Tupinambis merianae (tegu lizard)	Low temperature in winter	Whole animal	NA	NA	NA	[116]
Rana ridibunda (frog)	High temperature in summer	Liver	NA	TBARS↑, PC↓	SOD↑, CAT↓	[144]
Acrocephalus sechellensis (seychelles warbler bird)	Low food availability	Blood	NA	hydroperoxides↑	NA	[145]
Perdicula asiatica (bird)	High temperature in summer	Lungs	NA	MDA↑	SOD↓, CAT↓	[146]
Hirundo rustica L. (Barn Swallow) bird	Summer with higher temperature	Liver	NA	Lipid hydroperoxide, GSSG↑	SOD, CAT↓, GPx	[147]
Hirundo rustica L. (Barn Swallow) bird	Pre egg laying time verses post egg laying time	Blood plasma	NA	MDA↑, PC↓	NA	[148]
Halichoerus grypus (Grey seal)	Summer (No variation in temp)	Whole animal	NA	NA	NA	[149]
Ovis aries (Rasa Aragonesa rams)	Breeding season with high temperature	Semen	NA	NA	SOD↑, CAT↑, GPx	[150]
Bos Taurus (simmental bulls)	High temperature in summer	Semen	NA	TBARS↑, PC↑	GPx↑ (highest in autumn)	[151]
(Kuroge washu) Japanese Black cow	High temperature in summer	Blood	NA	TBARS↑	SOD↓, GPx↓	[152]
-do-	Short photoperiod exposure in winter	-do-	NA	TBARS↑	NA	[152]
Rattus norvegicus (Wistar rats)	High temperature in summer	Erythrocytes	NA	TBARS↑	SOD↓, GPx↓	[153]
Guinea-pigs	High temperature in summer	Heart	O_2-↑	NA	SOD↓	[154]
Rattus norvegicus (Wistar rats)	High temperature in summer	Heart	O_2-↑		SOD↓	[154]
Homo sapiens	High temperature in summer	--	NA	8-isoprostane↑	SOD↓	[154]

ROS: Reactive oxygen species; OS: Oxidative stress; MDA: Malondehyde; TBARS: Thiobarbituric acid reactive substances; PC: Protein carbonylation; AOE: Antioxidant enzymes; SOD: Superoxide dismutase; CAT: Catalase; GPx: Glutathione peroxidase; GSSG: Oxidised glutathione; NA: Not analysed. ↓ or ↑ symbols are used to indicate decrease or increase of the parameters with the corresponding season, respectively. Parameter with no symbol (↓ or ↑) indicates "no change" in the same with respect to the season.

1). Such observations refuse the general idea that long or short term adaptations such as aestivation or altered redox regulatory system do not protect the animals from OS under rise in temperature condition in summer

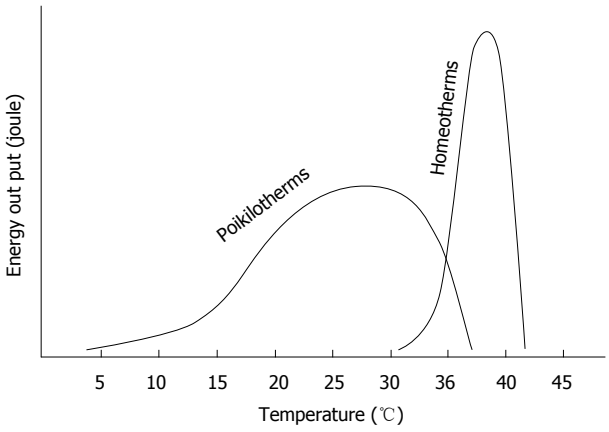


Figure 3 A proposed energy production system under rise in temperature condition in poikilotherms *vs* homeotherms. Schematic comparison showing a sustained energy (joule) output between a poikilotherm and homeotherm as a function of core body temperature. The homeotherms have a much higher energy output range but can only function over a very narrow range of body temperatures (30 ℃-40 ℃ in rats). However, poikilotherms have a much lower energy output range but can function over a very wide range of body temperatures (5 ℃-40 ℃ in house lizards). Since, the energy output range is different in different animals, values are not provided in Y-axis[159].

season. So, these animals also can be susceptible to OS and ageing under rise in environmental temperature.

Poikilothermy vs homeothermy under rise in environmental temperature condition

In relation to the present context of the production of toxic ROS under the elevated environmental temperature condition, the susceptibility of homeotherms than poikilotherms offers a debate. Poikilotherms are the organisms whose body temperature varies considerably with the ambient environmental temperature. Many terrestrial and aquatic ectotherms are categorized under this group. Vertebrates, specifically fish, amphibians, and reptiles, as well as a large number of invertebrates are studied under the group poikilotherms. The naked mole-rat is believed to be the only poikilothermic mammal[155-157]. On the other hand, homeotherms can thermoregulate for a stable internal body temperature regardless of environmental heating. However, the thermoregulation capacity works in homeotherms upto deviation from a certain range from their ambient environmental temperature. For example, homeothermy cannot work at 15 ℃ or 40 ℃ in a homeotherm who lives in ambient temperature of about 30 ℃ (Figures 3 and 4). Homeotherms maintain constant body temperatures through behavioral mechanisms alone *i.e.,* behavioral thermoregulation. Many reptiles use this strategy. For example, desert lizards are remarkable in that, they maintain near-constant activity temperatures that are often within a degree or two of their lethal critical temperatures[157]. Temperature regulatory mechanisms act through the autonomic nervous system and are largely controlled by the hypothalamus of the brain, which responds to stimuli from nerve receptors in the skin, *e.g.,* more active sweating in response to continued heat and an increase in subcutaneous fat deposits in response to continued cold[158].

In mammals including humans, temperature regulation represents the balance between heat production from metabolic sources and heat loss from evaporation (perspiration) and the processes of radiation, convection, and conduction[159]. In a cold environment, body heat is conserved first by constriction of blood vessels near the body surface and later by waves of muscle contractions, or shivering, which serve to increase metabolism. Shivering can result in a maximum fivefold increase in metabolism[158]. Therefore, this mechanism may not be sufficient for a naked person to increase the metabolic rate to replace heat lost to the environment at below 40 °F (4 ℃). Another heat-conserving mechanism observed in goose bumps, or piloerection. It raises the body hairs; although not especially effective in humans, in animals, it increases the thickness of the insulating fur or feather layer[159]. However, it is a time dependant and species specific response that can save the animal from the cold environment. On the other hand, in a warm environment, heat must be dissipated to maintain body temperature. In humans, increase in surface blood flow, especially to the limbs, acts to dissipate heat at the surface. At environmental temperatures above 34 ℃, or at lower temperatures when metabolism has been increased by work, heat must be lost through the evaporation of the water in sweat. People in active work may lose as much as 4 quarts per hour for short periods[158,159]. However, when the temperature and humidity are both high, evaporation is slowed, and sweating is not effective. Most mammals do not have sweat glands but keep cool by panting (evaporation through the respiratory tract) and by increased salivation and skin and fur licking[158]. A low rate of acclimatization in homeotherms occurs when they are continuously exposed to heat or cold. In extremes cases, under high environmental temperature or continuous exposure of animals to increase in state of environmental temperature may result in failure to maintain normal body temperature in animals[158]. The above discussed mechanism may not work properly under gradual and long term exposure of homeotherms to not only increase but also decrease in either side of their ambient environmental temperature. Under increase in body temperature, death may result due to heat exhaustion or uncontrolled hyperthermia. Under high temperature, homeotherms also adopt some physiological balance to decrease their metabolic rate. It indicates that under elevated temperature, homeotherms may dissipate their body heat upto certain extend in a time dependant manner. The main metabolic response, *i.e.,* sustained energy output in poikilotherms (for example in lizard) in comparison to homeotherms (for example in mouse) over a wide range of temperature (4-43 ℃) becomes different. In the former one, the energy output may show a stead increase up to 25 ℃-30 ℃ followed by a sharp fall in

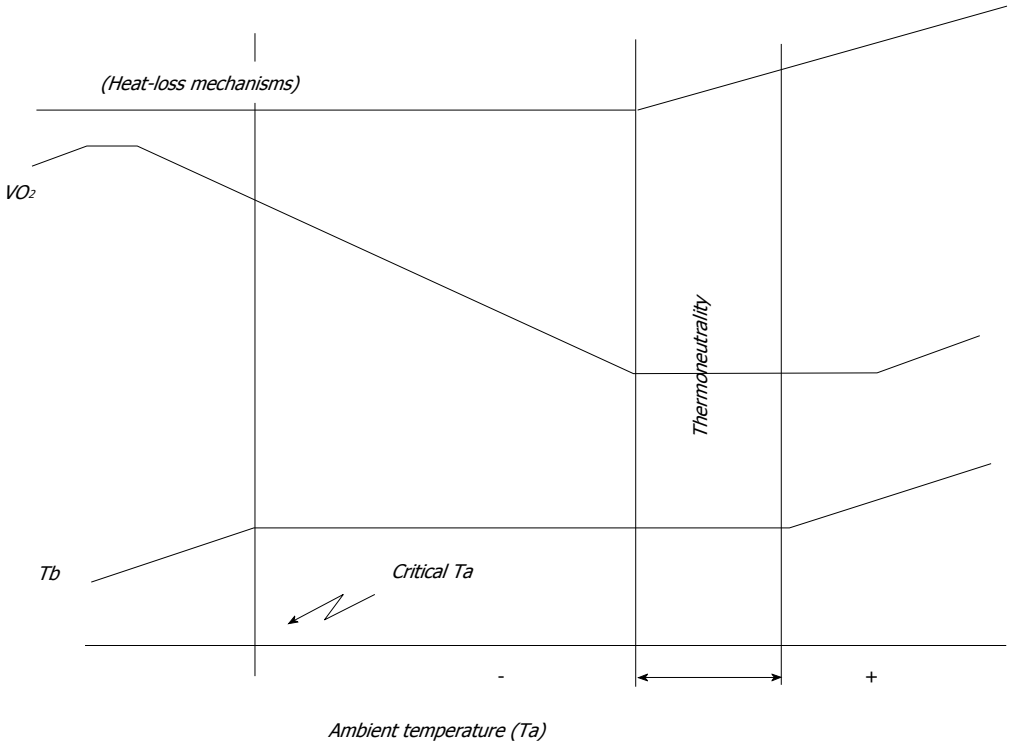


Figure 4 Relation among O_2 consumption increases in ambient temperature and thermoregulation in animals. When ambient Ta decreases below thermoneutrality, VO_2 increases maintaining body Tb. When thermogenesis does not suffice, Tb begins to fall (critical Ta). The ability to maintain a thermoneutral range is mostly due to an increase in heat dissipation. Eventually, with further increases in Ta, heat-loss mechanisms will not prevent a rise in Tb, which will also lead to a rise in VO_2[162]. Ta: Temperature; VO_2: Oxygen consumption; Tb: Temperature.

35 ℃-40 ℃. While in the later *i.e.,* in homeotherms, the temperature range on energy out put is very narrow being 30 ℃ to 40 ℃ with a peak at about 38 ℃[158-160] (Figure 2). The homeotherm has a much higher output, but can only function over a very narrow range of body temperatures and such situation may arrive under a gradual and long term exposure of homeotherms to increase in environmental temperature. So, in homeotherms, elevated temperature with elevation in high energy output can be correlated primarily with the increase in metabolism and secondarily with the increase in ATP production rate coupled with rapid electron transfer in ETC. It may then favor electron leakage at complex enzymes, *i.e.,* at complex Ⅰ and Ⅲ and production of ROS[36] (Figure 2). On the other hand, although the metabolic rate of homeotherms is proposed to be faster in colder temperatures than in warmer temperatures since they have to create their own warmth to maintain constant body temperature[160,161], the change in temperature from cold to warm may also cause a rise in the metabolic rate in homeotherms. Because their oxygen needs will increase as they regulate their body temperature. Several studies also confirm that elevated temperature causes increase in O_2 consumption in mammals[6] (Figure 4). It is noteworthy to mention that in context dependant manner, either increase or decrease in O_2 consumption may result when habitat temperature is changed especially when elevated (which may arrive due to global warming). On the other hand, due to the altered O_2 concentration in environment or due to altered metabolic status of homeotherms, they may experience hypoxia or hyperxia under elevated or reduced environmental temperature condition. Both hyperxia and hypoxia are known to induce ROS and OS in animals including homeotherms[6,41]. Altogether, higher or lower oxygen consumption, increased metabolism, increase in energy demand at elevated temperature may increase the risk of increase in ROS production. This is because, all the above processes are known to be positively correlated with ROS generation[6,41,162]. Therefore, with a much higher energy output only over a very narrow range of body temperatures (35 ℃-40 ℃), homeotherms are also entitled to experience high ROS level in their cells under elevated temperature conditions. Therefore, under continuous and gradual increase in environmental temperature, both the homeotherms and poikilotherm may experience OS and that can affect their longevity.

CONCLUSION

According to Houghton *et al*[163], the world is projected to experience an approximate doubling of atmospheric CO_2 concentrations to around 700 ppm accompanied by a 1.4 ℃-5.8 ℃ rise in mean global temperatures in the year 2100. These climatic changes would greatly alter the survivability and longevity of animals in general and poikilotherms in particular. Production of active

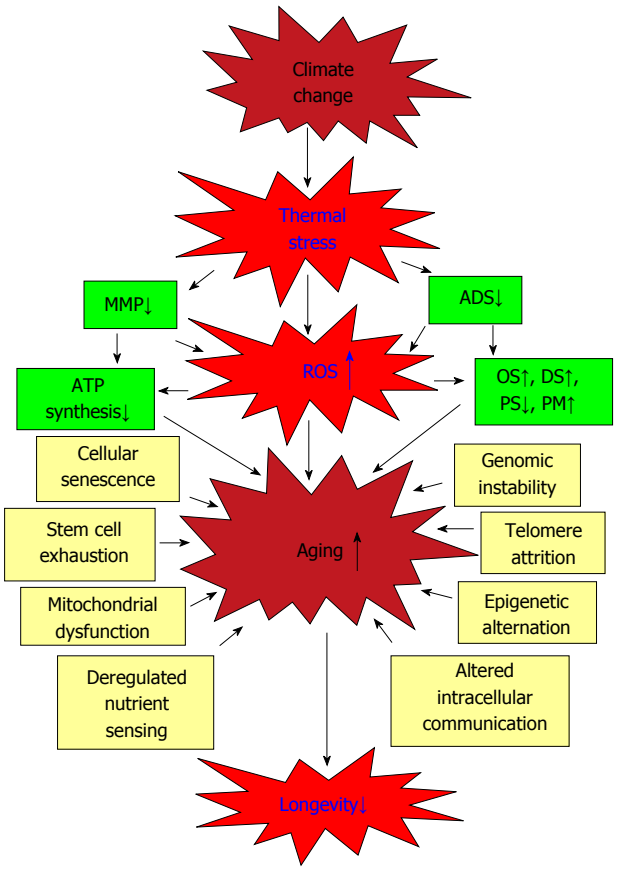


Figure 5 Possible correlation between global warming and longevity modulated by oxidative metabolism in animals. Climate change is responsible to increase the mean global temperature which may lead to produce ROS in susceptible animals. Increase IT can also be responsible to produce ROS *via* diminishing ADS and MMP. This in turn may increase aging in animals by elevating oxidative stress. Other vital processes such as decrease in ATP synthesis, increasing the chance to DS, PS and PM in animals can lead to additional ROS production and thereby, shortening their longevity. Due to thermal stress, physiological processes such as genomic instability, telomere shortening and mitochondrial dysfunction can also lead to elevate aging in animals which may ultimately decrease their longevity. ROS: Reactive oxygen species; IT: In temperature; ADS: Antioxidant defence system; MMP: Mitochondrial membrane potential; DS: Disease susceptibility; PS: Proteostasis; PM: Protein misfolding.

oxygen species influenced by rise in environmental temperature due to climatic changes may be one of the major reasons that are expected to influence the normal physiology and longevity of animals (Figure 5). Risks of climatic change especially global warming on longevity of animals through an increase in risk of disease susceptibility, protein misfolding, lowering ATP generation and several other factors (Figure 5) that boost aging in animals can not be ignored. Therefore, understanding the physiological impacts of global warming in relation to longevity of animals including human being would be one of the major challenges to the biologists of the present millennium.

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API2-MALT1 oncoprotein promotes lymphomagenesis *via* unique program of substrate ubiquitination and proteolysis

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Abstract

Lymphoma of mucosa-associated lymphoid tissue (MALT lymphoma) is the most common extranodal B cell tumor and accounts for 8% of non-Hodgkin's lymphomas. Gastric MALT lymphoma is the best-studied example and is a prototypical neoplasm that occurs in the setting of chronic inflammation brought on by persistent infection or autoimmune disease. Cytogenetic abnormalities are commonly acquired during the course of disease and the most common is chromosomal translocation t(11;18)(q21;q21), which creates the API2-MALT1 fusion oncoprotein. t(11;18)-positive lymphomas can be clinically aggressive and have a higher rate of dissemination than t(11;18)-negative tumors. Many cancers, including MALT lymphomas, characteristically exhibit deregulated over-activation of cellular survival pathways, such as the nuclear factor-κB (NF-κB) pathway. Molecular characterization of API2-MALT1 has revealed it to be a potent activator of NF-κB, which is required for API2-MALT1-induced cellular transformation, however the mechanisms by which API2-MALT1 exerts these effects are only recently becoming apparent. The API2 moiety of the fusion binds tumor necrosis factor (TNF) receptor associated factor (TRAF) 2 and receptor interacting protein 1 (RIP1), two proteins essential for TNF receptor-induced NF-κB activation. By effectively mimicking ligand-bound TNF receptor, API2-MALT1 promotes TRAF2-dependent ubiquitination of RIP1, which then acts as a scaffold for nucleating and activating the canonical NF-κB machinery. Activation occurs, in part, through MALT1 moiety-dependent recruitment of TRAF6, which can directly modify NF-κB essential modulator, the principal downstream regulator of NF-κB. While the

intrinsic MALT1 protease catalytic activity is dispensable for this canonical NF-κB signaling, it is critical for non-canonical NF-κB activation. In this regard, API2-MALT1 recognizes NF-κB inducing kinase (NIK), the essential upstream regulator of non-canonical NF-κB, and cleaves it to generate a stable, constitutively active fragment. Thus, API2-MALT1 harnesses multiple unique pathways to achieve deregulated NF-κB activation. Emerging data from our group and others have also detailed additional gain-of-function activities of API2-MALT1 that extend beyond NF-κB activation. Specifically, API2-MALT1 recruits and subverts multiple other signaling factors, including LIM domain and actin-binding protein 1 (LIMA1) and Smac/DIABLO. Like NIK, LIMA1 represents a unique substrate for API2-MALT1 protease activity, but unlike NIK, its cleavage sets in motion a major NF-κB-independent pathway for promoting oncogenesis. In this review, we highlight the most recent results characterizing these unique and diverse gain-of-function activities of API2-MALT1 and how they contribute to lymphomagenesis.

Key words: Oncogene; Fusion oncoprotein; Lymphoma; Chromosomal translocation; Ubiquitination; Apoptosis; Nuclear factor-κB; Caspases

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Core tip: We summarize the identification of novel API2-mucosa-associated lymphoid tissue (MALT) 1-interacting proteins that uniquely mediate the cellular effects of the fusion oncoprotein but not wild-type API2 or MALT1. API2-MALT1 recruits receptor interacting protein 1 and tumor necrosis factor (TNF) receptor associated factor 2, which normally function downstream of the TNF receptor, and utilizes these proteins to communicate unregulated canonical nuclear factor-κB (NF-κB) in a manner that does not depend on the protease activity of MALT1. Simultaneously, NF-κB inducing kinase is recruited to API2-MALT1 and is proteolytically cleaved by the MALT1 protease domain to generate a stable, non-canonical NF-κB-activating fragment. Finally, LIM domain and actin-binding protein 1 is similarly recruited and cleaved as an API2-MALT1 specific target and its cleavage mediates an NF-κB-independent mechanism of oncogenesis. Additional factors, including SMAC and BCL10, may also play key roles as API2-MALT1 binding partners and downstream signaling factors. Thus, the API2-MALT1 fusion utilizes a distinct set of protein-protein interactions to leverage multiple, divergent mechanisms and achieve potent oncogenic reprogramming of affected B cells.

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INTRODUCTION

Extranodal marginal zone B-cell lymphoma of mucosa-associated lymphoid tissue (MALT lymphoma) is a prototypical cancer that occurs at sites affected by chronic inflammation due to persistent pathogenic infection or autoimmune disease[1]. The best-studied example of MALT lymphoma affects the gastric mucosa and occurs in the setting of *Helicobacter pylori* infection. Lymphomas of this type are commonly treated with antibiotics to eradicate the causative microorganism, which prevents further antigenic stimulation of the oncogenic B cells. However, in a subset of MALT lymphomas, acquired genetic abnormalities relieve the neoplastic cells of their dependence on the inflammatory milieu and induce a state of stimulus-independent growth[2].

Interestingly, some MALT lymphomas arise in an atypical fashion and occur in the absence of underlying chronic inflammation or autoimmune disorders. These lymphomas typically harbor the most common and first-described genetic abnormality in MALT lymphoma, namely the chromosomal translocation t(11;18)-(q21;q21), which fuses a portion of the inhibitor of apoptosis 2 (*API2*; also known as *cIAP2*) and the MALT lymphoma translocation gene 1 (*MALT1*) genes creating an expressed fusion protein known as API2-MALT1[3-5]. This chromosomal translocation is specific to MALT lymphoma, and when it occurs, it is generally seen as the sole cytogenetic abnormality[6,7]. The fact that API2-MALT1-positive lymphomas arise in the absence of inflammatory conditions that typically predispose to the development of MALT lymphoma suggest that the fusion itself is the oncogenic driver. In addition, t(11;18)-positive lymphomas are associated with treatment resistance and a higher tendency to disseminate[8-11].

While many chromosomal breakpoints have been identified, the resultant API2-MALT1 chimeric proteins invariably contain the amino terminus of API2, which includes three intact baculovirus IAP repeat (BIR) domains, and the carboxy terminus of MALT1, which contains immunoglobulin (Ig)-like domains that also mediate protein-protein interactions (Figure 1)[12]. Also invariably included in the fusion is the enzymatically active "caspase-like" protease domain of MALT1. The proteolytic activity of MALT1 has been well-documented, but only a few natural substrates are known and our understanding of the overall impact of MALT1 proteolytic activity to cell signaling and physiology is still unfolding[13].

Wild-type API2 is a member of the IAP family of anti-apoptotic proteins and also contains a C-terminal caspase recruitment domain (CARD) that is not present in the commonest API2-MALT1 chimeras[14]. More importantly, API2 possesses a really interesting new gene (RING) domain that is *never* included in API2-MALT1 chimeras; the RING domain of API2 possesses E3 ubiquitin ligase activity and mediates covalent conjugation of ubiquitin to interacting proteins, which either targets the substrate for degradation *via* the proteasome or modulates the

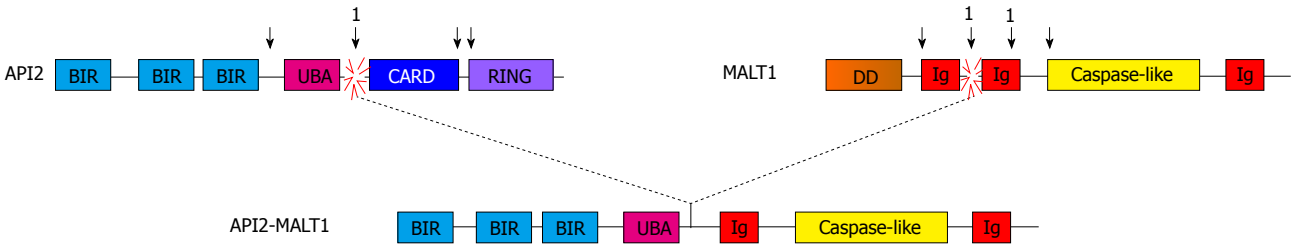


Figure 1 Schematic illustration of the API2-mucosa-associated lymphoid tissue 1 fusion oncoprotein. The most commonly occurring API2-MALT1 fusion is depicted. Recurrent breakpoint positions for both MALT1 and API2 are indicated by arrowheads. The most common breakpoint for API2, by far, is indicated by 1 (approximately 93%). There are two relatively common breakpoints for MALT1, both indicated by asterisks (approximately 42% and 32%, respectively). CARD: Caspase recruitment domain; DD: Death domain; Ig: Immunoglobulin-like; BIR: Baculovirus inhibitor of apoptosis repeat; UBA: Ubiquitin-associated domain; RING: Really interesting new gene; MALT: Mucosa-associated lymphoid tissue.

function of the substrate protein[15]. Wild-type MALT1 contains an amino-terminal death domain (DD) and an additional Ig-like domain; the DD is never present in API2-MALT1 fusion proteins and this N-terminal Ig-like domain is only rarely included[13]. MALT1 is a crucial mediator of antigen receptor-induced activation and proliferation in lymphocytes and also mediates cellular signaling in other immune cells downstream of immunoreceptor tyrosine-based activation motif -containing receptors. Additionally, MALT1 has been found to mediate inflammatory responses to agonistic activation of various G protein-coupled receptors in non-immune cells[12].

MALT1 couples to these various receptors *via* interaction with B-cell CLL/lymphoma 10 (BCL10), an adaptor protein, and members of the CARD and membrane-associated guanylate kinase-like (MAGUK) domain-containing protein (CARMA) family of proteins (Figure 2). Formation of a receptor-induced complex consisting of the CARMA, Bcl10, and MALT1 proteins, known as the CBM signalosome, results in oligomerization of MALT1 and its coupling to downstream signaling mediators to effect a response[12]. The best-studied signaling pathway that is activated by the CBM signalosome is that which controls nuclear factor-κB (NF-κB), a family of transcription factors that mediates the response to a growing cadre of diverse extracellular substances including growth factors, antigenic stimuli, peptide hormones, and chemotactic agents. There are actually two principal pathways for activating NF-κB subunits: The canonical (or classical) and non-canonical (or alternative) pathways (Figure 2)[16]. Both depend on stimulus-specific activation of kinase cascades that trigger degradation of NF-κB-inhibitory proteins and free the active transcription factor dimers; canonical dimers typically comprise RelA (p65) and p50 whereas non-canonical dimers comprise RelB and p52 (Figure 2). Active NF-κB dimers translocate to the nucleus and bind consensus sequences in the promoters of many genes to rapidly induce expression of proteins that drive proliferation, survival, and differentiation of cells of the immune system, all of which must be properly and appropriately controlled to minimize the risk of tissue damage and disease. Indeed, genetic defects that impair proper activation of NF-κB in lymphocytes can result in immunodeficiency, while aberrant hyper-activation of NF-κB is associated with autoimmune disease and various cancers. The precise mechanisms governing NF-κB activation are described in greater detail in the legend to Figure 2. In addition, many comprehensive reviews of NF-κB signaling have been published[16-19], including reviews focused on how the CBM signalosome interacts with the NF-κB machinery[20,21], and the reader is also referred to these for a more detailed treatment of the subject.

In this review, we will summarize the unique, gain-of-function properties that have been described recently for the API2-MALT1 fusion oncoprotein. In particular, we will highlight the contributions of the API2 moiety which, through its BIR protein interaction domains, creates a special platform for downstream signaling and works together in surprising fashion with the protease domain of the MALT1 moiety.

UNIQUE PROPERTIES OF API2-MALT1: AUTO-OLIGOMERIZATION

Like wild-type MALT1, the API2-MALT1 fusion protein is able to promote activation of NF-κB[22-24]. In both cases, it is thought that oligomerization of the MALT1 C-terminus is critical for downstream communication with the NF-κB machinery. However, while wild-type MALT1 must rely on its association with the CARMA and BCL10 components of the CBM signalosome for stimulus-induced oligomerization, API2-MALT1 is able to auto-oligomerize *via* heterotypic association between the BIR1 domain of the API2 moiety and the C-terminal caspase-like domain of MALT1[25,26]. Juxtaposing these two protein components imparts oncogenic potential to API2-MALT1, *per se*, that the wild-type counterparts are unable to achieve and depends on the presence of an intact BIR1 domain. Because the homo-oligomerization of API2-MALT1 occurs constitutively, and is not influenced by any known regulatory processes such as those which govern assembly and disassembly of the CBM signalosome, the presence of API2-MALT1 in a cell induces exceptionally potent and sustained NF-κB activation. This high level of NF-κB activity is associated with protection

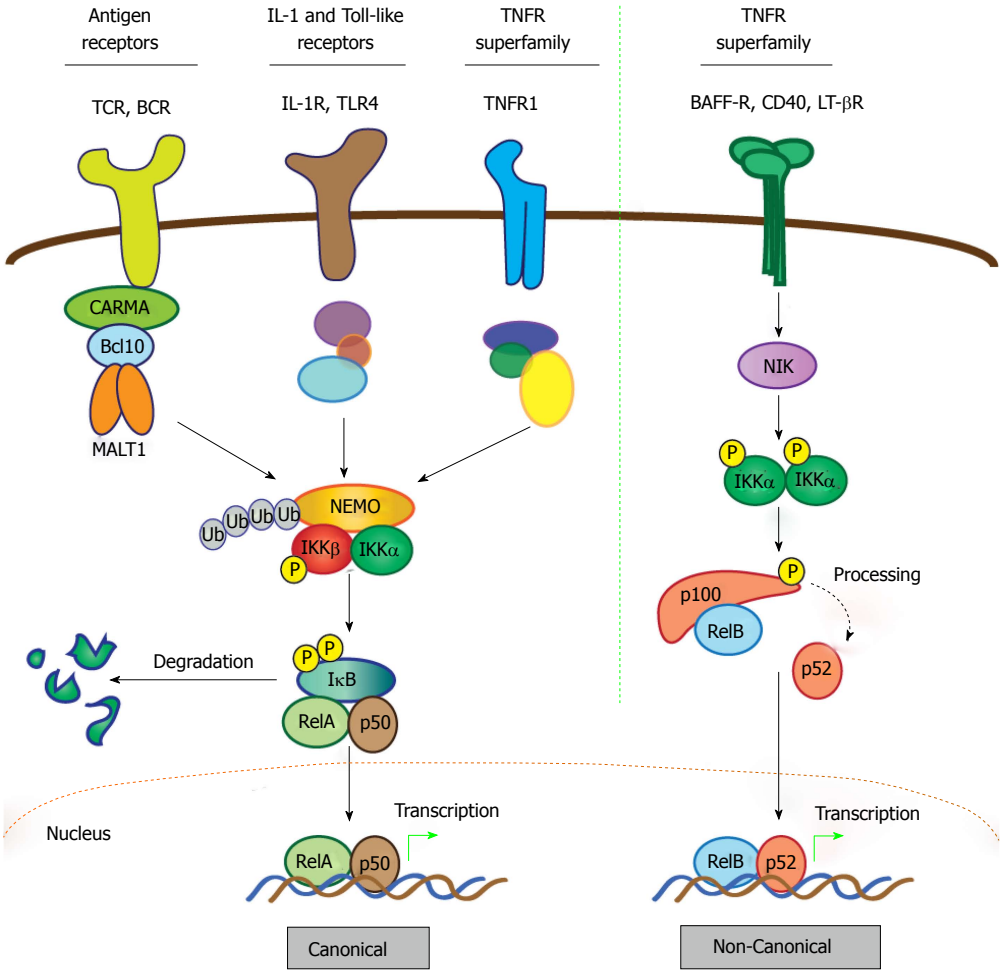


Figure 2 Activation of nuclear factor-κB: Canonical and non-canonical pathways. The NF-κB family comprises five members characterized by the presence of a Rel homology domain, which mediates DNA binding and dimerization. To allow prompt and efficient activation in response to stimuli, NF-κB dimers are sequestered in the cytoplasm in an inactive form *via* interaction with IκB proteins. Activation of NF-κB quickly follows ligand-induced stimulation of a variety of receptors, including TNF, IL-1, Toll-like, and BCR and TCR. The two principal signaling mechanisms that lead to activation of specific NF-κB heterodimers are the canonical and non-canonical pathways. In the case of canonical signaling, receptor-mediated signaling is transmitted *via* recruitment and/or activation of various upstream signaling components that promote activation of the IKK complex, which consists of catalytic kinase subunits IKKα and IKKβ and a regulatory scaffold protein called NF-κB essential modulator (NEMO; also called IKKγ). Once the IKK complex is activated, the steps directing canonical NF-κB stimulation are generally shared, regardless of the upstream stimulus. Selectivity is thought to reside mostly in the unique, "private" pathways utilized by distinct receptors for communication with the IKK complex. In the case of antigen receptors, assembly of the CBM signalosome is critical for this to occur. Upon activation of the IKK complex, the IKKβ kinase subunit phosphorylates IκBα, marking it for lysine (K)48-linked ubiquitination and subsequent degradation *via* the proteasome, which represents the commitment step for canonical NF-κB activation. Newly freed NF-κB heterodimers, most commonly RelA (p65) and p50, translocate to the nucleus where they bind target sites within the promoters of a vast array of responsive genes to induce expression. Notably, the gene encoding IκBα is directly regulated by NF-κB and its synthesis serves as a negative feedback loop, thus attenuating and resetting the canonical NF-κB machinery. In contrast to the multitude of stimuli and signaling intermediates that activate canonical NF-κB, a relatively small number of cell-surface receptors promote non-canonical NF-κB activation. These include the BAFF, LT-β, and CD40 TNF superfamily receptors. Stimulation of non-canonical NF-κB centers on stabilization and activation of the critical upstream regulator, NIK. In resting cells, NIK is targeted for K48-linked ubiquitination and proteasomal degradation by a complex of proteins that includes cIAP1, cIAP2, TRAF2 and TRAF3, which together function as a "destruction complex" and constitutively represses NIK activity. Ligand binding to selective TNF superfamily receptors promotes degradation of TRAF3, the protein which links NIK to cIAP1/2 and TRAF2, thereby freeing NIK from the destruction complex and allowing its stabilization and activation. Then, NIK phosphorylates and activates the IKKα kinase subunit, which in turn phosphorylates the NF-κB2 precursor p100, targeting it for partial proteasomal degradation to p52. Like IκBα, p100 contains C-terminal ankyrin repeats that are responsible for cytoplasmic retention of RelB/p100 complexes in unstimulated cells. Freed RelB/p52 dimers also undergo nuclear translocation and drive transcription of appropriate genes in a manner analogous to the canonical RelA/p50 heterodimer complexes. Unlike canonical NF-κB signal termination, negative regulation of non-canonical NF-κB is less well-characterized. It appears to depend on reciprocal IKKα-dependent phosphorylation of NIK on C-terminal serine residues, which leads to TRAF/cIAP complex-independent NIK destabilization. However, more work is needed to fully define the mechanism involved in attenuating the non-canonical NF-κB signaling pathway. CARMA: CARD and membrane-associated guanylate kinase-like domain-containing protein; NF-κB: Nuclear factor-κB; MALT: Mucosa-associated lymphoid tissue; IκB: Inhibitor of NF-κB; IL: Interleukin; IKK: IκB kinase; BAFF: B-cell activating factor belonging to the TNF family; LT: Lymphotoxin; NIK: NF-κB-inducing kinase; BCR: B-cell receptor; TCR: T-cell antigen receptor.

against apoptotic stimuli, and importantly, confers anchorage-independent growth to NIH-3T3 fibroblasts, underscoring the transformative potential of API2-MALT1 and marking it as a *bone fide* oncogene. It is important to note that these effects are dependent on the fusion of these two protein moieties and result from the invariant inclusion of the BIR domains from wild-type API2 and the protease and Ig-like domains of wild-type

MALT1. These seminal findings provided an excellent framework on which to interrogate in greater detail how the API2 and MALT1 fusion partners contribute to the overall potential of API2-MALT1 and offer explanations for the advanced clinical nature of t(11;18)-positive lymphomas. In particular, these observations prompted exploration of whether heterotypic protein interactions, in addition to this homotypic interaction, occur *via* either the API2 or MALT1 domains to affect function of the overall fusion protein.

UNIQUE PROPERTIES OF API2-MALT1: ASSEMBLY OF NOVEL DOWNSTREAM EFFECTOR COMBINATIONS

Smac/DIABLO and BCL10

In addition to auto-oligomerization, the API2 moiety of API2-MALT1 also mediates other critical protein-protein interactions. The API2 BIR domains interact with multiple signaling proteins that play important roles as regulators of cellular apoptosis and NF-κB activity, and these interactions likely contribute to API2-MALT1-dependent promotion of cell survival. For example, second mitochondrial activator of caspases (Smac; also known as DIABLO) is a mitochondrial protein that is released into the cytoplasm in response to pro-apoptotic stimuli and binds to the API2 moiety of API2-MALT1[27]. Smac can promote apoptosis by binding to X-linked inhibitor apoptosis, which then liberates caspases from inhibition and allows apoptosis to proceed[28]. It has been proposed that the binding of Smac to the API2 moiety of API2-MALT1 sequesters it and prevents it from performing this function, which is critical to API2-MALT1's ability to suppress Smac-dependent apoptosis[27]. However, the precise molecular mechanism by which API2-MALT1 interferes with Smac-dependent apoptosis is not yet fully understood, nor is it known how the MALT1 moiety of the fusion contributes to this process.

One study demonstrated that like Smac, BCL10 also binds to the API2 moiety of the API2-MALT1 fusion[29]. The authors first found that wild-type API2 interacts with BCL10 through its BIR region and ubiquitinates BCL10 *via* its RING E3 ubiquitin ligase domain. They then showed that API2-mediated ubiquitination targets BCL10 for proteasomal degradation. In contrast to wild-type API2, the API2-MALT1 fusion, regardless of specific translocation breakpoint, always lacks the API2 RING domain and therefore does not posses E3 ubiquitin ligase activity. The authors thus demonstrated that the API2-MALT1 fusion retains the ability to bind BCL10 *via* the BIR region but fails to ubiquitinate BCL10. This leads to stabilization and upregulation of BCL10 in t(11;18)/API2-MALT1 positive lymphomas and could theoretically sensitize cells to prosurvival signaling through the B cell receptor. The role of the MALT1 moiety in API2-MALT1-dependent stabilization/upregulation of BCL10, and the contribution of BCL10 upregulation to the oncogenic potential of API2-MALT1, has yet to be determined.

Receptor interacting protein 1, TNF receptor associated factor 2 and TNF receptor associated factor 6

The BIR domains of wild-type IAP proteins, including API2, mediate unique interactions with members of the TNF receptor associated factor (TRAF) family of signaling proteins to modulate NF-κB activation in the setting of tumor necrosis factor (TNF) receptor stimulation[30-33]. Here, the activated TNF receptor recruits the adaptor TNF receptor associated DD protein (TRADD), which in turn binds TRAF2 and receptor interacting protein 1 (RIP1)[34,35]. TRAF2 also recruits wild-type API2 (cIAP2) and cIAP1 proteins, which together mediate the non-degradative (K63-linked) poly-ubiquitination of RIP1 (Figure 3)[15,31,33,36]. Since TRAF2 and the IAP proteins each contain similar E3 ubiquitin ligase (RING) domains, it remains somewhat unclear which protein is the critical ligase for RIP1 ubiquitination, or if all three work in concert. However, in isolation, each is capable of catalyzing the ubiquitination given the right conditions so it is thought that each may play an active role[37]. Next, the poly-ubiquitin chains on RIP1 create a docking site for the NF-κB essential modulator (NEMO) subunit of the IKK complex, promoting canonical NF-κB activation[37-41].

Since the API2 moiety of API2-MALT1 retains the BIR domains, the possibility existed that this portion of the fusion protein recruits TRAF2, and although the API2 RING domain is missing from the API2-MALT1 fusion, the recruited wild-type TRAF2 might mediate RIP1 ubiquitination (Figure 3). Indeed, TRAF2 binding occurs through the BIR1 domain of the API2 moiety and deletion of this motif from the oncoprotein compromises its ability to activate NF-κB[26]. Further, deletion of the RING domain of TRAF2 inhibits API2-MALT1-induced NF-κB activation[26]. To further characterize the essential nature of TRAF2 and the manner in which it contributes to API2-MALT1-induced effects, we turned again to the TNF receptor signaling complex as a potential analogous model. We determined that, somewhat akin to TRADD, API2-MALT1 can recruit RIP1 through an interaction that is independent of both the BIR1 domain and TRAF2, and that this is required for API2-MALT1-induced canonical NF-κB activation (Figure 3)[42]. API2-MALT1 expression also promotes ubiquitination of RIP1 and recruitment of NEMO, but only in the presence of wild-type TRAF2 and its intact RING domain. Mutants of API2-MALT1 that fail to recruit TRAF2 cannot induce RIP1 ubiquitination, do not recruit NEMO or activate NF-κB, and cannot promote transformation of NIH-3T3 cells[42].

Although the API2 moiety binds both RIP1 and TRAF2, expression of the API2 moiety alone is insufficient to promote either RIP1 ubiquitination or NF-κB activation[42]. Therefore, the MALT1 moiety must provide additional necessary functionality. However, MALT1 protease activity, the principal activity ascribed to the MALT1 C-terminus, is not required since disabling the catalytic domain by site-directed mutagenesis has no effect on RIP1 ubiquitination. Since the C-terminus of MALT1 is also known to bind TRAF6, another RING-containing E3 ligase, we considered the possibility that

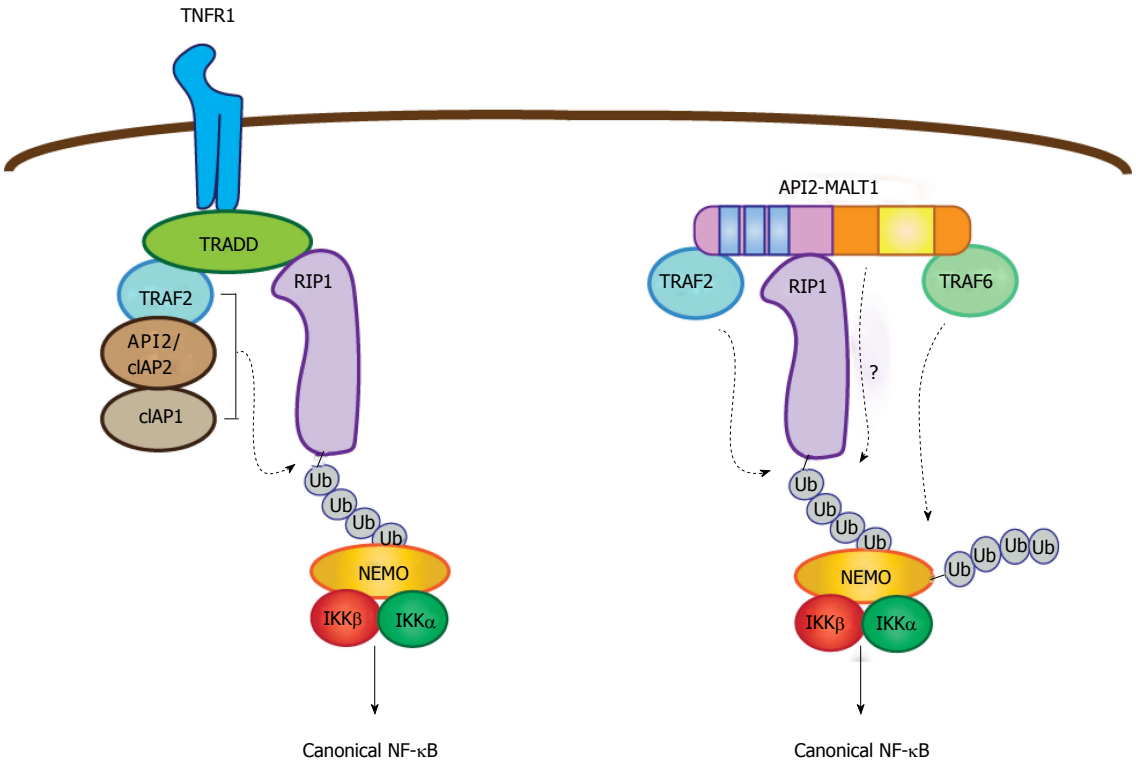


Figure 3 Parallel pathways for canonical nuclear factor-κB activation mediated by tumor necrosis factor receptor and API2-mucosa-associated lymphoid tissue 1. In the context of TNF receptor activation, TNF receptor associated death domain protein brokers the assembly of a complex containing three E3 ubiquitin ligases (API2/cIAP2, cIAP1, and TRAF2), which together promote RIP1 ubiquitination. This then serves as a platform for recruitment of NEMO. In parallel fashion, the API2 moiety of API2-MALT1 binds TRAF2 and RIP1 to promote RIP1 ubiquitination, but in this case the ubiquitination event seems to be mediated only by TRAF2. Importantly, the MALT1 moiety facilitates the ubiquitination of RIP1 through an as of yet undefined mechanism. Recruitment of NEMO *via* the RIP1 ubiquitin scaffold allows MALT1 moiety-associated TRAF6 to promote NEMO ubiquitination, IKK complex activation and subsequent canonical NF-κB activation. TRAF: TNF receptor associated factor; NEMO: NF-κB essential modulator; RIP1: Receptor interacting protein 1; TRADD: TNF receptor associated death domain protein; IKK: IκB kinase; MALT: Mucosa-associated lymphoid tissue; TNF: Tumor necrosis factor; NF-κB: Nuclear factor-κB.

TRAF6 recruitment to the API2-MALT1 fusion might represent the critical event and that TRAF6 might work together with TRAF2 to mediate the RIP1 ubiquitination; but this is not the case either since site-directed mutagenesis of the TRAF6 binding sites within API2-MALT1 has little effect on RIP1 ubiquitination[42]. Taken together, these results suggest that there is some, still-undetermined functionality of the MALT1 moiety within the API2-MALT1 fusion protein that allows the API2 moiety to direct efficient TRAF2-dependent RIP1 ubiquitination. Further work will be required to reveal the nature of this enabling activity.

Nevertheless, it is important to underscore that maximal NF-κB induction does appear to depend on TRAF6 recruitment to API2-MALT1, since NF-κB activity is significantly dampened upon mutation or deletion of the C-terminal TRAF6 binding sites[42,43]. This is because TRAF6 appears to direct a pathway of K63-linked NEMO ubiquitination that is parallel to the TRAF2-dependent pathway of RIP1 ubiquitination. Thus, we speculate that API2-MALT1 coordinates a sequential process whereby RIP1 recruitment and TRAF2-dependent ubiquitination occurs first and provides a platform for subsequent NEMO recruitment and TRAF6-dependent ubiquitination (Figure 3). By working together in this fashion, the tethered API2 and MALT1 moieties appear to provide an especially potent activating signal for the IKK complex.

To fully understand this complex process of API2-MALT1 driven substrate ubiquitination, it is also important to identify the essential E2 ubiquitin conjugating enzymes that are involved. Originally, API2-MALT1 expression was shown to promote deregulated ubiquitination of NEMO and NF-κB activation in a manner that depended on the E2 enzyme, ubiquitin conjugating enzyme 13 (Ubc13)[25]. This is analogous to the situation for wild-type MALT1, whereby CBM-dependent NF-κB activation in response to antigen receptor engagement requires both Ubc13 and TRAF6-mediated ubiquitination of NEMO[44]. Thus, it appears that Ubc13 plays a major role in API2-MALT1-mediated canonical NF-κB activation, but additional investigation is necessary to definitively conclude this supposition and determine if additional E2 conjugating enzymes are involved. In conclusion, considerable progress has been made in understanding the elaborate mechanisms by which API2-MALT1 brokers IKK complex activation. In addition to utilizing a MALT1 centric mechanism for ubiquitinating NEMO, the fusion protein also appears to adopt and exploit a mechanism that is ordinarily used by the TNF receptor for ubiquitination of RIP1.

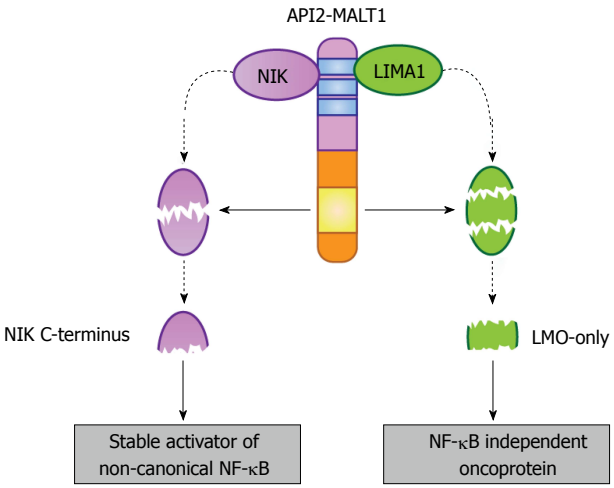


Figure 4 Selective substrates of the API2-mucosa-associated lymphoid tissue 1 fusion oncoprotein. The API2 moiety recruits at least two proteins (NIK and LIMA1), making them available as targets for the MALT1 protease domain (yellow box) preserved in the MALT1 moiety. In the case of NIK, site-specific cleavage frees a stable, catalytically active fragment that promotes deregulated non-canonical NF-κB activation. This fragment is stable because it lacks the C-terminal domains necessary for its recruitment to the NIK "destruction complex". In the case of LIMA1, site-specific cleavage at two sites not only destroys the tumor suppressor function of the full-length LIMA1 protein but also liberates a central, LMO-domain only fragment that displays gain-of-function oncogenic activity. MALT: Mucosa-associated lymphoid tissue; NF-κB: Nuclear factor-κB; NIK: NF-κB inducing kinase; LIMA: LIM domain and actin-binding protein 1.

UNIQUE PROPERTIES OF API2-MALT1: RECRUITMENT AND CLEAVAGE OF UNIQUE PROTEOLYTIC SUBSTRATES

Recent studies have highlighted a particularly intriguing aspect of API2-MALT1 function, namely its ability to recruit and proteolytically cleave substrates that are normally not recognized as substrates by the protease domain of wild-type MALT1. Evidence suggests that this occurs because the API2 moiety provides substrate binding functionality, bringing these substrates into close proximity of the MALT1 proteolytic active site. To date, two such substrates have been identified, but we speculate that more may exist and could be revealed through detailed proteomic screens designed to capture unique API2-MALT1 binding partners.

NIK

Early studies investigating the protease dependent-function of API2-MALT1 established that optimal activation of NF-κB required the intact MALT1 protease domain of the fusion oncoprotein[23,24]. Because both canonical and non-canonical NF-κB dimers bind overlapping DNA consensus sequences[45], we reasoned that perhaps the protease activity of API2-MALT1 was preferentially more important for one or the other of these pathways. By assessing the subcellular distribution of NF-κB subunits in response to either wild-type or protease-dead API2-MALT1 expression, we found no difference in the nuclear translocation of the canonical NF-κB subunit RelA (p65); that is, protease-dead API2-MALT1 was just as capable as wild-type API2-MALT1 at promoting RelA translocation[46]. However, translocation of the non-canonical NF-κB subunit p52 was much less effective in response to expression of protease-dead API2-MALT1, and this mutant was unable to promote processing of p100 to p52. This suggested to us the possibility that API2-MALT1 protease activity may influence the initial steps of non-canonical NF-κB activation. Because the fusion oncoprotein is able to interact with components of the NF-κB inducing kinase (NIK) destruction complex, including TRAF2, we hypothesized that perhaps API2-MALT1 was sequestering and/or triggering degradation of one or more of the components in a protease-dependent manner, leading to NIK stabilization. Instead, we found that API2-MALT1 was able to directly associate with NIK *via* the API2 moiety, bringing it into close proximity to the MALT1 protease domain and thereby making it an unexpected cleavage target[46]. We then determined that MALT1 triggers cleavage of NIK at Arg325, creating a stable, catalytically-active C-terminal NIK fragment (Figure 4). Furthermore, we found that suppressing NIK levels in API2-MALT1-expressing B cells impaired non-canonical NF-κB activation and blocked expression of downstream targets, specifically those involved in apoptosis resistance and B-cell adhesion, sensitizing API2-MALT1-expressing cells to dexamethasone-induced cell death and blocking vascular cell adhesion molecule -1-dependent adhesion. Importantly, interrogation of primary MALT lymphoma patient samples revealed the presence of cleaved NIK and enhanced p100 processing only in API2-MALT1 translocation-positive specimens. Furthermore, gene expression analyses of two independent MALT lymphoma tumor collections established that API2-MALT1-positive tumors are enriched in expression of non-canonical NF-κB target genes[46]. NIK is thus far known as a selective cleavage substrate for API2-MALT1; wild-type MALT1 appears unable to cleave NIK, likely because MALT1 has no mechanism for recruiting NIK and therefore has limited opportunity to position it in proximity to its proteolytic active site. These results uncovered the first substrate of the MALT1 protease that is specifically targeted by the API2-MALT1 fusion oncoprotein. In addition, the results underscored how the two moieties of the fusion oncoprotein work together in gain-of-function fashion, with the API2 moiety recruiting an unnatural substrate, and the MALT1 moiety mediating enzymatic cleavage of that substrate.

LIM domain and actin-binding protein 1

The majority of the literature on API2-MALT1 has focused on the mechanisms by which API2-MALT1 subverts cellular signaling proteins to manifest robust and sustained NF-κB activation, thereby promoting MALT lymphomagenesis. Recently, however, a novel NF-κB-independent, but MALT1 protease domain-dependent mechanism of API2-MALT1-mediated oncogenesis was revealed[47]. Mass spectrometric analysis of imm-

unoprecipitated complexes from cells expressing either wild-type or catalytically inactive API2-MALT1 identified a novel association with the tumor suppressor, LIM domain and actin-binding protein 1 (LIMA1), also known as epithelial protein lost in neoplasm. As for NIK, the LIMA1 interaction was mapped to the API2 moiety of API2-MALT1. Also similar to the situation for NIK, the recruitment allows MALT1 protease-dependent cleavage of LIMA1, but in this case cleavage occurs in two foci, thereby releasing a central LIM-domain only (LMO) fragment (Figure 4).

Since LIMA1 acts as a tumor suppressor, its expression in B lymphocytes serves to reduce growth, proliferation, colony formation, and adhesion to extracellular matrix. Experimentally silencing LIMA1 was sufficient to reverse these effects and cause enhanced cell proliferation, colony formation, and B cell adhesion, effects that mimic API2-MALT1 over-expression[47]. Interestingly, API2-MALT1 expression was even more potent at promoting cell proliferation than was LIMA1 silencing, which led to the hypothesis that generation of the LMO cleavage fragment might unmask a pro-tumorigenic property of the LMO fragment. Indeed, engineered overexpression of the LMO cleavage fragment alone led to increased cell proliferation, colony formation, and fibronectin-mediated adhesion[47]. Thus, it appears that API2-MALT1 dependent LIMA1 cleavage serves the dual purpose of depleting a tumor suppressor from the cell while simultaneously generating an oncogenic LMO cleavage fragment.

In contrast, enforced expression of a cleavage resistant LIMA1 was sufficient to suppress API2-MALT1-dependent growth, proliferation, and colony formation. Further, cleavage resistant LIMA1 was capable of blocking API2-MALT1-dependent tumorigenesis in a murine xenograft model[47]. Importantly, the cleavage of LIMA1 was detected in API2-MALT1-positive MALT lymphoma patient samples, providing strong clinical evidence that, like NIK cleavage, this occurs in human tumors. Taken together, these studies revealed that API2-MALT1-dependent cleavage of LIMA1 contributes to the overall mechanism of action of API2-MALT1-induced lymphomagenesis, and broadens our appreciation of API2-MALT1 as a fusion oncoprotein that affects cell biology through mechanisms that go beyond NF-κB activation. Further investigation is required to determine if LIMA1 is ever targeted for cleavage by wild-type MALT1, but from what we have learned regarding NIK cleavage, we suspect that LIMA1 is only targeted *via* the combined actions of the API2 and MALT1 moieties. Supporting this notion, overexpression of wild-type MALT1 is insufficient to reverse the tumor suppressive effects of LIMA1.

CONCLUSION

The t(11;18) translocation, identified only in a subset of MALT lymphomas, creates the API2-MALT1 fusion oncoprotein and is associated with clinically aggressive disease. Molecular characterization of the mechanisms by which API2-MALT1 exerts its effects has revealed two general strategies of cellular protein subversion: (1) recruitment and exploitation of unique combinations of signaling proteins as a mechanism for unregulated downstream NF-κB activation; and (2) recruitment and cleavage of unnatural substrates, generating substrate fragments that possess gain-of-function oncogenic activity. Like other fusion oncoproteins (*e.g.*, BCR-ABL, NPM-ALK) each of these mechanistic strategies requires the presence and concerted activities of both fusion partners. Fully understanding the breadth and scope of API2-MALT1-dependent interactions may inform design of targeted therapies to block associations that arm API2-MALT1 with the means to perturb cellular signaling pathways and promote lymphomagenesis.

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Development of regional specificity of spinal and medullary dorsal horn neurons

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Abstract

Extensive studies have focused on the development and regionalization of neurons in the central nervous system (CNS). Many genes, which play crucial roles in the development of CNS neurons, have been identified. By using the technique "direct reprogramming", neurons can be produced from multiple cell sources such as fibroblasts. However, understanding the region-specific regulation of neurons in the CNS is still one of the biggest challenges in the research field of neuroscience. Neurons located in the trigeminal subnucleus caudalis (Vc) and in the spinal dorsal horn (SDH) play crucial roles in pain and sensorimotor functions in the orofacial and other somatic body regions, respectively. Anatomically, Vc represents the most caudal component of the trigeminal system, and is contiguous with SDH. This review is focused on recent data dealing with the regional specificity involved in the development of neurons in Vc and SDH.

Key words: Regionalization; Development; Central nervous system; Trigeminal subnucleus caudalis; Spinal cord; Pain

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Core tip: The region-specific control of proliferation, survival and differentiation of neural precursors plays a critical role in development of the central nervous system (CNS). Recent data demonstrated that locally released ninhydrin-reacting small molecules are involved in the region-specific regulation of neuronal development in trigeminal subnuleus caudalis and spinal dorsal horn, which are CNS regions involved in pain signal transmission. These findings not only provide novel insights for understanding CNS regionalization and functional

organization, but also raise important questions for future investigations that are needed for the clarification of neuroregenerative processes and for the development of new approaches for treating neurodegenerative diseases and pain.

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INTRODUCTION

Neurons in the central nervous system (CNS) are regionalized and organized through their circuitry as groups performing specific activities. Functional differences among neuronal groups in the CNS of adult animals are found to be predetermined as early as when the neuroepithelium develops[1-5]. Extensive investigations have revealed that the region-specific control of proliferation, survival and differentiation of neural precursors plays a critical role in the development of the CNS[1-5]. The precursor cells are sensitive to environmental cues which control the expression of region-specific phenotypes[1-5]. However, understanding the region-specific regulation of CNS neurons is still one of the biggest challenges in neuroscience.

The dorsal region of gray matter in the medulla includes subnucleus caudalis (Vc) of the spinal tract nucleus of the trigeminal system[6-8]. Morphologically, Vc is contiguous with the spinal dorsal horn (SDH); this was first noted by Cajal[8]. Indeed, both SDH and Vc have a laminated structure, and other aspects of their cytoarchitecture and cellular elements are essentially the same. Furthermore, both Vc and SDH also share many physiological similarities[6,7,9,10]. Recent findings in regards of the region-specific regulation of neuronal development in Vc and SDH are reviewed in this article.

REGIONALIZATIONS IN THE CNS

During development of the CNS the formation of regional patterning of the neural tube along anteroposterior and dorsoventral axes is one of the earliest events. At this step, hundreds of distinct neuronal subtypes are generated from a single layer of multipotent neuroepithelial progenitor cells with a positional identity (see reviews[3-5]). The telencephalon, where the cerebral cortex and the basal ganglia originate, is initially regionalized. Based on findings in research of the development of the cortex, it has been proposed that the structure patterning is intrinsically encoded in progenitors[5,11,12]. It is found that interneurons related clonally tend to cluster together and act synchronously once they reach the cortex[13,14]. The area identity has been established before any functional diversification is apparent[15]. Although it has also been found that the areal specificity can be affected by afferent connections in a neuronal activity-dependent manner[5,15], a stronger genetic prepatterning is indicated by findings that the region-specific thalamocotical connections are maintained while the mutations of the genes Emx2, COUP-TFI and Sp8 cause changes in cortical area identity[16-18].

Data obtained from cultures of neural progenitors *in vitro* have revealed a high degree of plasticity in cell fate choices[19-22]. Mature neurons can be produced from multiple cell sources such as fibroblasts by using the technique "direct reprogramming" or "trans differentiation"[19-23]. The sequential generation of projection neurons observed *in vivo* can also been found in cultures of neuron precursors *in vitro*[24]. Taken together, it has been proposed that genetic factors play primary roles in neuronal development from progenitor cells, while the environmental factors acting *in vivo* are secondary[3-5].

Developmental changes may also occur in non-neural cells in the CNS such as glial cells including both astrocytes and microglial. Astrocytes are the most abundant cell type in the mammalian CNS and have been viewed as relatively homogeneous, interchangeable and the least migratory. Recently, however, there is accumulating evidence of morphological and molecular differences between astrocytes in different brain regions[25,26]. Culture and transplantation experiments have also demonstrated functional differences among astrocytes from different brain regions[25,27,28]. These differences may be related to astrocyte derivation from regionally patterned radial glial cells[29].

The spatial patterning in the spinal cord is organized by neural progenitors with position identities, which create a segmental template that underlies neuronal diversity[25]. In the spinal cord, astrocytes are also developed in a position-dependent manner and regionalized in a segmental template[25,30]. By providing positional guidance cues for neuronal migrating and axon path finding, astrocytes are involved in the formation of neuronal circuits[25,31]. Several genes encoding extracellular matrix components or axon and cell migration factors have been found to be expressed differentially in astrocytes in the dorsal *vs* ventral spinal cord[32]. This suggests that regional astrocytic subtypes may encode positional functions during development.

THE REGION-SPECIFIC REGULATION OF NEURONAL DEVELOPMENT IN Vc *VS* SDH

Vc represents the most caudal component of the trigeminal system. Both Vc and SDH contain second-order sensory neurons receiving low- and/or high-threshold primary afferent inputs from the orofacial region and other somatic regions of the body, respectively. Most small-diameter nociceptive primary afferents terminate in the superficial laminae of SDH and Vc. Nociceptive neurons in the superficial and deeper laminae relay

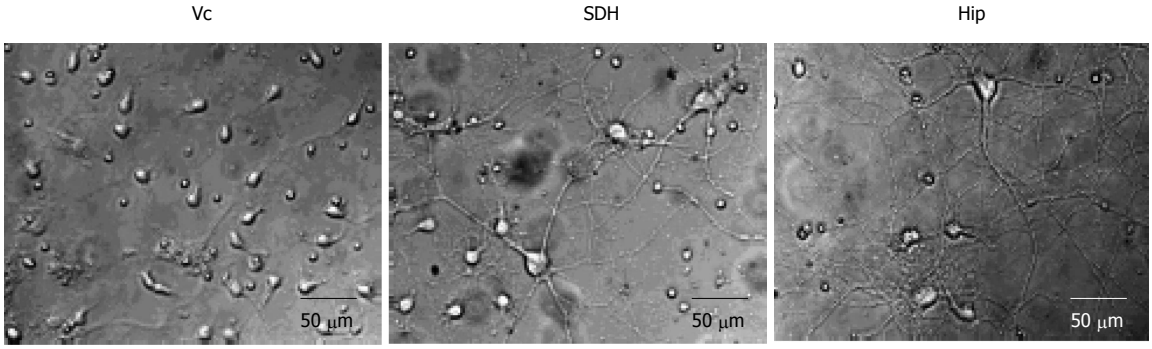


Figure 1 Neuronal growth in trigeminal subnucleus caudalis, spinal dorsal horn and hippocampus cultures. Examples of images were recorded at day 14 after plating. Vc: Trigeminal subnucleus caudalis; SDH: Spinal dorsal horn; Hip: Hippocampus.

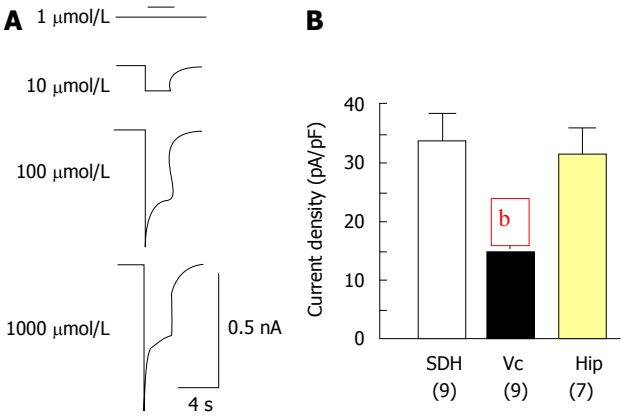


Figure 2 Whole-cell responses mediated by glutamate. A: Examples of current traces recorded in Vc neurons. The currents were evoked with glutamate at concentrations as indicated; B: Summary data (mean ± SEM) of peak current density evoked with 1000 μmol/L of glutamate respectively from Vc, SDH and Hip neurons. Values in brackets indicate number of neurons tested. $^{b}P < 0.01$, independent *t*-test, indicate significant differences detected in Vc *vs* that in SDH or Hip neurons. Vc: Trigeminal subnucleus caudalis; SDH: Spinal dorsal horn; Hip: Hippocampus.

the nociceptive input to higher brain centers[6,7,9,10,33]. According to their mechanoreceptive field properties, sensory neurons in Vc and SDH have been classified into low-threshold mechanoreceptive neurons which only respond to innocuous stimuli (*e.g.*, tactile); wide-dynamic range neurons which respond to both innocuous and noxious stimulation; and nociceptive-specific neurons (NS) which only respond to noxious stimuli[6,7,9,10,33]. Astrocytes and microglial in Vc and SDH have been found to modulate the activity of the nociceptive neurons[34,35].

However, despite the several morphological and functional similarities between Vc and SDH noted above, there are some differences. For example, the distribution of afferent fibers releasing substance P and calcitonin gene related peptide in Vc of adult animals shows a different pattern than that in SDH, a dual representation of peripheral tissues can be found in the rostral and caudal regions of Vc, and there is a transitional zone between Vc and trigeminal subnucleus interpolaris (of the trigeminal spinal tract nucleus) that has a special role in orofacial sensorimotor function and appears to have no clear parallel in the SDH or spinal system[33,36,37]. In addition, it was recently found that cultured neurons from Vc of rat embryos (18 d gestation) grew much slower, the number of primary processes was less and the length of the processes was shorter when compared with cultured SDH and hippocampus (Hip) neurons[38]. Moreover, two weeks after plating for culture, Vc neurons also remained separated while the processes of a large number of neurons in Hip and SDH cultures had merged and formed networks[38] (Figure 1). A question raised by these findings is: What is the functional significance of these differences between Vc and SDH or Hip neurons?

To answer this question, glutamate-mediated whole-cell currents in cultured Vc neurons were recorded at day 14 after plating[38]. All neurons tested showed glutamate concentration-dependent responses (see an example in Figure 2A). However, the current density of Vc neurons was significantly lower than that of SDH or Hip neurons (Figure 2B). Furthermore, it was found that spontaneous miniature excitatory post-synaptic currents (mEPSCs) could be detected in over 80% of SDH and Hip neurons recorded while less than 30% of Vc neurons tested showed mEPSC activity. Detailed analyses showed that the mean frequency and peak amplitude of mEPSCs recorded in cultured Vc neurons were around 0.98 Hz and 25 pA. Most mEPSCs recorded in Vc neurons had both AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) and NMDA (N-methyl-D-aspartate) receptor-mediated components with decay time constants around 4.8 ms and 94 ms, respectively. All of these parameters calculated from mEPSCs recorded in Vc neurons were similar to those found in SDH and Hip neurons[38-41].

To understand the mechanisms which may underlie the differences observed in the development of Vc *vs* SDH or Hip neurons, cell viability in cultures were examined[38]. Embryonic Vc and SDH tissues containing similar proportions of live cells (11% in Vc and 12% in SDH) were initially plated, and live cells in both Vc and SDH cultures increased. However the increase in live cells in Vc was significantly less than that in SDH (Figure 3). At 14 d after plating, the percentage of live cells in

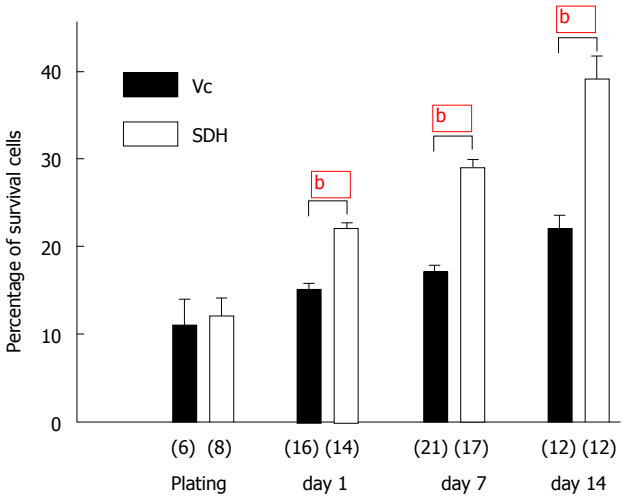


Figure 3 Cell viability in in trigeminal subnucleus caudalis and spinal dorsal horn cultures. Bars show the percentage values (mean ± SEM) of live cells in Vc and SDH cells detected during and after plating as indicated. $^{b}P < 0.01$ (independent *t*-test) when compared with that in SDH cultures. Numbers in brackets indicate the number of dishes tested. Vc: Trigeminal subnucleus caudalis; SDH: Spinal dorsal horn; Hip: Hippocampus.

Vc culture increased only to 22% whereas that in SDH increased to 39%. These data suggest the possibility that cell death occurs more in developing Vc than that in SDH. To confirm this possibility TUNEL (a terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling) was used to detect cell death in cultures of Vc and SDH. Cell death occurring in young and mature neurons was determined by TUNEL with co-labeling of Tuj1 and NeuN antibody, respectively. Cell death occurring in astroglial cells was determined by TUNEL with co-labeling of GFAP antibody. It was found that at 14 d after plating more mature neurons underwent cell death in Vc than SDH whereas cell death in young neurons or astroglial cells appeared similarly in these two regions[38].

A particularly interesting finding in this study[38] was that the difference in cell survival in Vc *vs* SDH was still maintained in cultures either with neurobasal media supplemented with B27, β-FGF2 and L-glutamine [which minimizes the growth of glial cells (< 15%)[42]] or with MEM supplemented with 10% fetal bovine serum and insulin. Altering the concentration of β-FGF2 or insulin added into culture medium did not produce significant effects on the difference in cell survival between Vc and SDH. However, co-culture experiments where the tissue of one region was cultured in a tissue environment of another region showed that that the growth of Vc neurons co-cultured with SDH tissues was significantly promoted (Figure 4): (1) when compared with Vc neurons not co-cultured with SDH, the Vc neurons co-cultured with SDH had more and longer processes which merged and formed networks around 14 d after plating; and (2) the glutamate-mediated current density of the Vc neurons co-cultured with SDH increased and more neurons showed spontaneous mEPSC activity. In contrast, when compared with SDH neurons not co-cultured with Vc, the growth of SDH neurons co-cultured with Vc was significantly inhibited. The glutamate-mediated current density and the occurrence of spontaneous mEPSC activity reduced. These data strongly suggest that some factors which may inhibit or stimulate neuronal growth, are released in a region-specific manner, and thereby differentially regulate the development and function of Vc and SDH.

Many factors may be involved in the Vc-specific regulation, *e.g.*, growth factors including the epidermal growth factors[43], fibroblast growth factors (FGFs)[44-47], transforming growth factors[48], nerve growth factors[49], insulin[50,51], and members of the bcl-2 and caspase gene families[52]. However, the differences in neuronal growth observed in Vc *vs* SDH are similar, irrespective of the concentration of β-FGF2 or insulin supplemented[38], indicating their unlikely involvement in the differential growth regulation of Vc *vs* SDH. Since a group of non-protein ninhydrin-reacting small molecules (NF) isolated from human plasma (by column chromatography using Sephadex G-25 followed by G-15) has been shown to be involved in the regulation of neuronal growth[53-55], NF in Vc, SDH and Hip-conditioned media were investigated for their possible involvement[38]. Consistent with findings in human plasma, two ninhydrin components (NF1 and NF2) could be classified from media used for cell culture. To determine whether NF1 and NF2 isolated from conditioned medium used for Vc, SDH or Hip culture might produce different effects, cell viability in cortical cultures treated with the isolated NF1 or NF2 was also examined in this study[38]. When compared to cultured cortical cells without any treatment, no significant change in cell viability of cortical cells was observed following the application of either non-ninhydrin-reacting fractions isolated from conditioned culture media or NF1 and NF2 isolated from non-conditioned culture medium. However, application of NF1 isolated from Hip- or SDH-conditioned culture medium significantly enhanced cell viability in cortical cultures but application of NF1 isolated from Vc-conditioned medium produced no significant effect. Application of NF2 isolated from Vc-conditioned medium (Vc-NF2) significantly inhibited the growth and viability of cortical cells; only 20% of the cells remained alive in cortical cultures with a 24-h-treatment of Vc-NF2. However, NF2 isolated from either Hip- or SDH-conditioned medium produced no such effect. Thus, these findings strongly suggest that locally released ninhydrin-reacting small molecules may underlie the developmental differences between cultured Vc neurons and SDH neurons.

To obtain insights into whether Vc-NF2 may also be released in a region-specific manner *in vivo*, Vc, SDH or Hip slices were prepared from rats at day 1 after birth, and the media used for culturing the slices were sampled at 3-4 d after slice plating[38]. Like its effects on cortical cells in culture, NF1 from Hip or SDH slice-conditioned medium was found to enhance cell viability and increase the number of live cells in cortical cultures, but NF1 isolated from Vc slice-conditioned

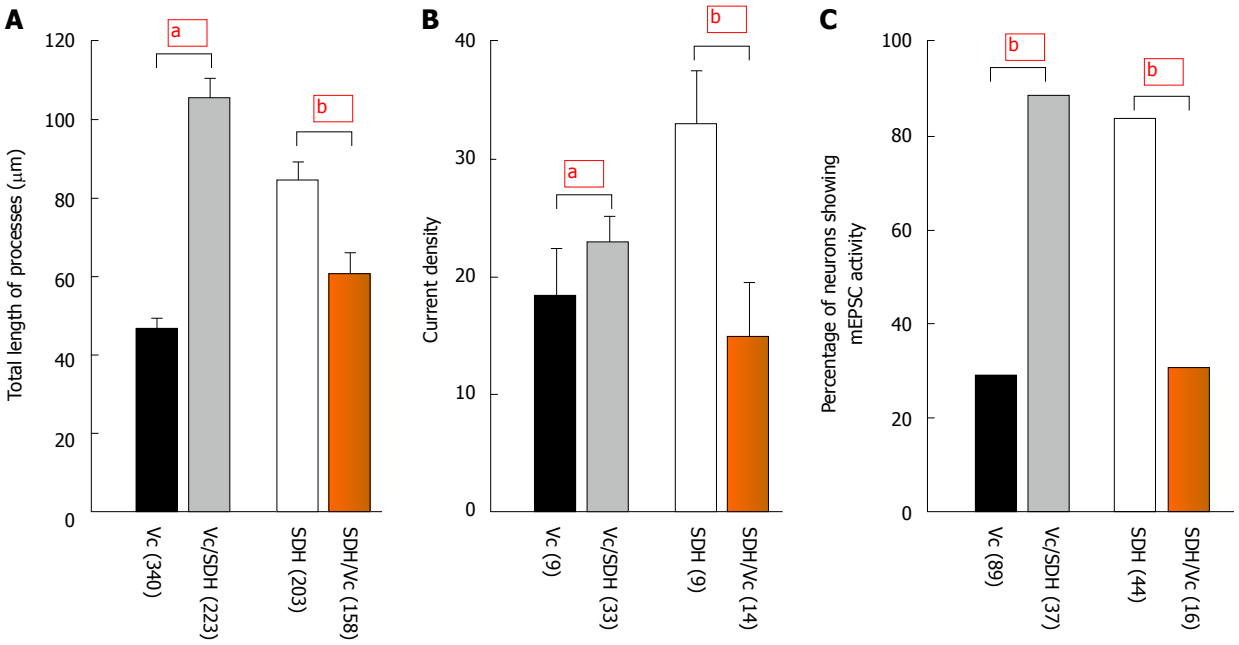


Figure 4 Effects of co-culture. The bar graph shows summary data (mean ± SEM) of the total length of processes at day 7 after plating (A), current density (B) and occurrence of mEPSC activity (C) recorded at day 14 after plating. Vc/SDH: Vc cells co-cultured with SDH; SDH/Vc: SDH cells co-cultured with Vc; $^{a}P < 0.05$, $^{b}P < 0.01$ (independent *t*-test in A and B, Fisher's exact test in C) when compared with those in co-cultures. Numbers in brackets indicate the number of neurons tested. Vc: Trigeminal subnucleus caudalis; SDH: Spinal dorsal horn. mEPSC: Miniature excitatory post-synaptic current.

medium had no significant effect. NF2 isolated from Vc slice-conditioned medium inhibited cell growth and viability, whereas NF2 isolated from Hip or SDH slice-conditioned medium did not induce such changes. Thus, these findings have provided further evidence that Vc-NF2 and SDH-NF1 are important factors involved in region-specific regulation in the dorsal horn *ex vivo*. The finding of consistent effects on neuronal growth induced by Vc-NF2, SDH-NF1 or Hip-NF1 isolated from conditioned media either for embryonic or postnatal cultures excludes the possibility that the differences observed in neuronal development of Vc *vs* SDH or Hip might be induced by the different culture methods used.

It has been shown that different subtypes of neurons such as interneurons and projection neurons in SDH mature at different rates[56,57]. Thus, the differences documented in the neuronal development and functions of Vc *vs* SDH in animals of the same ages and under the same conditions[38] could conceivably be also related to differences in the developmental stage of the CNS regions. However, whether interneurons and projection neurons in Vc mature at different rates is still unknown. A rostrocaudal gradient of inhibitory factors which are diffusible and released from the ventral horn have been found in the developing spinal cord[58-60]. Although more detailed investigations are still needed, the findings that less trophic and more growth-inhibitory factors may be released locally from Vc in comparison to SDH or Hip indicate that these locally released factors may be involved also in the regulation of the maturation rate of neurons of different subtypes and/or in different CNS regions.

FUTURE RESEARCH DIRECTIONS

In the CNS, the functional specificities of neurons are predetermined at a very early stage of development. The region-specific control of proliferation, survival and differentiation of neural precursors plays a critical role. The ninhydrin-reacting small molecules in fraction NF1 or NF2 isolated from conditioned medium used for Vc, SDH or Hip culture induced significant changes in neuronal growth while those from unconditioned medium did not, confirming that specific molecules are released by neural tissues in culture[38]. These data have: (1) demonstrated that the involvement of locally released factors in the region-specific regulation of neuronal development in Vc and SDH which are CNS regions critically involved in pain signal transmission; (2) provided novel insights for understanding CNS regionalization and functional organization; and (3) raised several important questions for future investigations that are needed for the clarification of neuroregenerative processes and for the development of new therapeutic approaches for treating neurodegenerative diseases and pain.

To determine how the ninhydrin-reacting small molecules are released in Vc and SDH and from what types of cells

As noted above, neuronal growth in cultures with neurobasal medium with the addition of B27, β-FGF2 and L-glutamine, was significantly improved and glial cells were much reduced in Vc, SDH and Hip cultures when compared with those in cultures with MEM supplemented with 10% fetal bovine serum and insulin. Nonetheless, neurons in Vc cultures still showed signifi-

cantly slower growth, lower glutamate receptor activity and more cells undergoing death when compared with those in SDH and Hip cultures. Thus, there is the possibility that the growth-trophic and growth-inhibitory molecules such as in SDH-NF1 and Vc-NF2 may be released from neurons as well as from glial cells. Studies are needed to determine how and from what types of cells these molecules are released in Vc and in SDH. The characterization of intrinsic and extrinsic signals underlying the regulation of the release of the growth-trophic factor SDH-NF1 and the growth-inhibitory factor Vc-NF2 will be key for research advances aiming to understand mechanisms underlying the region-specific regulation of neuronal development in Vc and SDH.

To determine how and what types of cells may be regulated by the locally released molecules in NF1 or NF2 in Vc and SDH

Different neuronal populations have been found to be differentially regulated during development[1,2,49,61,62]. The data obtained in experiments of TUNEL with co-labeling of GFAP, NeuN or Tuj1 antibody showed that at day 7 after plating, significantly more NeuN- and GFAP-stained Vc cells showed TUNEL when compared with those in SDH, but no such difference was noted between Tuj1-labeled neurons in Vc *vs* SDH[38]. At day 14 after plating, there were still more neurons showing NeuN co-labeling with TUNEL in Vc but no difference between Vc and SDH could be found in neurons showing Tuj1 co-labeling or in astroglial cells showing GFAP co-labeling. These findings therefore imply that molecules in Vc-NF2 may selectively target intracellular signaling pathway(s) developed in mature neurons and thereby causing neuronal death. The cellular types in Vc and SDH are heterogeneous. In Vc and SDH, glutamate and GABA are the principal excitatory and inhibitory transmitters, respectively. Genetically, whether neurons will develop to a glutamatergic or GABAergic cells depends upon the expression of homeobox genes *Tlx3*, *Tlx1* and *Lbx1*[63-65]. Thus, characterization of the development of Vc and SDH cells with gene-related labeling needs to be conducted to clarify which neuronal and astrogial cell types in Vc, SDH or Hip may be regulated by locally released NF1 or NF2.

As noted above, many fewer Vc neurons in culture showed spontaneous EPSC activity and the glutamate-mediated whole-cell current density was significantly low, when compared with those in neurons in SDH or Hip cultures[38]. Co-cultures of Vc with SDH significantly increased the glutamate-mediated whole-cell current density and mEPSC occurrence in Vc whereas co-culture of SDH with Vc significantly reduced the glutamate-mediated whole-cell current density and mEPSC occurrence in SDH neurons. Although locally released Vc-NF2 and SDH-NF1 have been found to be important factors underlying the alteration of cell growth in Vc and SDH, it still needs to be determined if the glutamate-mediated whole-cell current density and mEPSC activity may also be directly modulated by bath applied Vc-NF2 or SDH-NF1.

Moreover, although experimental data have demonstrated that locally released Vc-NF2 and SDH-NF1 play important roles in the region-specific regulation of neuronal growth *ex vivo*[38], there is a need for studies *in vivo* comparing the rate of growth, expression of glutamate receptors and cell death of Vc *vs* SDH neurons. The characterization of the effects of locally released factors *in vivo* still represents an important future research direction. Neurons in Vc and SDH play crucial roles in pain and related sensorimotor functions. Studies addressing whether and how the locally released factors may affect nociceptive and sensorimotor functions in the trigeminal and spinal systems are particularly important for understanding pathophysiological conditions expressing pain and/or sensorimotor dysfunction.

To determine the chemical nature of SDH-NF1 and Vc-NF2

Two fractions called fractions V3A and V3[54,55] containing non-protein, ninhydrin-reacting small molecules can be isolated by using size exclusion chromatography on Sephadex G-25 followed by Sephadex G-15 from human plasma. Furthermore, the fraction V3A is found to have insulin-like activity, while fraction V3 is an inhibitor of insulin action[54,55]. Fraction V3A contains chiro-inositol and galactosamine, and fraction V3 contains myoinositol 1,2-cyclic phosphate and galactosamine. The myoinositol 1,2 cyclic phosphate group is essential for the inhibitory action of fraction V3[54,55]. V3A applied to hippocampal or cortical cultures significantly increases neuronal growth while V3 inhibits neuronal growth[53]. Non-protein, ninhydrin-reacting small molecule fractions (NF1 and NF2) can be isolated from culture media by using the same size exclusion chromatography as that used to isolate fractions V3 and V3A from human plasma. Interestingly, the elution profiles of NF1 and NF2 are very similar to those of fractions V3 and V3A from human plasma[38]. However, so far the chemical nature of the fractions NF1 and NF2 remains unknown. Thus, the identification of the chemical structures of molecules in Vc-NF2 and SDH-NF1 should be a major focus of future research in order to develop useful agents to selectively block the effect of Vc-NF2 or SDH-NF1. These agents should be very useful tools not only for investigating region-specific regulation in areas of the CNS such as Vc and SDH, but also for developing new approaches to diagnose and treat neurodegenerative diseases, pain and sensorimotor dysfunction.

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MINIREVIEWS

Regulation of RNA binding proteins in trypanosomatid protozoan parasites

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Abstract

Posttranscriptional mechanisms have a critical role in the overall outcome of gene expression. These mechanisms are especially relevant in protozoa from the genus *Trypanosoma*, which is composed by death threatening parasites affecting people in Sub-saharan Africa or in the Americas. In these parasites the classic view of regulation of transcription initiation to modulate the products of a given gene cannot be applied. This is due to the presence of transcription start sites that give rise to long polycistronic units that need to be processed cotranscriptionally by trans-splicing and polyadenylation to give mature monocistronic mRNAs. Posttranscriptional mechanisms such as mRNA degradation and translational repression are responsible for the final synthesis of the required protein products. In this context, RNA-binding proteins (RBPs) in trypanosomes have a relevant role as modulators of mRNA abundance and translational repression by associating to the 3′ untranslated regions in mRNA. Many different RBPs have been proposed to modulate cohorts of mRNAs in trypanosomes. However, the current understanding of their functions lacks a dynamic view on the different steps at which these RBPs are regulated. Here, we discuss different evidences to propose regulatory events for different RBPs in these parasites. These events vary from regulated developmental expression, to biogenesis of cytoplasmic ribonucleoprotein complexes in the nucleus, and condensation of RBPs and mRNA into large cytoplasmic granules. Finally, we discuss how newly identified posttranslational modifications of RBPs and mRNA metabolism-related proteins could have an enormous impact on the modulation of mRNA abundance. To understand these modifications is especially relevant in these parasites due to the fact that the enzymes involved could be interesting targets for drug therapy.

Key words: Trypanosoma; Posttranscriptional gene expression; Ribonucleoprotein complexes; RNA-binding protein; Developmental regulation; Sleeping sickness; Posttranslational modification; Phosphorylation; Chagas disease

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Core tip: We discuss several ways to regulate the function of RNA-binding proteins in trypanosomes. We highlight the propensity of these proteins to engage in interactions with other proteins and RNA, resulting in the formation of large reversible aggregates induced by environmental stress. Finally, the possible role of posttranslational modifications on the function of these proteins is discussed in the context of recent high-throughput proteomic evidences.

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INTRODUCTION

The synthesis of the required protein products, and their levels, demands coordinated mechanisms to regulate gene expression in all organisms. These mechanisms operate in eukaryotic cells at the level of transcription initiation and/or mRNA processing, localization, stability or translational efficiency. As an exception to the rule, a group of protozoan parasites belonging to the genus *Trypanosoma* have evolved towards regulating gene expression without classical transcription initiation control. Within these parasites are *T. brucei*, the causative agent of Sleeping Sickness in Sub-Saharan Africa[1], and *T. cruzi*, causing Chagas Disease in the Americas[2]. These parasites have complex life cycles, which involve an hematophagous insect vector and a vertebrate host, each one hosting different life forms with unique characteristics and metabolisms[3]. Unfortunately, there are no effective vaccines against these parasites, and current drug treatments are highly toxic, present low tolerance and require long patient compliance[4]. Although the current drug treatments can be effective during the acute stage of the infection, there is a need for newer, safer and more effective treatments against these neglected diseases.

One peculiarity of these parasites is that their genomes have protein-coding genes organized into large directional gene clusters[5,6], which lack canonical RNA polymerase Ⅱ promoters. Instead, histone variants and epigenetic regulation seem to mark the boundaries of yet undefined transcription start sites for these long transcriptional units[7,8]. The resultant polycistronic immature mRNAs are then processed by coordinated trans-splicing and polyadenylation events, giving rise to mature monocistronic mRNAs[9]. However, protein-coding sequences in each trypanosomatid transcriptional unit are not functionally related as they are in bacterial operons[5]. From this point onwards, posttranscriptional regulation of gene expression heavily contributes to the levels of the protein products that are synthesized, depending on the cell's requirements. The expression of functionally related mRNAs seems to be controlled in a coordinated fashion as posttranscriptional regulons[10]. Within this view, structural motifs enriched in 3' untranslated regions seem to govern the fate of the mRNA molecule inside the cell. Different transcripts carrying the same signature motifs are likely to be regulated in a similar way[11]. These structural motifs serve as ligands for different RNA-binding proteins (RBPs) that associate with the mRNA, forming a ribonucleoprotein (RNP) complex, which is also composed by many other proteins that interact with different parts of the transcript. Consequently, the presence or absence of different RBPs, which in turn can recruit other factors to the RNP complex, likely dictates the fate of the mRNA inside the cell. RBPs interact with mRNA motifs using dedicated functional domains such as the RNA-Recognition Motif (RRM), the Zinc Finger domains, the Pumilio domain and the ALBA domain, to name the most relevant domains found in trypanosomatid RBPs[12].

Trypanosomes have been the muse for the identification of novel biological mechanisms such as RNA trans-splicing, mitochondrial RNA editing, and antigenic variation[13], allowing us to understand their similarities and differences with higher eukaryotic organisms. Identification and characterization of RBPs in trypanosomes started almost 20 years ago. However, it was the concerted sequencing and annotation of their genomes 10 years ago that handled the field with the tools to characterize factors and deeply describe these mechanisms in trypanosomes[14,15]. While *T. cruzi* lacks some the components required for a functional RNA interference (RNAi) machinery, in *T. brucei* mRNA levels can be easily downregulated by this mechanism[16], making this organism the workhorse in the field. The usefulness of omics-approaches in trypanosomes has also allowed the identification of the mRNA molecules affected when a certain RBP is down or upregulated[17]. This, added to the current ability to identify the associated mRNA molecules of a given RBP by RNA-crosslinking and sequencing technologies[18], can give a deeper understanding of the role of the RBP under study. However, our current understanding of RBP function in trypanosomes is rather static, and is far from reflecting the dramatic changes in posttranscriptional gene expression these protozoa suffer during their life cycles. In their mammalian and insect hosts, these parasites adopt different life forms that are adapted to environmental changing conditions. Thus, any single factor modulating gene expression in trypanosomes needs to be regulated in order to cope with the changing developmental and environmental conditions they face.

Here, we will expose the yet poorly explored regulation of the function of trypanosomatid RBPs, emphasizing on those involved in the modulation of cytoplasmic mRNA levels and translation. Regulation is probably exerted at different levels: RBP expression for a given developmental stage, localization-mediated

Table 1 Developmentally expressed RNA-binding proteins in trypanosomes confirmed by experimental data

RBP	Stage	Ref.
T. brucei		
P34	Procyclics	[23]
P37	Bloodstream	[23]
ZFP1	Procyclics	[26]
ZC3H20	Procyclics	[78]
Alba 3	Tsetse fly except transition stages in proventriculus	[79]
Alba 4	Tsetse fly except transition stages in proventriculus	[79]
RBP6	Metacyclics, epimastigotes	[29]
RBP10	Bloodstream	[28]
hnRNPF/H	Mainly bloodstream	[80]
T. cruzi		
UBP2	Mostly epimastigotes, low in amastigotes	[25]
RBP3	Epimastigotes	[81]
RBP4	Epimastigotes	[81]
RBP19	Amastigotes	[82]
ZFP2	Downregulated in metacyclic trypomastigotes	[83]

RBP: RNA-binding protein.

function including nucleocytoplasmic shuttling and nuclear RNA-binding, condensation and sequestering into RNP complexes, and posttranslational modifications (PTMs). We aim to analyze the possible additional layers of complexity that RBPs bring to the gene expression game in trypanosomes.

Developmental regulation of RBP expression

The simplest way to regulate the function of a protein in a time manner is to prevent or promote its synthesis. This is especially relevant for trypanosomes, which alternate between different life forms in their insect and mammalian hosts during which gene expression seems to be regulated essentially at the posttranscriptional level. In these parasites, certain developmental programs were confirmed to be orchestrated by RBPs[12]. Originally, the developmentally regulated expression of each RBP was detected using specific antibodies (Table 1), although current high-throughput approaches rely on the analysis of the transcriptome and the proteome[19-22]. While the latter approaches can give an extensive amount of information on the regulated abundance of mRNAs and proteins, including RBPs, biochemical or genetic validation for each RBP is required to confirm developmental regulation. The first reported case of developmentally regulated RBPs in *T. brucei* was from P34/P37[23]. These are highly similar RRM-containing RBPs that are required for the assembly of the 60S ribosomal subunit[24], being P34 expressed in procyclics and P37 expressed in bloodstream forms[23]. In *T. cruzi*, the RRM-containing RBP UBP2 was the first to show developmental regulation, being expressed in replicative epimastigotes and amastigotes, while it is almost undetectable in cell-derived trypomastigotes[25]. It remains to be determined if overexpression or the lack of expression of P34/P37, UBP2 or other proteins listed in Table 1, can compromise the developmental program of a trypanosome.

Hendriks *et al*[26] demonstrated that genetic ablation of ZFP1 in *T. brucei* prevented the correct repositioning of the kinetoplast, a specific configuration for the mitochondrial DNA present in trypanosomes and leishmanias[27]. This phenotype was associated with the incomplete morphological differentiation from bloodstream to procyclic form *T. brucei* parasites[26], suggesting a role for ZFP1 in *T. brucei* bloodstream form differentiation. RBP10, which is another RRM-containing protein, showed to be enriched in bloodstream-form *T. brucei* cells[28]. Knock-down of RBP10 by RNAi in bloodstream forms resulted in the downregulation of transcripts with high abundance in this stage, while overexpression of RBP10 in procyclics revealed an increased abundance of bloodstream specific transcripts[28]. Hence, manipulation of RBP10 protein levels seems to modulate the abundance of specific developmentally regulated transcripts, although there was no commitment to a differentiation event. RBP6-mediated developmental differentiation in *T. brucei* is probably the most relevant finding regarding developmental expression[29] (Figure 1A). A genome wide high-throughput RNA-seq analysis revealed RBP6 transcripts in *T. brucei* parasites from the proventriculous were enriched 13-times relatively to procyclics from the midgut. When Kolev *et al*[29] overexpressed RBP6 in cultured procyclic cells, they observed developmental stages that are normally observed in the insect vector and not in culture, namely long and short epimastigotes and metacyclic trypomastigotes (Figure 1A). RBP6-overexpressing parasites in culture showed the hallmarks of undergoing metacyclogenesis, and could be used to infect mice, showing the full potential of an infective form. All these examples reveal the role of developmentally regulated RBPs in the modulation of the abundance of life form-specific transcripts, pointing to these and other RBPs as master factors regulating developmental programs.

Regulation of RBP localization

A potential way to regulate the function of an RBP is to limit where in the cell it can associate to its RNA target. Although we are beginning to understand the complexity of RBP-mediated posttranscriptional regulation of gene expression, current models often describe static events, where the RBP is functional once it is associated with the RNA. For many RBPs directly involved in nuclear RNA metabolism, or for those showing high sequence identity to characterized factors involved in conserved nuclear mechanisms, a nuclear localization would make sense for the expected function. This has been the case for the LA protein[30], the exoribonuclease XRNE involved in pre-rRNA processing[31], the nuclear cap binding protein CBP20[32], the Splice-Leader RNP complex associated Sm proteins[33], or Lsm proteins[34], to name a few. Notwithstanding this, nuclear localization for other functionally uncharacterized RBPs like ZFP8 in *T. cruzi* requires further understanding of the function of the protein to assign it to a nuclear function[35]. This

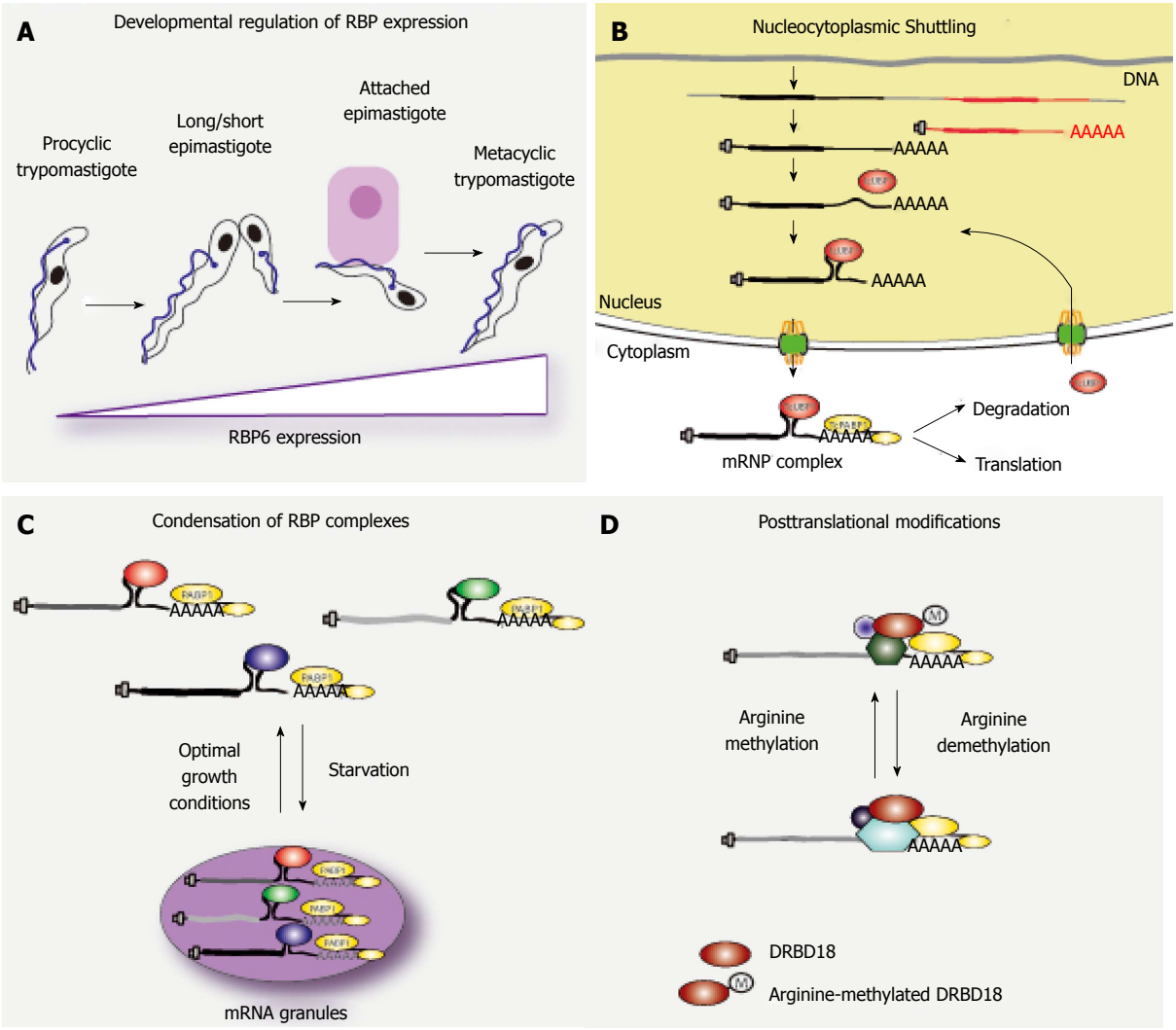


Figure 1 Regulation of RNA-binding proteins function in trypanosomes. Each panel summarizes the proposed mode of regulation of the function of RNA-binding proteins in trypanosomes.

was the case of the nuclear protein RBP33 in *T. Brucei*, which was suggested to be involved in the regulation of nuclear gene silencing[36]. Nuclear localization signals in trypanosomes are difficult to detect because they are not usually predicted by the algorithms developed for mammalian proteins[30,37], adding another layer of complexity to the characterization of RBPs targeted to the nucleus.

For most models of cytoplasmic RBP function there is no information on where and when the RNP complex is formed. This is especially relevant in posttranscriptional regulation, which is believed to happen mostly in the cytoplasm, but has a main actor (the mRNA) with a nuclear history. In *T. brucei* bloodstream forms, most mRNA half-lives are under 20 min, with few long lived mRNAs with half lives of more than 2 h[17]. In *Saccharomyces cerevisiae*, expression dynamics of RBPs showed an increased protein abundance and higher half-life as compared to non-RBP proteins[38]. If this is also the case for trypanosomes, it could suggest that a single RBP molecule might complete more than one cycle of RNP complex biogenesis and degradation. In mammalian cells, RNP complex remodeling and recycling of RBPs has been studied from a nuclear perspective, with many factors associating to mRNA in the nucleus, travelling together through the nuclear pore complex and returning to the nucleus for another event of RNP biogenesis[39]. It is clear that analyzing a snapshot for the localization of an RBP will tell where the molecule spends most of its time, but will fail to determine whether it spends time in other cellular locations albeit transiently. The evidence of RBP nucleocytoplasmic shuttling in trypanosomes points to a possible nuclear step for proper cytoplasmic function[40]. This hypothesis arose from the behavior of six single RRM-containing RBPs (UBP1-2, RBP3-6) in *T. cruzi*[37]. In this case, these cytoplasmic proteins reversibly accumulated in the nucleus when cells were stressed with sodium arsenite. Although this is not a physiological stressor, its use allowed the nuclear accumulation of these RBPs, events that would have been undetected under normal conditions[40]. Active transcription and an intact RRM were required for

Table 2 List of components identified in starvation mRNA granules in trypanosomes

Component	Organism
poly(A)	*T. brucei, T. cruzi*
UBP1	*T. brucei, T. cruzi*
UBP2	*T. cruzi*
RBP3	*T. cruzi*
RBP4	*T. cruzi*
RBP5	*T. cruzi*
RBP6b	*T. cruzi*
PABP1	*T. brucei, T. cruzi*
PABP2	*T. brucei, T. cruzi*
DRBD3	*T. brucei*
DRBD4	*T. brucei*
ALBA1	*T. brucei*
ALBA2	*T. brucei*
ALBA3	*T. brucei*
ALBA4	*T. brucei*
ZFP3	*T. brucei*
DHH1	*T. brucei, T. cruzi*
XRNA	*T. brucei, T. cruzi*
SCD6	*T. brucei*
PBP1	*T. brucei*
MKT1	*T. brucei*
CAF1	*T. brucei*
NOT1	*T. brucei*
NOT5	*T. brucei*
UPF1	*T. brucei*
DBP1	*T. brucei*
AGO1	*T. brucei*
eIF4G1	*T. brucei*
eIF4G4	*T. brucei*
Serine-threonine kinase receptor-associated protein	*T. brucei*
mRNA cap guanine-N7 methyltransferase	*T. brucei*
Phosphatase	*T. brucei*
Methyltransferase	*T. brucei*

UBP1 nuclear accumulation, suggesting dependence on nuclear availability of newly synthesized mRNAs for this to happen[37]. Colocalization of UBP1 with target transcripts and poly (A) mRNA in the nucleus suggests that arsenite could be inhibiting nuclear export of mRNA. In the same line of evidence, the cytoplasmic DRBD3 in *T. brucei* showed a similar response to arsenite stress[41], showing that similar events of nucleocytoplasmic shuttling could be affected by arsenite in both species. Also in *T. brucei*, PABP2 could accumulate in the nucleus under a combined stress of heat shock and sinefungin addition[42], a drug that prevents trans-splicing of mRNA by inhibiting Cap4 formation of Splice leader molecules[43]. All these evidences point towards a nuclear mRNA-binding event taking place during mRNP biogenesis (Figure 1B). It remains to be determined if this nuclear event is required for the function of these RBPs in the cytoplasm.

Regulation of RBP interactions and condensation in RNP complexes

In the cytoplasm of trypanosomatid cells mRNA molecules are associated with general and specific RBPs. It is believed that these specific RBPs will dictate the fate of the mRNA[44]. Thus, mRNA molecules and RBPs are distributed in RNP complexes throughout the cytoplasm. However, these RNP complexes can be very different in protein composition from each other, with proteins colocalizing in some foci but not in every one of them[45].

Under starvation conditions in *T. cruzi* and *T. brucei*, large cytoplasmic granules are formed as a result of the condensation of different RNP complexes[46]. Several evidences suggest that these are not genuine Stress Granules, and consequently were named mRNA granules, since these were characterized based on the dynamics of the mRNA[46]. The list of proteins colocalizing with mRNA granules is in expansion (Table 2), since these structures have been a valuable tool to involve a putative RBP in mRNA metabolism in trypanosomes. No ribosomal proteins from the 40S or 60S subunits were detected in mRNA granules in *T. cruzi*[46], suggesting that the transcripts accumulating in these structures have already exited translation. In *Saccharomyces cerevisiae*, metabolic enzymes also reorganize from a homogenous distribution in the cytoplasm to reversible granule-like assemblies under starvation[47]. In this work it was proposed that the recruitment of enzymes to cytoplasmic foci during the nutrient deprivation period could function as a reservoir of critical cellular components for cell-cycle reentry once nutrients are readily available. This would empty the cytoplasm of these proteins, and would prevent their function due to condensation in granules. This hypothesis is especially relevant in trypanosomes for proteins with enzymatic activity like the exoribonuclease XRNA, which is involved in 5′ to 3′ enzymatic digestion of highly unstable and developmentally regulated mRNA[48] (Figure 1C). XRNA is in fact a component of starvation mRNA granules, where it does not seem to be promoting mRNA degradation. In fact, the transcripts stored in mRNA granules in trypanosomes are intact, since these do possess a poly(A) tail and the characteristic mini-exon at the 5′ end[46,49]. This, added to the fact that transcripts in granules can reenter translation if a new source of nutrients is available[50], suggests that these structures can play a role as reservoirs of transcripts during a physiological stress like starvation. This setup would provide fast templates for synthesis of cellular components that would allow differentiation or cell-cycle re-entry.

Current evidence failed to identify a single RBP required for the formation of mRNA granules, although the overexpression of SCD6 is sufficient to induce the formation of aggregates similar to starvation granules, which contain mRNA, DHH1 and eIF4E[49]. However, depletion of SCD6 by RNAi in *T. brucei* did not affect the formation of starvation mRNA granules[51], suggesting that redundant factors might be involved in the formation of these structures. Of the experimentally validated components of these structures, many contain RNA-binding domains (RBD), such as the RRM or ALBA domain, or contain domains involved in interactions with other mRNA metabolism-related proteins (Table 2). Very recently, Fritz *et al*[52] developed a very ingenious approach to purify starvation induced mRNA granules

that is based on the release of these structures from the subpellicular microtubule cage after cytoskeleton depolymerization, followed by mass spectrometry (MS)[52]. By this approach, they could detect many proteins already identified in mRNA granules, and many new components, 17 of which could be efficiently validated by eYFP-fusion protein colocalization to mRNA granules markers. These included RNA metabolism related proteins, as well as one phosphatase, one methyltransferase, and four hypothetical proteins (Table 2).

Other domains accompanying RBDs in RBPs can provide a scaffold for protein interactions and additional regulation[53]. This is the case of low complexity (LC) sequences, defined by low information content due to the repetition of a few amino acids. LC sequences enriched in RBPs and mRNA metabolism related proteins resemble prion-like domains, allowing these proteins to self-assemble. For instance, the yeast Lsm4 protein, a core P-body component, appears to be involved in granule formation by self-aggregation of its Q/N rich domain[54]. Reijns *et al*[55] identified 20 yeast P-body proteins with above average Q/N content, and demonstrated a reduced association to these structures when these sequences were removed from Ccr4p, Pop2p and Dhh1p. In trypanosomes, proteins like UBP1, UBP2, RBP3 and RBP6, which contain Q-rich sequences, are found in starvation mRNA granules[46]. In spite of this, the presence of a functional RRM is necessary and sufficient for the localization of UBP1 to these structures, showing a direct association to transcripts in starvation mRNA granules for this RBP[46]. Q or G-rich sequences were also identified in many other RRM-type RBPs[56], and a poly-Q sequence has been proposed to be a motif involved in the interaction of MKT1 with several mRNA-related proteins[57]. In mammalian cells, the LC sequence of Fused in Sarcoma (FUS) is able to form reversible hydrogel droplets *in vitro*, which can trap other LC sequence-containing molecules[58]. These sequences are predicted to form intrinsically unstructured domains, and were shown to orchestrate the dynamic assembly of RNP granules in yeasts and mammalian cells through homotypic and heterotypic associations[59]. Thus, the polymeric association of different RBPs in RNP complexes seems to control the dynamic assembly of RNA granules. It is therefore the presence of LC sequences, or a combination of these with an RBD, that could be regulating the function of an RBP, and probably of the bound mRNA, through their recruitment to RNP granules.

To add a layer of complexity, Han *et al*[60] demonstrated that phosphorylation of FUS LC sequence inhibits its association to homotypic hydrogel droplets. The hypothesis arising from these results is that regulatory phosphorylations can prevent LC sequence-containing RBPs to transition into the RNA granule, thus regulating RNP formation and composition[61]. This hypothesis guides our review to probably the most important, yet the less explored, way to modulate the function of an RBP in trypanosomes.

Regulation by PTMs

PTM of proteins has shown to increase the functional diversity of the proteome by the covalent addition of functional groups, peptides or proteins[62]. Amongst the many PTM a protein can suffer, we will discuss here those that were shown to modify cytoplasmic or nuclear RBPs in trypanosomes, namely phosphorylation, SUMOylation, and arginine-methylation. To understand the role these PTMs on RBPs, and many other proteins, will have a huge impact in trypanosomes, since these modifications involve one or more enzymes that could be used as targets for pharmacological intervention for these parasitic diseases.

It was more than 20 years ago when the first RBP with PTMs was identified in *T. brucei*. This was the case of Nopp44/46, which is an RBP belonging to a family composed by several members with different number of RGG repeats[63]. Nop44/46 is phosphorylated on serine and tyrosine residues, and has been proposed to be required for formation of the large ribosomal subunit in *T. brucei*[64]. Numerous proteomic approaches have recently been used to identify phosphorylated proteins in *T. brucei* and *T. cruzi* (Table 3), since phosphorylation events are especially relevant due to their impact in a broad spectrum of processes and regulatory functions[65]. In *T. brucei*, the phosphoproteome of the bloodstream form was the first to be explored based on phosphopeptide enrichment coupled to MS[66]. In this study, serine, threonine and tyrosine phosphorylated residues were detected, showing conserved phosphorylation sequence motifs in regulatory kinases, suggesting that phosphorylation-based signaling mechanisms are conserved in trypanosomes[66]. However, a previous genome wide survey for protein kinases in *Leishmania*, *T. brucei* and *T. cruzi* showed these parasites lack receptor-linked tyrosine and tyrosine kinase-like kinases, but instead posses dual specificity kinases[67]. Urbaniak and collaborators[68] reported a quantitative phosphorylation study of the *T. brucei* bloodstream and procyclic forms by SILAC labeling. The results from this work revealed a significant number of phosphorylated ZFPs, RRM-type RBPs, and mRNA metabolism-related proteins (Table 3), some of which showed lifecycle specific regulation of phosphorylation status. In this work it was proposed that the dynamic phosphorylation of RBPs could have the potential to modulate gene expression, as these proteins could be acting as ultimate effector molecules of some of the trypanosomatid signaling cascades[68]. *T. cruzi* has also been explored extensively at the phosphoproteomic level[69-71], revealing many new and already *T. brucei* identified phosphorylated RBP and related proteins (Table 3). In a very interesting study, Queiroz *et al*[71] performed a comparative proteomic and phosphoproteomic analysis during amastigogenesis, the transition from non-replicating and infective trypomastigotes to replicating non-infective amastigotes. From these analyses several

Table 3 RNA-binding proteins and mRNA metabolism-related proteins with identified posttranslational modifications in trypanosomes

RBP	*T. brucei*	*T. cruzi*	Posttranslational modification
PUF domain			
PUF1	Tb927.10.4430	TcCLB.397937.10/TcCLB.508625.160	Phosphorylated in *T. brucei*[68] Phosphorylated in *T. cruzi*[71]
PUF2	Tb927.10.12660	TcCLB.507831.110	Phosphorylated in *T. brucei*[66,68] Phosphorylated in *T. cruzi*[69]
PUF3	Tb927.10.310	TcCLB.508787.30/TcCLB.468005.9	Phosphorylated in *T. brucei*[66,68]
PUF4	Tb927.6.820	TcCLB.509399.190/TcCLB.510073.30	Phosphorylated in *T. brucei*[66,68]
PUF6	Tb927.10.15870	TcCLB.510125.10	Phosphorylated in *T. brucei*[66,68] Phosphorylated in *T. cruzi*[71] Sumoylated in *T. cruzi*[73]
PUF7	Tb927.11.14960	TcCLB.508445.99/TcCLB.511715.100	Sumoylated in *T. cruzi*[73]
PUF9	Tb927.1.2600	TcCLB.506563.10/TcCLB.503869.40	Phosphorylated in *T. brucei*[68] Arginine-methylated in *T. brucei*[75]
PUF10	Tb927.11.6740	TcCLB.506773.130/TcCLB.508799.70	Arginine-methylated in *T. brucei*[75]
PUF11	Tb927.11.10810	TcCLB.503757.30/TcCLB.503719.39	Phosphorylated in *T. brucei*[68] Arginine-methylated in *T. brucei*[75]
RRM domain			
PABP1	Tb927.9.9290	TcCLB.506885.70	Phosphorylated in *T. brucei*[68] Phosphorylated in *T. cruzi*[70,71] Sumoylated in *T. cruzi*[73]
PABP2	Tb927.9.10770	TcCLB.508461.140	Phosphorylated in *T. brucei*[68]
UBP1	Tb927.11.500	TcCLB.507093.220	Phosphorylated in *T. cruzi*[84]
RBP3	Tb927.11.530	TcCLB.507093.250	Phosphorylated in *T. brucei*[68]
RBP8	Tb927.7.320	TcCLB.504165.20/TcCLB.508981.20	Phosphorylated in *T. brucei*[68]
RBP9	Tb927.11.12120	TcCLB.511127.10/TcCLB.511481.70	Phosphorylated in *T. brucei*[68]
RBP10	Tb927.8.2780	TcCLB.508989.30/TcCLB.509569.120	Phosphorylated in *T. brucei*[66,68]
RBP12	Tb927.10.12740	TcCLB.511277.200	Phosphorylated in *T. brucei*[68] Phosphorylated in *T. cruzi*[71]
U1 snRNP (RBP24)	Tb927.8.4830	TcCLB.508837.140/TcCLB.511383.30	Phosphorylated in *T. brucei*[68]
RBP28	Tb927.3.1030	TcCLB.511871.110/TcCLB.511863.20	Phosphorylated in *T. brucei*[68] Arginine-methylated in *T. brucei*[75]
RBP29	Tb927.10.13720	TcCLB.511277.580	Phosphorylated in *T. cruzi*[69,71]
RBP30	Tb927.5.1750	TcCLB.505229.20/TcCLB.510823.20	Phosphorylated in *T. brucei*[68]
RBP33	Tb927.8.990	TcCLB.503733.50/TcCLB.508569.90	Phosphorylated in *T. brucei*[68] Arginine-methylated in *T. brucei*[75]
RBP35	Tb927.9.12360	TcCLB.510741.40	Phosphorylated in *T. brucei*[68] Phosphorylated in *T. cruzi*[70]
RBP38	Tb927.11.5850	TcCLB.508641.180/TcCLB.508515.30	Phosphorylated in *T. brucei*[68]
RBP42	Tb927.6.4440	TcCLB.509167.140	Phosphorylated in *T. cruzi*[71]
RBSR1	Tb927.9.6870	TcCLB.506625.70	Phosphorylated in *T. brucei*[68] Phosphorylated in *T. cruzi*[69]
RBSR4	Tb927.10.3500	TcCLB.510265.40/TcCLB.510311.50	Phosphorylated in *T. brucei*[68] Arginine-methylated in *T. brucei*[75]
DRBD2	Tb927.9.13990	TcCLB.510755.120/TcCLB.508413.50	Phosphorylated in *T. cruzi*[69]
DRBD5	Tb927.6.3480	TcCLB.469785.40/TcCLB.507025.50	Phosphorylated in *T. brucei*[68]
DRBD7	Tb927.4.400	TcCLB.507873.30/TcCLB.510689.60	Phosphorylated in *T. brucei*[68]
DRBD10	Tb927.11.16020	TcCLB.507037.20/TcCLB.508707.80	Phosphorylated in *T. brucei*[68]
DRBD11	Tb927.3.3940	TcCLB.503683.30/TcCLB.509999.120	Phosphorylated in *T. brucei*[68]
DRBD13	Tb927.8.6650	TcCLB.506399.40/TcCLB.509243.20	Phosphorylated in *T. brucei*[68]
DRBD17	Tb927.8.710	TcCLB.507649.100/TcCLB.508567.100	Phosphorylated in *T. brucei*[68] Arginine-methylated in *T. brucei*[75]
DRBD18	Tb927.11.14090	TcCLB.511727.190	Arginine-methylated in *T. brucei*[75]
HNRNPH	Tb927.2.3880	TcCLB.511109.130/TcCLB.504157.10	Phosphorylated in *T. brucei*[68] Phosphorylated in *T. cruzi*[70,71] Sumoylated in *T. cruzi*[73]
TRRM1	Tb927.2.4710	TcCLB.509317.60/TcCLB.511621.50	Phosphorylated in *T. brucei*[66,68] Phosphorylated in *T. cruzi*[69] Sumoylated in *T. cruzi*[73]
TRRM3	Tb927.3.3670	TcCLB.506989.100/TcCLB.510149.140	Arginine-methylated in *T. brucei*[75]
PPCTI	Tb927.5.3750	TcCLB.503619.20/TcCLB.511647.40	Phosphorylated in *T. brucei*[68]
MRD1	Tb927.8.4170	TcCLB.509561.110/TcCLB.503897.90	Phosphorylated in *T. brucei*[68]
TSR1	Tb927.8.900	TcCLB.509607.30/TcCLB.503715.10	Phosphorylated in *T. brucei*[68]
NRBD1/2 (P34/P37)	Tb927.11.14000/Tb927.11.14020	TcCLB.511727.270/TcCLB.511727.290	Arginine methylated in *T. brucei*[75]
Conserved	Tb927.10.7030	TcCLB.506779.100/TcCLB.511153.100	Arginine-methylated in *T. brucei*[75]
Conserved	Tb927.11.7310	TcCLB.506779.100/TcCLB.511153.100	Arginine-methylated in *T. brucei*[75]
Conserved	Tb927.11.14090	TcCLB.511727.190	Arginine-methylated in *T. brucei*[75]
Conserved	Tb927.11.6240	TcCLB.506297.230/TcCLB.510101.80	Arginine-methylated in *T. brucei*[75]
Zinc finger domains			
ZFP1	Tb927.6.3490	TcCLB.511511.6	Phosphorylated in *T. brucei*[66,68]
ZFP3	Tb927.3.720	TcCLB.509719.69/TcCLB.509231.39	Phosphorylated in *T. brucei*[68]
ZC3H5	Tb927.3.740	TcCLB.511867.10/TcCLB.507775.10	Phosphorylated in *T. brucei*[68]
ZC3H7	Tb927.3.1340	TcCLB.509233.210	Phosphorylated in *T. brucei*[68]
ZC3H8	Tb927.3.5250	TcCLB.510143.120/TcCLB.508409.310	Phosphorylated in *T. brucei*[68] Arginine-methylated in *T. brucei*[75]
ZC3H11	Tb927.5.810	TcCLB.507305.40	Phosphorylated in *T. brucei*[68,85]
ZC3H12	Tb927.5.1570	TcCLB.510819.119/TcCLB.506739.99	Phosphorylated in *T. brucei*[86]
ZC3H13	Tb927.5.1580	No homologue	Phosphorylated in *T. brucei*[66,68]

ZC3H17	Tb927.7.930	TcCLB.508879.10/TcCLB.508215.10	Phosphorylated in *T. brucei*[68]
ZC3H18	Tb927.7.2140	TcCLB.511807.160	Phosphorylated in *T. brucei*[68]
ZC3H20	Tb927.7.2660	TcCLB.503567.9/TcCLB.506859.204	Phosphorylated in *T. brucei*[68]
ZC3H21	Tb927.7.2670	TcCLB.506859.230/TcCLB.511817.10	Phosphorylated in *T. brucei*[68]
ZC3H22	Tb927.7.2680	TcCLB.506859.240/TcCLB.511817.20	Phosphorylated in *T. brucei*[68]
ZC3H23	Tb927.7.4980	TcCLB.509149.20/TcCLB.508175.350	Phosphorylated in *T. brucei*[68]
ZC3H28	Tb927.9.9450	TcCLB.506885.200/TcCLB.510729.220	Phosphorylated in *T. brucei*[68] Phosphorylated in *T. cruzi*[70]
ZC3H29	Tb927.9.9520	TcCLB.510729.210/TcCLB.506885.204	Phosphorylated in *T. brucei*[68]
ZC3H30	Tb927.10.1540	TcCLB.506977.110	Phosphorylated in *T. brucei*[68]
ZC3H31	Tb927.10.5150	TcCLB.506009.10/TcCLB.510295.59	Sumoylated in *T. cruzi*[73]
ZC3H32	Tb927.10.5250	TcCLB.503795.10 /TcCLB.506679.10	Phosphorylated in *T. brucei*[68]
ZC3H34	Tb927.10.12330	TcCLB.507787.140/TcCLB.507625.70	Phosphorylated in *T. brucei*[68] Arginine-methylated in *T. brucei*[75]
ZC3H35	Tb927.10.12740	TcCLB.511263.30/TcCLB.507831.40	Phosphorylated in *T. brucei*[68]
ZC3H37/38	Tb927.10.12780	TcCLB.507831.20/TcCLB.511267.20/ TcCLB.511263.50	Phosphorylated in *T. brucei*[68]
ZC3H39	Tb927.10.14930	TcCLB.508895.50	Phosphorylated in *T. brucei*[68]
ZC3H40	Tb927.10.14950	TcCLB.508895.60/TcCLB.506211.60	Phosphorylated in *T. brucei*[66,68] Phosphorylated in *T. cruzi*[69,71]
ZC3H41	Tb927.11.1980	TcCLB.508355.330/TcCLB.508357.9	Phosphorylated in *T. brucei*[68]
ZC3H43	Tb927.11.7450	TcCLB.511151.20/TcCLB.508241.90	Phosphorylated in *T. brucei*[68]
ZC3H44	Tb927.11.7890	TcCLB.506933.50	Phosphorylated in *T. brucei*[68]
ZC3H46	Tb927.11.16550	TcCLB.507089.30/TcCLB.504085.70	Phosphorylated in *T. brucei*[68]
ZC3H47	Tb927.6.4960	TcCLB.506945.210	Phosphorylated in *T. brucei*[68] Phosphorylated in *T. cruzi*[69]
Conserved	Tb927.11.3970	TcCLB.509229.90/TcCLB.506733.140	Phosphorylated in *T. brucei*[68]
Conserved	Tb927.9.14120	TcCLB.510759.100/TcCLB.506999.120	Arginine-methylated in *T. brucei*[75]
Other			
NOP44/46	Tb927.8.760	TcCLB.510859.17/TcCLB.510859.10	Phosphorylated in *T. brucei*[68] Arginine-methylated in *T. brucei*[75]
SMD2	Tb927.2.5850	TcCLB.508667.49/TcCLB.511189.80	Phosphorylated in *T. brucei*[68]
DCL2	Tb927.3.1230	No homologue	Phosphorylated in *T. brucei*[68] Arginine-methylated in *T. brucei*[75]
CAF1	Tb927.6.600	TcCLB.511827.60/TcCLB.510535.60	Phosphorylated in *T. brucei*[68]
Not1	Tb927.10.1510	TcCLB.509247.30	Phosphorylated in *T. cruzi*[70]
XRNA	Tb927.7.4900	TcCLB.507817.80	Phosphorylated in *T. cruzi*[69]
ALBA3	Tb927.4.2040	TcCLB.510877.40	Phosphorylated in *T. brucei*[68] Arginine-methylated in *T. brucei*[75]
ALBA4	Tb927.4.2030	TcCLB.510877.30	Phosphorylated in *T. brucei*[68] Arginine-methylated in *T. brucei*[75]
eIF4E	Tb927.11.11770	TcCLB.508827.30	Phosphorylated in *T. brucei*[68] Phosphorylated in *T. cruzi*[70]
	Tb927.6.1870	TcCLB.509037.40/TcCLB.421959.10	Phosphorylated in*T. brucei*[68]
eIF4G2	Tb927.9.5460	TcCLB.508277.340/TcCLB.506445.20	Phosphorylated in *T. brucei*[68]
	Tb927.11.10560	TcCLB.510285.100/TcCLB.504827.130	Phosphorylated in *T. brucei*[68]
eIF4G5	Tb927.8.4500	TcCLB.508989.90	Phosphorylated in *T. brucei*[68]

RBP: RNA-binding protein; RRM: RNA-recognition motif.

RBPs and mRNA-related proteins were identified as being developmentally regulated between the final morphological stages, or during the transformation process[71]. To note was that several RBPs also showed developmental phosphorylation, suggesting that these modifications could be regulating the function of these proteins. It has been suggested that phosphorylation may induce global conformational changes in an RBP, either promoting or inhibiting protein-protein or protein-RNA interactions allosterically[72]. Thus, regulatory phosphorylation events in RBPs could have an enormous impact on gene expression regulation in trypanosomes due to the prevalence of posttranscriptional mechanisms in these parasites.

In another study Bayona *et al*[73] reported the functionality of the small ubiquitin-like modifier (SUMO) pathway in *T. cruzi*. The addition of SUMO is a covalent and reversible modification that can usually affect a protein's normal function. Initially SUMOylation was believed to be only involved in nuclear events such as nucleocytoplasmic transport, DNA replication, gene transcription and DNA damage response, but with the identification of numerous other targets it now seems that SUMOylation can affect numerous processes at both sides of the nuclear envelope[74]. Within the many proteins modified by SUMO addition in *T. cruzi*, some belong to the RBP category (Table 3)[73]. Given that this type of modification is usually very unstable and it is lost during typical experimental manipulations, adequate experimental approaches will be required in the future to address the effect of SUMOylation on RBPs in trypanosomes.

Last but not least, the potential of arginine-methylation was explored by a global proteomic analysis in *T. brucei*[75]. In this work, methylated arginine residues were identified mainly in glycine-rich contexts, as described in other organisms. The functional classification of proteins derived from this work revealed an interesting amount of proteins involved in mRNA metabolism (Table 3)[75], and other related proteins. The relevance of this finding in *T. brucei* is highlighted by the impact that arginine-methylation can have on protein-protein and protein-nucleic acid interactions and subcellular localization of proteins[76]. DRBD18, a double RRM-containing RBP, was initially identified as having three methyl-arginines[75]. In a posterior characterization of DRBD18 in *T. brucei*, methylmimetic or hypomethylated mutants were expressed in parasites

downregulated for endogenous DRBD18. Both mutants exhibited a differential modulation of the *T. brucei* transcriptome, clearly showing opposing effects of due to methylarginine content[77]. Surprisingly, these differences did not seem to be regulated at the level of subcellular localization or affinity for RNA molecules, since both mutants showed similar association to RNA molecules in the cytoplasm. Instead, the methylation state of DRBD18 arginine residues seemed to modulate the interactions of these DRBD18 variants with other protein factors involved in mRNA metabolism, since they showed to associate to different RNP complexes (Figure 1D). Thus, differential arginine-methylation was proposed to shape the composition of DRBD18 RNP complexes, and thus to modulate the fate of the associated mRNA[77].

CONCLUSION

Although the above-described events might seem yet unrelated, future work might provide evidences to connect different mechanisms to regulate the function of an RBP in trypanosomes. This is especially relevant for localization, aggregation and interaction events that might be regulated by posttranslational modifications. Given that RBPs do not have enzymatic activities, and hence cannot be tested as druggable targets, it is tempting to speculate that the enzymes (kinases, phosphatases, SUMO E3 ligases and methyltransferases) responsible for these posttranslational modifications might be indeed good candidates for drug therapy. Approaches targeting any of these protein modifications are likely to have a very high impact for these neglected diseases, either by discovery of new drugs or by drug repurposing.

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Engineered magnetic core shell nanoprobes: Synthesis and applications to cancer imaging and therapeutics

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Abstract

Magnetic core shell nanoparticles are composed of a highly magnetic core material surrounded by a thin shell of desired drug, polymer or metal oxide. These magnetic core shell nanoparticles have a wide range of applications in biomedical research, more specifically in tissue imaging, drug delivery and therapeutics. The present review discusses the up-to-date knowledge on the various procedures for synthesis of magnetic core shell nanoparticles along with their applications in cancer imaging, drug delivery and hyperthermia or cancer therapeutics. Literature in this area shows that magnetic core shell nanoparticle-based imaging, drug targeting and therapy through hyperthermia can potentially be a powerful tool for the advanced diagnosis and treatment of various cancers.

Key words: Magnetic core shell nanoparticles; Magnetic resonance imaging; Cancer therapeutics; Drug delivery; Hyperthermia

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Core tip: Magnetic core shell nanoparticles have recently gained a lot of interest due to its excellent design and applicability to various fields of research including biomedical sciences. The core shell particle contains a highly magnetic core surrounded by a thin shell of desired material, the choice being dependent on the application. The applicability of core shell nanoparticles in the area of *in vivo* imaging, drug delivery and therapeutics in the form of hyperthermia have been discussed along with the various procedures for synthesis of these useful nanoprobes.

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INTRODUCTION

Nanoparticles are emerging as a prospective biomedical tool with applications in diagnostics, specific drug

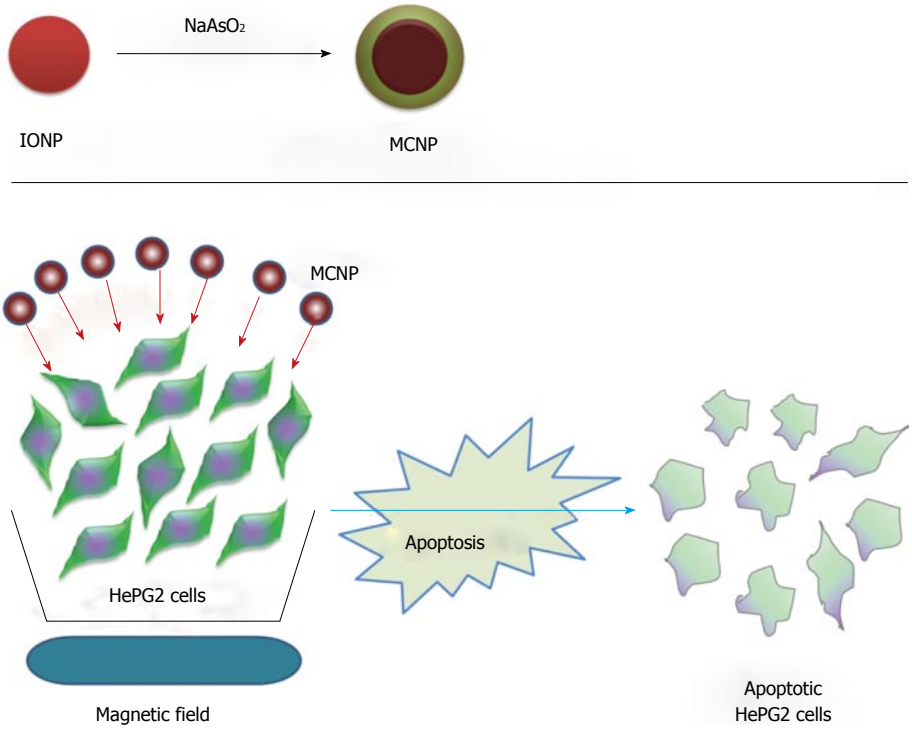


Figure 1 Schematic showing synthesis of magnetic core shell nanoparticle and their application towards targeting the cancer cells. Upper panel shows generation of MCNPs and lower panel represents magnetically facilitated arsenite delivery using MCNPs to kill cancerous cells through apoptosis. MCNP: Magnetic core shell nanoparticle; IONP: Iron oxide nanoparticle.

delivery and therapeutics of diseases. Among these, magnetic nanomaterials with a core shell structure has been of interest for a long time primarily owing to their vast applications for example in magnetic data recording devices, in sensors, in catalytic reactions and in various biomedical applications encompassing tissue imaging for diagnosis, drug delivery and therapeutic management of diseases[1-4]. Attachment of biomolecular markers to the surface of magnetic nanoparticles has been used to demonstrate that magnetic nanoparticles have the potential to deliver noxious drugs at a specific location in the body accurately[5], which in effect can lead to design of highly specialized bio-probes for diagnostic imaging[6,7]. These advances have encouraged the development of biocompatible magnetic nanomaterials endowed with ultra-sensitive imaging potential in order to find wide applications in targeted as well as non-invasive *in vivo* medical imaging.

One such example could be Au coated magnetic nanoparticles, a complex system that has drawn a lot of attention[8]. Au coating is responsible for more effective stabilization of the magnetic nanoparticles, particularly metallic nanoparticles with high magnetic moment under toxic biological environment. The magnetic nanoparticles can readily be functionalized by Au because of its well-developed Au-S chemistry; the gold coating provides plasmonic properties to the magnetic nanoparticles. This renders the composite core/shell nanoparticles exceedingly attractive for multidisciplinary applications comprising of magnetic, optical, and biomedical applications. Another example could be arsenicals which have been known for thousands of years to be carcinogenic or poisonous despite their therapeutic or beneficial effects which are observed at a low dose. The United States Food and Drug Administration has granted approval of arsenic trioxide (ATO) as an effective leading edge treatment for relapsed and/or refractory APL patients. Further, several reports emphasize the success of arsenic in a diverse hematological malignancies for example promonocytic leukemia, chronic myelogenous leukemia, multiple myeloma, T-cell leukemia, and a wide array of cancers derived from solid tumors such as renal cell carcinoma, neuroblastoma, glioblastoma, gastric, hepatocellular, head and neck, cervical, prostate and breast cancers[9-15]. ATO either alone or in combination with other drugs has been used for each disease. The soluble most toxic and naturally prevalent form of arsenic ($NaAsO_2$) has been shown to induce apoptosis in human malignant melanoma cells (A375) *in vitro*. Enhanced generation of reactive oxygen species, mitochondrial membrane potential damage and caspase activation are the critical mediators of apoptosis[16]. Extensive clinical application has been limited by: (1) diverse sensitivity of tumor cells to a higher dose of arsenic; and (2) less sensitive cell sensitization needing up to 10 times higher concentration of arsenic entailing the risk of arsenic-induced side effects[17-19] (illustrated in Figure 1). Cancers, especially solid tumours, at their advanced stage are therefore difficult to manage by arsenic

administration in its present form. Nanotechnology, especially the core shell magnetic nanoparticles, has been shown to have advantages over others in terms of using effective lower dose of arsenite with minimal side effect and higher efficacy[20]. In this context, the present review highlights the synthesis and applications of diverse magnetic core shell nanoparticles in diagnostics especially imaging and in therapeutics.

TYPES OF MAGNETIC CORE-SHELL NANOPARTICLES

The core shell nanoparticle is a type of nanoparticle consisting of a core or inner matter and a shell or outer coating material. Various types of core shell nanoparticles with different combinations in close interaction have been prepared with distinctive use. The combinations can be inorganic/inorganic, inorganic/organic and organic/inorganic materials. Generally magnetic core-shell nanoparticles can be classified into two following types.

Magnetic oxide core shell

The maghemite or magnetite magnetic nanoparticle has a relatively inert surface composition which generally does not permit strong covalent bond formation with functional molecules. The use of shell of silica onto the surface of magnetic nanoparticles has been shown to enhance the reactivity of magnetic nanoparticles[21]. The silica shell can be readily customized through the formation of covalent bonds with a variety of surface functional groups[22]. These silica-functionalized magnetic nanoparticles can be used to conjugate with some fluorescent dye molecules through the formation of covalent bond[23].

Ferrite nanoparticle clusters, comprising of about 80 maghemite super paramagnetic oxide nanoparticles per bead with silica shell, have a number of benefits over metallic nanoparticles. These can be summed up as: (1) greater chemical and thermodynamic stability; (2) precise size distribution; (3) greater colloidal stability; (4) adjustability of the magnetic moment with the nanoparticle cluster size; (5) retention of super paramagnetic properties irrespective of the cluster size of nanoparticles; and (6) straightforward covalent functionalization by the silica surface.

Metallic magnet core shell

The core material of magnetic naoparticles may be deactivated by mild oxidation, use of surfactants and/or polymers[24]. In an oxidative surroundings, antiferromagnetic CoO layer was formed onto the surface of the Co nanoparticle. Lately, the exchange bias synthetic protocol has been used to generate Co core CoO shell nanoparticles having gold outer shell[25]. Also, much interest has been generated in nanoparticles with a magnetic core consisting either of elementary iron or cobalt with a nonreactive shell made of grapheme, which have been synthesized recently[26]. The advantages compared to ferrite or elemental nanoparticles are: (1) higher magnetization; and (2) higher stability in acidic and basic solutions as well as organic solvents.

Synthesis of magnetic core shell nanoparticles

Magnetic core shell nanoparticle synthesis involves mainly two steps: First is the synthesis of magnetic nanomaterials and after that coating of the magnetic nanomaterials with desired organic or inorganic materials according to the requirement. The synthesis can be affected by a variety of combinations including inorganic/organic, organic/inorganic and inorganic/inorganic materials in close interaction. The selection of shell materials in core-shell nanoparticles is largely decided by the end application and utility. Generally silica, different types of metal and nonmetallic oxides, polymer and drug molecules are employed as coating materials. Thus here the main important task is the synthesis of magnetic nanomaterials (core). These core nanomaterials can be synthesized by the following four methodologies.

CO-PRECIPITATION TECHNIQUE

Iron oxide (Fe_3O_4 or γ-Fe_2O_3) nanoparticles could be prepared by the slow addition of a base into a mixture of aqueous Fe^{2+}/Fe^{3+} salt solutions under an inert atmosphere at elevated temperature or room temperature depending upon requirement. This method is termed as co-precipitation technique. The composition and morphology of the magnetic nanoparticles prepared by co-precipitation method is highly dependent on the precursor salt and reaction conditions such as temperature, pH and ionic strength of reaction system. The quality of the magnetite nanoparticles is fully reproducible if the synthetic conditions are fixed. The experiment showed that the magnetic saturation values of magnetite nanoparticles are lower than bulk value (90 emu/g). Under ambient conditions, magnetite nanoparticles are not very stable. These nanoparticles are readily oxidized to a more stable maghemite form or dissolved in an acidic pH. Being a ferrimagnet, oxidation process is less problematic. The conversation of magnetite to maghemite is accomplished by forming acidic dispersion, followed by iron (Ⅲ) nitrate addition. Now this maghemite particle shows chemical stability through a wide range of pH.

Although the magnetite particles are transformed into maghemite nanoparticles at the initial stage, the experimental challenge in the formation of Fe_3O_4 involving co-precipitation technique lies in controlling narrow particle size distribution. As particle size and morphology largely depend on blocking temperature, a broad range of particle size distribution would be due to the fluctuation of blocking temperature, which results in irregular magnetic activity. Nanoparticles obtained by co-precipitation technique are polydisperse in nature.

Recently, considerable approaches in synthesizing

monodisperse magnetite nanoparticles having diverse sizes and morphology have been finished by the exploitation of various organic stabilizers and reducing agents. For instance, the stabilization of magnetite nanoparticles having dimensions of 3-11 nm was done by applying an aqueous solution of 1 wt% polyvinlyalcohol (PVA).

On the other hand chainlike clusters precipitate of magnetite nanoparticles could be formed by the application of PVA having 0.1 mol% carboxyl groups as stabilizing agent[27]. The above fact shows that the appropriate surfactant choice is a key matter to stabilize the particle. Formation of magnetite by the use of trisodium citrate in an alkaline pH, followed by successive oxidation at 90 ℃ for 30 min with iron (Ⅲ) nitrate produces size-tunable maghemite nanoparticles. The adjustment of the molar ratio of metal ions (Fe^{2+}/Fe^{3+}) and citrate ions is the main key to vary the particle size from 3 to 8 nm[28]. The special effect of carboxylate and hydroxy carboxylate ions on the preparation of iron oxides or oxyhydroxides has been investigated elaborately[29]. Deprotonated carboxy and deprotonated α-hydroxy groups are essentials for the formation of surface complexes[30]. The advanced investigations have revealed that the best stabilization of magnetic Fe_3O_4 nanoparticles was done by oleic acid[31,32].

Thermal decomposition

Synthesis of magnetic particles with desired shape and size generated from the idea of quality semiconductor nanocrystals and oxide-nanoparticles synthesis involving thermal decomposition technique using non-aqueous media[33-35]. Organometallic compounds on thermal decomposition in high-boiling organic solvents with stabilizing surfactants produce monodisperse magnetic nanocrystals with smaller size[36,37]. In this process, the frequently used surfactants are fatty acids, oleic acid[38], and hexadecylamine[39]. Metal acetylacetonates [M(acac)n] (M = Fe, Mn, Co, Ni, Cr; *n* = 2 or 3, acac = acetylacetonate), metal cupferronates (MxCupx) [M = metal ion; Cup = N-nitrosophenylhydroxylamine, $C_6H_5N(NO)O$-] or carbonyls[40] can be used as organometallic precursors. The size and shape of magnetic nanocomposite can be regulated by varying the ratio of the starting reagents including organometallic compounds, surfactant, and solvent. The reaction temperature, time and aging period are also vital for the precise control of size and shape of magnetic nanoparticles. In case of the metal in zero valent state, for example in carbonyls, thermal decomposition primarily tends to the creation of the metal, however a two-step procedure is often used to make oxide nanoparticles. For example, at 100 ℃, iron pentacarbonyl is capable of decomposing into a mixture of oleic acid and octyl ether, followed by the addition of a mild oxidant like trimethylamine oxide $(CH3)_3NO$ at a higher temperature, produces monodisperse γ-Fe_2O_3 nanocrystals of approximately 12 nm in size[41]. A precursor with cationic metal centers on decomposition produces oxide nano. For example, Fe_3O_4 is formed on decomposition of $Fe(acac)_3$ in the presence of oleoylamine, 1,2-hexadecanediol and oleic acid in phenol ether[37,42]. The pyrolysis of metal fatty acid salts (such as salts of decanoic acid, lauric acid, myristic acid, palmitic acid, oleic acid, stearic acid) in a non-aqueous solution (octadecene, n-eicosane, tetracosane, or a mixture of octadecene and tetracosane) generated size and shape controlled magnetic oxide nanocrystal[43]. This method provides nearly monodisperse Fe_3O_4 nanocrystals having a wide range of size adjustable capacity (4-50 nm) with controlled shapes, including dots and cubes. This method has successfully employed for the synthesis of Cr_2O_3, MnO, Co_3O_4, and NiO magnetic nanocrystals. Variation of the reactivity and concentration of the precursors is the key factor to control the size and shape of the nanocrystals. Variation of concentration and chain length of the fatty acids determine the reactivity of the materials. Generally faster reaction rate associates with the shorter chain length. Alcohols or primary amines are often employed to speed up the reaction rate and decrease the reaction temperature.

Hyeon *et al*[41] employed a similar thermal decomposition procedure for the synthesis of monodisperse iron oxide nanoparticles[27]. They have generated an iron oleate complex *in situ* by using iron (Ⅲ) chloride and sodium oleate which were then decomposed between 240 ℃ and 320 ℃ in different solvent systems like 1-hexadecene, 1-octadecene, 1-eicosene, octyl ether or trioctylamine. In this process particle size is determined by the temperature of decomposition and period of aging. Here aging was an important and necessary step for the generation of iron oxide nanoparticles. The nanoparticles prepared by this root can be dispersed in a variety of organic solvents alongside hexane and toluene. Iron pentacarbonyl and the iron oleate complex on decomposition at different temperatures produce monodisperse iron nanoparticles (6-15 nm) which can again be oxidized to magnetite[44]. This process is comparable with seed-mediated growth and explained by the classical LaMer mechanism. Hyeon synthesis involves thermal decomposition of iron pentacarbonyl at a moderately low temperature and the decomposition of the iron oleate complex at a higher temperature leading to the formation of iron oxide nanoparticles which disperse easily in organic solvents.

In biotechnology application, magnetic nanoparticles which are water soluble are more advantageous. This requirement led to the preparation of water soluble Fe_3O_4 nanocrystals with $FeCl_3 \cdot 6H_2O$ as an iron source and 2-pyrrolidone as a coordinating solvent under refluxing condition (245 ℃)[45]. In this method, the mean particle size is controlled at 4, 12, and 60 nm, respectively, when the reflux times are 1, 10, and 24 h. With increasing reflux time, change of shapes of the particles from spherical to cubic morphologies was observed. Recently, water-soluble magnetite nanoparticles have been synthesized using one-pot synthesis under analogous reaction states with the addition of a surface capping agent like α,ω-dicarboxyl-terminated poly (ethylene

glycol)[46]. These nanoparticles are exploited as magnetic resonance imaging (MRI) contrast agents for the diagnosis of cancer.

Metallic nanoparticles can also be prepared by thermal-decomposition method. The metallic nanoparticles have a large number of advantages over other metal oxide nanoparticles owing to their larger magnetization. Thermal breakdown of [$Fe(CO)_5$] in the presence of polyisobutene in decalin in a nitrogen atmosphere at 170 ℃ produces metallic iron nanoparticles[47]. Depending on the $Fe(CO)_5$/polyisobutene ratio, the size of the particle can be adjusted from 3 to 10 nm, with a polydispersity of approximately 10%. Susceptibility measurements revealed that the iron nanoparticles prepared by this way can be easily oxidized by exposure to air. This oxidation can generate a marginal increase of particle sizes approximately by a factor of 1.3. Iron nanocubes can be synthesized by the breakdown of $Fe[N(Si(CH_3)_3)_2]_2$ with H_2 in the presence of hexadecylammonium chloride or hexadecylamine and oleic acid at 150 ℃[48]. The edge-length of the nanocubes varied from 7 nm to 8.3 nm along with the varying relative concentrations of amine and acid ligand. These nanocubes can accumulate into expanded crystalline superlattices by way of their crystallographic axes aligned.

Cobalt nanoparticles can also be prepared by the thermal-decomposition method. Their shape and morphology both can be controlled by this method[49]. Cobalt nanodisks can also be prepared by thermal-decomposition of a cobalt carbonyl precursor[50,51]. The high-temperature reduction of noncarbonyl organometallic complexes produces cobalt nanorods[52,53] and nickel nanorods[54]. For example, decomposition of [$Co(H_3\text{-}C_8H_{13})(h_4\text{-}C_8H_{12})$] with H_2 in anisole at 150 ℃ in the presence of a combination of hexadecylamine and a fatty acid (lauric, octanoic, or stearic acid) produces monodisperse ferromagnetic cobalt nanorods. The variation of diameter and length of the cobalt nanorods largly depends upon different acids used[53].

For easy handling and application under oxidizing conditions, air-stable magnetic nanoparticles are very important. The thermolysis of $Co_2(CO)_8$ in the presence of alkyl-aluminum compounds produces monodisperse colloidal cobalt nanoparticles[54]. The Co particles can be regulated in the size-range of 3-11 nm, by changing the alkyl chain length of these organo aluminum complexes. Air-stable particles can be synthesized by mild surface oxidation of the cobalt nanoparticles with synthetic air. The oxidation step is necessary as saturation magnetization of the CoO particles decays rapidly while exposed to air subsequent to the peptization with the surfactant KorantinSH.

Magnetic alloy nanoparticles have many benefits over other magnetic nanoparticles owing to their high magnetic anisotropy, enhanced magnetic susceptibility and large coercivities[55]. Currently metal phosphides have generated a lot of scientific interest in nanotechnology and chemistry beside $CoPt_3$ and FePt[56-59]. For ferromagnetism, magnetoresistance, and magnetocaloric effects, hexagonal iron phosphide and allied materials have been rigorously studied[60,61]. Recently FeP and MnP nanoparticles have been synthesized from the reaction of iron(Ⅲ) acetylacetonate and manganese carbonyl, respectively, with tris(trim ethylsilyl)phosphane at elevated temperatures[62,63]. Antiferromagnetic FeP nanorods were prepared by the thermal decomposition of a precursor/surfactant mixture solution[64]. Thermal decomposition of permanently supplied iron pentacarbonyl in trioctylphosphane using a syringe pump produces discrete iron phosphide (Fe_2P) nanorods.

Microemulsion techniques

Thermodynamically stable isotropic liquid mixture, where the micro-domain of either or both liquids is stabilized by an interfacial surfactant film, is called microemulsion[64]. In case of water-in-oil microemulsions, the aqueous phase is dispersed as micro size droplets bounded by a monolayer of surfactant molecules in the continuous hydrocarbon phase. The molar ratio of water to surfactant determined the size of the reverse micelle[65]. Mixing of two identical water-in-oil microemulsions including the preferred reactants results in continuous collision, and break again, finally forming a precipitate in the micelles[66]. Addition of solvent like acetone or ethanol to the microemulsions produces a precipitate, which can be collected by filtration or centrifugation. In this concept, a microemulsion served as a nanoreactor for the generation of nanoparticles.

Metallic Co, Co/Pt alloy, and gold-coated Co/Pt nanostructures have been prepared using this microemulsion technique in reverse micelles of cetyltrimethlyammonium bromide, with 1-butanol as co-surfactant in octane oil phase[66]. Using microemulsion technique, spinel ferrites can be synthesized. Water-in-toluene inverse micelles and sodium dodecylbenzenesulfonate (NaDBS) as surfactant were employed to prepare $MnFe_2O_4$ nanoparticles of sizes 4-15 nm[67]. A clear aqueous solution of $Mn(NO_3)_2$ and $Fe(NO_3)_3$ has been employed for this synthesis. An NaDBS aqueous solution is administered to the metal salt solution, followed by addition of a huge volume of toluene when reverse micelles are formed. The size of the resulting $MnFe_2O_4$ nanoparticles was determined by the volume ratio of water and toluene. A sol-gel reaction was employed to prepare iron oxide nanorods through reverse micelle formation from oleic acid and benzyl ether, using $FeCl_3 \cdot 6H_2O$ as iron precursor and propylene oxide as a proton scavenger[68]. The shape of nanorods was controlled by the fluctuation of reaction temperature, environmental conditions, and hydration state of the gels during reflux or heating in tetralin.

The reaction of methylamine and *in situ* formation of cobalt and iron dodecyl sulfate (formed by combination of aqueous sodium dodecyl sulfate with iron chloride or cobalt acetate solution) produces cobalt ferrite fluid[69]. An increase of sodium dodecyl sulfate concentration and

a decrease of total reactant concentration decrease the size of the cobalt ferrite particles.

Although the microemulsion procedure has been used to prepare magnetic nanoparticles in a controlled fashion, the size and shape of the products vary over a wide range. Furthermore, compared to other methods the yield of nanoparticles is low in microemulsion technique. For the synthesis of material, large amounts of solvent are necessary. Moreover, the efficiency of the process is low and it is rather difficult to scale up.

Hydrothermal synthesis

A wide range of nanostructures can be synthesized by applying hydrothermal conditions. A liquid-solid-solution reaction has been employed for the synthesis of a diverse array of nanocrystals. The system employed for the synthesis consists of a solid- liquid-solution matrix containing metal linoleate (solid), an ethanol linoleic acid (liquid) and a water-ethanol solution at different reaction temperatures under hydrothermal conditions[70]. This approach is based on phase transfer and separation mechanism occurring at the liquid-solid-solution interfaces present during the synthesis. Using hydrothermal reduction, monodisperse, hydrophilic, single crystalline ferrite microspheres were synthesized. According to this process, a mixture of $FeCl_3$, sodium acetate, ethylene glycol, and PEG was stirred vigorously till a clear solution is formed, followed by sealing in a Teflon-coated stainless-steel autoclave, and heated to 200 ℃ for 8-72 h.

The above four synthetic methods have some advantages and disadvantages. Among the four roots co-precipitation is the preferred route in terms of simplicity of the synthesis. Thermal decomposition method can be considered the best in terms of control of size and morphology of the nanoparticles. As a substitute, microemulsions can also be employed to synthesize monodispersed nanoparticles having various morphologies. However, a large amount of solvent is needed in this method. Hydrothermal synthesis, although generates superior quality nanoparticles, is a comparatively little investigated method for the synthesis of magnetic nanoparticles. To date, magnetic nanoparticles are synthesized on a large scale by use of co-precipitation and thermal decomposition procedures.

MRI AND CANCER THERAPY

MRI, magnetic resonance tomography or nuclear MRI (NMRI) is an important non-invasive imaging technique to visualize internal structures of the body in detail by using the magnetic property of the various interacting magnetic nuclei present inside the body. When a person is kept under a powerful static magnetic field, the average magnetic moment of the magnetic nuclei present inside the body becomes aligned with the direction of that magnetic field. These magnetic nuclei upon excitation with another external electromagnetic field having the correct frequency (known as the resonance frequency) can flip the spin to the reverse direction by absorbing this radiation. This resonance frequency is generated by turning on the radio frequency current for a very short period. As soon as the electromagnetic field is turned off, the nuclei return to the original thermodynamic equilibrium position and the bulk magnetization re-aligns along the static magnetic field. During this relaxation phase, an electromagnetic radiation in the radio frequency range is emitted which is measured with receiver coils.

MRI signal strengths are influenced by the factors T1 (spin-lattice/longitudinal relaxation time), T2 (transverse relaxation time) and r (spin energy). To enhance the tissue contrast, several exogenous contrast agents like complexes of gadolinium(Ⅲ) and magnetic nanoparticles are injected intravenously. However, Gd(Ⅲ) complex system has some serious drawbacks regarding Gd(Ⅲ) ion exchange using endogenous metals like Zn, Cu and uptake of complexes in extravascular space. This problem can be overcome by employing monodisperse, cross-linked iron oxide (CLIO) nanoparticles as an MRI contrasting agent[71,72]. Owing to highly stable, non-toxic and high cellular uptake, CLIO has been widely used as an exogenous contrast agent[71,73,74].

Advancement of MRI contrast agents which can be frequently applied for biomedical imaging is a demanding assignment. This is primarily due to the prospect of a suitable MRI agent with: (1) ability to be synthesized in huge amount; (2) long shelf life; (3) proper biocompatibility; (4) hindrance to its aggregation in biological fluids; and (5) large relaxivity, which will bring about better contrast in biological imaging. FePt@Fe_2O_3 core-shell magnetic nanoparticles, for example, act as a T2 MRI contrast agent and at the same time as a carrier of drug and can therefore be employed in cancer management applications[75]. For the latter application FePt@Fe_2O_3 core-shell nanoparticles are at first synthesized followed by functionalization using polyethylene glycol (PEG). Further, efficient targeting of folate receptor positive tumor cells is mediated by using folic acid conjugated FePt@Fe_2O_3-PEG nanoparticles. The chemotherapy drug, doxorubicin (DOX), is finally attached to these nanoparticles through hydrophobic adsorption, to deliver the drug in a selective manner for killing of cancer cells. Use of these FePt@Fe_2O_3-PEG nanoparticles has been done for *in vivo* MRI, to generate tumor MR contrasts, which can be accumulated in a passive tumor or can be utilized for active tumor targeting. Moreover, FePt@Fe_2O_3-PEG did not reveal any noticeable toxicity in both *in vitro* and *in vivo* experiments. Therefore, PEGylated FePt@Fe_2O_3 core-shell nanoparticles can be exploited as a potential multimodal integrated therapeutic and diagnostic nanoplatform.

Recently DOX and magnetic nanoparticles were incorporated into antibody-conjugated poly-(D,L-lactide-co-glycolide) (PLGA) nanoparticles where DOX serves as an anticarcinogenic drug and Fe_2O_3 nanoparticles utilized

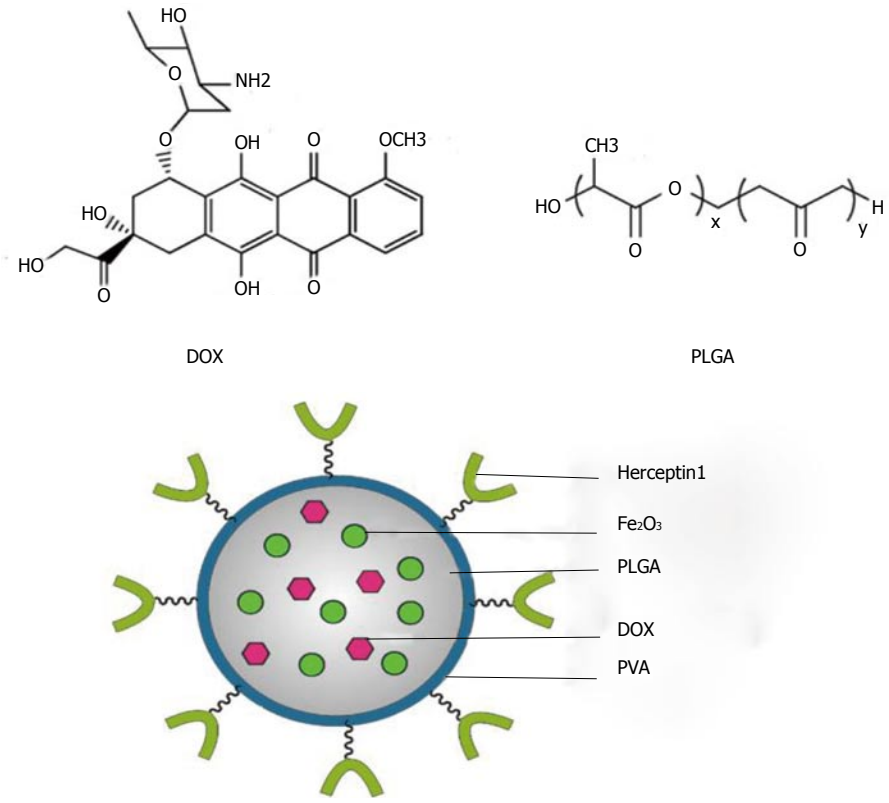


Figure 2 Use of magnetic nanoparticles embedded into poly-(D,L-lactide-co-glycolide) nanoparticles for diagnosis and treatment of cancer. DOX: Doxorubicin; PLGA: Poly-(D,L-lactide-co-glycolide); PVA: Polyvinlyalcohol.

as an imaging agent (Figure 2). This nanocomposite has been employed for the concurrent targeted iagnosis and management of breast cancer[76].

Recently, multimodal nano-materials have been exploited for immediate diagnosis and therapy. In this situation, simultaneous bio-imaging and drug delivery could be done by forming homogeneous core-shell composite particles through the integration of mesoporous silica with superparamagnetic monodisperse nanocomposite. For example, distinct, monodisperse, and perfectly size regulated core-shell mesoporous silica nanoparticles having a size < 100 nm with single Fe_3O_4 nanocrystals as cores (designated as Fe_3O_4@$mSiO_2$) were employed for synchronized MR/fluorescence imaging as well as drug delivery[77]. Multimodal imaging agents like magneto-fluorescent nanoparticles have been developed by creating optical imaging and MRI property simultaneously in the nanoparticles[78-81]. For example, glycine functionalized CLIO-Cy5.5 (CLIOGly) had a great affinity for activated macrophages, while the 3,3′,4,4′-benzophenontetracarboxylic dianhydride attached CLIO-Cy5.5 (CLIO-bentri) selectively interacted with latent macrophages.

APPLICATION OF MAGNETIC CORE-SHELL NANOPARTICLES IN DRUG DELIVERY

A frontline application of magnetic nanomaterials is as drug carriers wherein magnetic field induced drug delivery popularly known as "magnetic drug delivery" is the method of choice. The idea of magnetic drug delivery involves the injection of drug-loaded magnetic nanomaterials which are guided to the specific site by the influence of magnetic field gradient. These nanomaterials are held at the targeted site until the therapy is done, after which these are removed. By this process high local concentration of desired drug could be created, thus avoiding toxicity and other undesirable side effects on normal cells in other parts of the body. Although significant success has been achieved in *in vivo* experiments, so far, definite clinical studies are still lacking. Many basic concerns over magnetic drug delivery methods are yet to be deciphered, some of which are: (1) size regulated preparation and shelf-life of magnetic nanoparticles; (2) compatibility of the covering layers (polymer or silica); (3) binding of drug molecules; and (4) the physiological considerations[66,82].

APPLICATION OF MAGNETIC CORE-SHELL NANOPARTICLES IN HYPERTHERMIA

Magnetic nanoparticles can be used in the management of hyperthermia. This method is regarded as a complementary treatment to chemotherapy, radiotherapy and surgery in cancer[6,83]. Magnetic materials when subjected to exposure to an oscillating magnetic field,

lead to heat production through magnetic hysteresis loss, Neel-relaxation and Brown-relaxation mechanisms. This principle is employed in magnetic induction hyperthermia[47]. Induced currents are generated in metallic objects during the application of alternating magnetic field, leading to the generation of heat. Due to the collective magnetic behaviour of metals, this phenomenon is greatly enhanced in case of metals. As a result, magnetic fluid containing magnetic nanoparticles, upon exposure to an alternating magnetic field, become a powerful heat sources. The heat, generated by this way, destroys cancer cells due to its higher sensitivity to temperatures in excess of 41 ℃ compared to the normal cells.

Frictional forces due to the rotation of the particles in a medium of low viscosity (Brown-relaxation) or loss processes during the reorientation of the magnetization (Neel-relaxation) are responsible for the heating of magnetic oxide substances having poor electrical conductivity. Magnetic anisotropy is used to determine the losses from the reorientation of magnetization by means of the measurements of intrinsic magnetic properties. Due to the thermal fluctuations, re-magnetization process may occur for single domain particles. As the particle dimension is less, the barrier energy would also be less. The external energy may influence magnetic moment in exceeding the energy barrier.

Another loss type may arise in the case of ferro fluids in addition to the losses caused by magnetization rotation inside the particles. This is associated with the rotational Brownian motion of the magnetic elements. For this process, rotational friction within the suspension fluid of a definite viscosity dictates the energy barrier. The structural properties of the nanoparticles are mainly responsible for the amount of heat generation. Therefore, water soluble metal oxide synthesis in water dispersion medium with regulated shape and size is the challenging task in the advanced research.

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ORIGINAL ARTICLE

Basic Study

Arctic ground squirrel neuronal progenitor cells resist oxygen and glucose deprivation-induced death

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Abstract

AIM: To investigate the influence of ischemia/reperfusion on arctic ground squirrel (AGS) neuronal progenitor cells (NPCs), we subjected these cultured cells to oxygen and glucose deprivation.

METHODS: AGS NPCs were expanded and differentiated into NPCs and as an ischemia vulnerable control, commercially available human NPCs (hNPCs) were seeded from thawed NPCs. NPCs, identified by expression of TUJ1 were seen at 14-21 d *in vitro* (DIV). Cultures were exposed to control conditions, hypoxia, oxygen and glucose deprivation or glucose deprivation alone or following return to normal conditions to model reperfusion. Cell viability and death were assessed from loss of ATP as well as from measures of alamarBlue® and lactate

dehydrogenase in the media and from counts of TUJ1 positive cells using immunocytochemistry. Dividing cells were identified by expression of Ki67 and phenotyped by double labeling with GFAP, MAP2ab or TUJ1.

RESULTS: We report that when cultured in NeuraLife™, AGS cells remain viable out to 21 DIV, continue to express TUJ1 and begin to express MAP2ab. Viability of hNPCs assessed by fluorescence alamarBlue (arbitrary units) depends on both glucose and oxygen availability [viability of hNPCs after 24 h oxygen glucose deprivation (OGD) with return of oxygen and glucose decreased from 48151 ± 4551 in control cultures to 43481 ± 2413 after OGD, $P < 0.05$]. By contrast, when AGS NPCs are exposed to the same OGD with reperfusion at 14 DIV, cell viability assessed by alamarBlue increased from 165305 ± 11719 in control cultures to 196054 ± 13977 after OGD. Likewise AGS NPCs recovered ATP (92766 ± 6089 in control and 92907 ± 4290 after modeled reperfusion; arbitrary luminescence units), and doubled in the ratio of TUJ1 expressing neurons to total dividing cells (0.11 ± 0.04 in control cultures *vs* 0.22 ± 0.2 after modeled reperfusion, $P < 0.05$). Maintaining AGS NPCs for a longer time in culture lowered resistance to injury, however, did not impair proliferation of NPCs relative to other cell lineages after oxygen deprivation followed by re-oxygenation.

CONCLUSION: Ischemic-like insults decrease viability and increase cell death in cultures of human NPCs. Similar conditions have less affect on cell death and promote proliferation in AGS NPCs.

Key words: Neurogenesis; Neuronal progenitor; Hypoxia tolerance; Hibernation

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Core tip: Cultured arctic ground squirrel (AGS) neuronal progenitor cells (NPCs) resist cell death under conditions designed to model ischemia/reperfusion and instead show evidence of proliferation. Persistence of progenitor properties and hence the capacity to divide is a unique characteristic of AGS NPCs. Mechanisms that prolong neuronal progenitor properties may be targets to increase tolerance to cerebral ischemia/reperfusion in humans at risk of stroke and cardiac arrest.

Drew KL, Wells M, McGee R, Ross AP, Kelleher-Andersson J. Arctic ground squirrel neuronal progenitor cells resist oxygen and glucose deprivation-induced death. *World J Biol Chem* 2016; 7(1): 168-177 Available from: URL: http://www.wjgnet.com/1949-8454/full/v7/i1/168.htm DOI: http://dx.doi.org/10.4331/wjbc.v7.i1.168

INTRODUCTION

Failed delivery of oxygen and nutrients within the brain leads to neuronal pathology, disability and death in humans[1,2]. Arctic ground squirrels (AGS), a hibernating species, do not show detrimental effects of global cerebral ischemia *in vivo* that are typical of humans and other mammals[3,4]. Unlike other hibernating species[5], tolerance to modeled ischemia in AGS brain slices does not depend on the hibernating state and persists outside of the hibernation season[6,7]. We thus hypothesized that aspects of resistance to ischemia/reperfusion injury would be evident in neuronal progenitor cells (NPCs) derived from AGS. Because the effects of ischemia/reperfusion injury in NPCs are not well studied, we included human NPCs for comparison.

NPCs are cells derived from neural stem cells (NSCs) that have committed to a neuronal fate, but retain the capacity to divide[8]. Both NSCs and NPCs are found in adult brain and serve as pools of renewable neurons. In the adult brain, traumatic events including cerebral ischemia[9], epileptic seizures[10] and traumatic brain injury[11,12] promote neurogenesis. Though neurogenesis may involve proliferation of NSCs or NPCs evidence suggests that adult neurogenesis in the dentate gyrus of the hippocampus originates from restricted NPCs[13]. The fate of NPCs following ischemia/reperfusion is therefore significant to recovery from stroke and cardiac arrest.

Here we compared human and AGS NPCs, identified as cells that are nestin negative and TUJ1 positive, for vulnerability to oxygen and glucose deprivation *in vitro*. We report that hypoxic and ischemic-like insults decrease viability and increase cell death in cultures of human NPCs. Similar conditions fail to induce cell death in AGS NPCs.

MATERIALS AND METHODS

Cell culture

AGS NPCs were prepared as described previously[14] by thawing and seeding AGS NSCs onto a T-75 flask treated with poly-L-ornithine. Cells were allowed to proliferate using a DMEM/F12 growth media in the presence of bovine serum and bFGF (10 ng/mL) until 75% confluence. Cells were passaged and seeded onto 96-well plates (Biocoat, poly-L-Lysine coated, Becton Dickenson) at a low density (10000-20000 cells/well) and grown in differentiation media (lacking bFGF) for four days before switching to Neurobasal™ (Invitrogen) or NeuraLife™ (Lifeline Cell Technology) neuron maintenance media. Human NPCs were seeded from thawed NPCs (Clonexpress, Gaithersburg, MD) that originated from whole first trimester fetal brains. Cells were maintained for up to 14 d *in vitro* (DIV) in Neurobasal™ or up to 21 DIV in NeuraLife™ then fixed with 4% paraformaldehyde.

Hypoxia and oxygen glucose deprivation

Hypoxia with reoxygenation (O_2 dep w/reOx) or modeled ischemia with reperfusion [oxygen glucose deprivation (OGD) w/rep] was achieved as follows. Media (80%) was changed from maintenance media containing 25

mmol/L glucose to maintenance media containing 5 mmol/L glucose 24 h prior to substrate deprivation to better approximate *in vivo* glucose concentrations[15]. Substrate deprivation was initiated by removing 80% of media and replacing it with normoglucose (5 mmol/L glucose in maintenance media) or glucose deprived media (0 mmol/L glucose in maintenance media). Plates were then placed in normoxic or hypoxic conditions for 48 h.

Hypoxic conditions were achieved by placing plates in a Billups-Rothenberg chamber flushed with 95% N_2/5% CO_2 until the partial pressure of O_2 in the chamber was below 0.7% of atmospheric pressure then sealed. For normoxic conditions the chamber was left open for free gas exchange and placed in an incubator at 37 ℃ and 95% air (21% O_2)/5% CO_2. Humidity was maintained by placing water-soaked gauze on the bottom of the chamber. Following the hypoxic/normoxic period the plates were removed from their respective chambers and placed in an incubator at 37 ℃ and 95% O_2/5% CO_2 for 24 to 72 h. The media was never changed following the hypoxic/normoxic period, but glucose was returned to the media, when specified as OGD w/rep. In some experiments a small amount of media was removed for early analysis of lactate dehydrogenase (LDH) levels.

Glucose deprivation without oxygen deprivation (Gluc dep w/return) was achieved by treating plates as described for OGD except that plates were maintained in a normoxic environment throughout the treatment period. Following the return of glucose, oxygen, or oxygen and glucose, viability was assessed from cellular respiration monitored using an alamarBlue® assay and/or LDH levels in the media. After testing for alamarBlue® and LDH, the cultures were fixed and processed for immunocytochemistry. Human NPCs were treated in a similar manner and served as a positive control for NPCs derived from an ischemia vulnerable species.

Measures of cell viability and cell death

Media (100 μL) was assayed for LDH (Roche Diagnostics) as a measure of cell death, according to manufacturer's instructions. AlamarBlue® (Invitrogen), a redox indicator that yields a fluorescent signal in response to metabolic activity and is proportional to cell number under the conditions described, was assayed as a measure of cell viability. AlamarBlue® solution was added to 200 μL of media in each well of the 96 well plate 2 h prior to assay. Following 2 h at 37 ℃ and 95% air/5% CO_2, respiration was determined in the cells by removing 100 μL of media into an empty 96-well plate to measure a respiratory factor using a fluorescence plate reader.

ATP measurements

Immediately following OGD/normoxia or following the 24 h reperfusion period, media was removed from all wells and then 100 μL of buffer and substrates were added to cells according to manufacturer's directions (ATPlite Kit, PerkinElmer). The plates were agitated on an orbital shaker for 2 min. The lysate was transferred to a black 96-well plate and luminescence measured on a Wallac Victor2 Multiplate reader.

Immunocytochemistry

Cells in 96-well plates were fixed for 20 min with 4% paraformaldehyde and incubated with antibodies for Ki67, MAP2ab or TUJ1. The cells were rinsed numerous times with PBS (Ca^{2+}, Mg^{2+} free), the cells were blocked with Triton X-100 containing blocking buffer and then; β-tubulin polyclonal antibody TUJ1 (Covance Cat# PRB-435P), 1/2000 dilution; MAP2 (a and b) (Abcam Cat#ab3096), 1/300 dilution; GFAP (Abcam Cat#ab16997), 1/100; Nestin (Abcam Cat #ab7659), 1/200 and Ki67 (Abcam Cat #ab695093), 1/1000 dilution. The cells were again rinsed several times with PBS then secondary antibody (Alexa fluor 546 goat anti-rabbit IgG (H + L) (Life Technologies - Molecular Probes, Cat #A11010) was added along with Hoechst dye 33342 (Life Technologies - Molecular Probes Cat. # H3570). The abundance of immunoreactive cells was measured using the neuronal profiling software of the Cellomics Arrayscan instrument to assess total number of cells within each field (Hoechst positive cells) and number of neurons within each field with a neuronal process (TUJ1-positive and Hoechst-positive cells). Hoechst dye 33342 labeled nuclei of all cells. Ki67 antibody was used when identifying dividing cells of any type.

Statistical analysis

Data are shown as mean ± SD. Unless otherwise indicated, data were analyzed by one-way ANOVA followed by Holm-Sidak multiple comparisons or *t*-test for pairwise comparisons when data were normally distributed, and by Mann-Whitney rank sum test or Kruskal-Wallis one way ANOVA on ranks followed by Dunn's multiple comparisons when data were not normally distributed. Software for analysis included Excel 2010 and SigmaPlot (v. 11.0). Sample size (*n*) refers to the number of wells per treatment condition unless indicated otherwise. $P < 0.05$ was interpreted as statistically significant.

RESULTS

NPCs are identified in cultures of human cells by expression of TUJ1 and neurons by MAP2ab at 14 DIV (Figure 1). By contrast, AGS cultures begin as NSCs, identified by nestin-ir and an absence of GFAP-ir at 7 DIV (Figure 2). By 14 DIV, AGS cultures express TUJ1 and GFAP characteristic of proliferating progenitors committed to neuronal or astrocytic fates[8] and nestin is no longer expressed. When cultured in NeuraLife™ AGS cells remain viable out to 21 DIV, continue to express TUJ1 and begin to express MAP2ab (Figure 2).

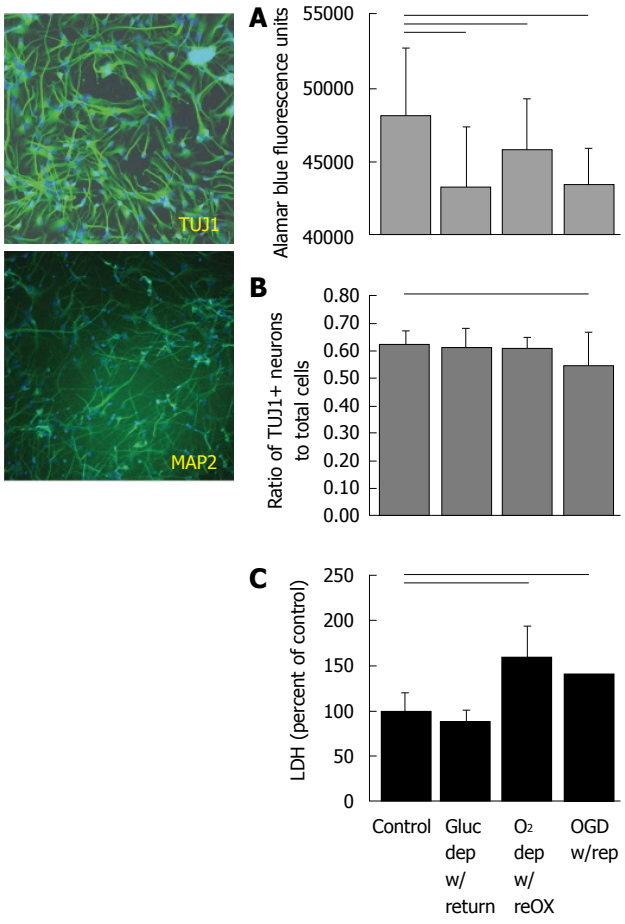


Figure 1 Cultures of human neuronal progenitor cells express TUJ1 and MAP2ab at 14 d *in vitro* (left) and die within 24 h of re-oxygenation (right). Cultures were deprived of oxygen and/or glucose for 48 h and resources were replaced for 24 h before assaying for viability (A), proportion NPC to total cells (B) or cell death (C). Horizontal lines indicate $P < 0.05$; ANOVA followed by a Holm-Sidak test (A, B) or Kruskal-Wallis ANOVA on ranks followed by Dunn's multiple comparisons (C). Mean ± SD. Only differences relative to control are shown. NPC: Neuronal progenitor cell.

Viability of human NPCs and neurons at 14 DIV depended on both glucose and oxygen availability, although combined deprivation of both substrates produced the most consistent decrease in viability and neuron numbers. Depriving human cultures of glucose, oxygen or oxygen and glucose produced a decrease in alamar Blue® fluorescence ($P < 0.001$; ANOVA, n = 72-96, Figure 1A). All treatments combined showed a significant decrease in the ratio of TUJ1+ NPCs to total cells ($P < 0.05$, ANOVA, n = 15-20, Figure 1B) although post-hoc analysis showed that only cultures exposed to OGD with reperfusion had significantly fewer NPCs to total cells compared to controls. Oxygen deprivation with reoxygenation and OGD with reperfusion produced a significant increase in LDH released into the media ($P < 0.05$, Kruskal-Wallis, ANOVA on ranks, n = 18-24) (Figure 1C).

By contrast, when AGS NPCs were exposed to oxygen deprivation with reoxygenation or OGD with reperfusion at 14 DIV, cell viability increased ($P < 0.001$, ANOVA, n = 5-6, Figure 2A), and the ratio of NPCs to total cells increased ($P < 0.01$, ANOVA, n = 9-12, Figure 2B) despite a small, but significant increase in LDH ($P < 0.001$, ANOVA, n = 6-9, Figure 2C). When deprived of glucose alone, LDH decreased relative to control and viability and neuron numbers remained unchanged. Viability of the cultured cells after a period of recovery was also evident from measures of ATP. Although AGS NPCs lost ATP within 40 h after oxygen or oxygen and glucose deprivation ($P < 0.001$, ANOVA, n = 6, Figure 3A), ATP returned to or exceeded control levels within 24 h of reintroducing resources ($P < 0.001$, ANOVA, n = 5-6, Figure 3B). Results are expressed as total ATP luminescence units because the ATP assay superseded cell counts.

The increase in viability and number of TUJ1 positive NPCs to total cells in the presence of cell death suggested that TUJ1 positive NPCs were proliferating in response to injury at a rate faster than other cell lineages. To test this hypothesis we identified dividing cells as those immunoreactive for Ki67, a cell cycle protein expressed during the G_1 phase of the cell cycle. As expected, the ratio of dividing (NPCs) (Ki67 and TUJ1 positive) to the total number of dividing cells (Ki67 positive) increased following oxygen deprivation and oxygen and glucose deprivation ($P < 0.01$, Kruskal-Wallis, n = 5-8, Figure 4A). The number of dividing neuronal progenitors remained constant while the number of total dividing cells decreased ($P < 0.001$, ANOVA, n = 6-8, Figure 4B).

We next asked if encouraging AGS NPCs to terminally differentiate by extending the number of DIV beyond 21 d would abolish ischemia-induced neurogenesis and tolerance to oxygen deprivation. Using a novel culture media (NeuraLife™, Lifeline Cell Technology, Frederick, MD) cultures remained viable beyond 3 wk and began to express MAP-2ab (Figure 2). After this additional week in culture, 48 h of hypoxia followed by 24 h of re-oxygenation produced an increase in LDH in the media ($P < 0.001$, t-test, n = 83-84, Figure 5A). However, the number of NPCs relative to total cells did not change (Figure 5B), and the extended culture period did not abolish neurogenesis. The number of dividing NPCs (cells positive for Ki67 and TUJ1) 24 h after re-oxygenation increased when compared to normoxia treated control cultures (Figure 5C). NPCs counts and evidence of neurogenesis persisted up to 72 h after re-oxygenation. By 48 and 72 h after re-oxygenation the ratio of NPCs to total cells increased slightly [48 h, Figure 6A, $P < 0.01$, t-test, n = 4 (averages of 6 replicates)] or remained the same (72 h, Figure 6C). In addition, the number of dividing NPCs increased (48 h, Figure 6B) or the decline in dividing NPCs was less than the decline in proliferation of other cell lineages (72 h, Figure 6D) ($P < 0.05$, t-test, n = 4, averages of 6 replicates).

Increased antioxidant capacity may protect AGS during periods of reperfusion during arousal from torpor[16-18]. We therefore asked if AGS cultures would resist injury induced by the pro-oxidant H_2O_2. AGS NPCs were in fact more sensitive to H_2O_2 than cultures of

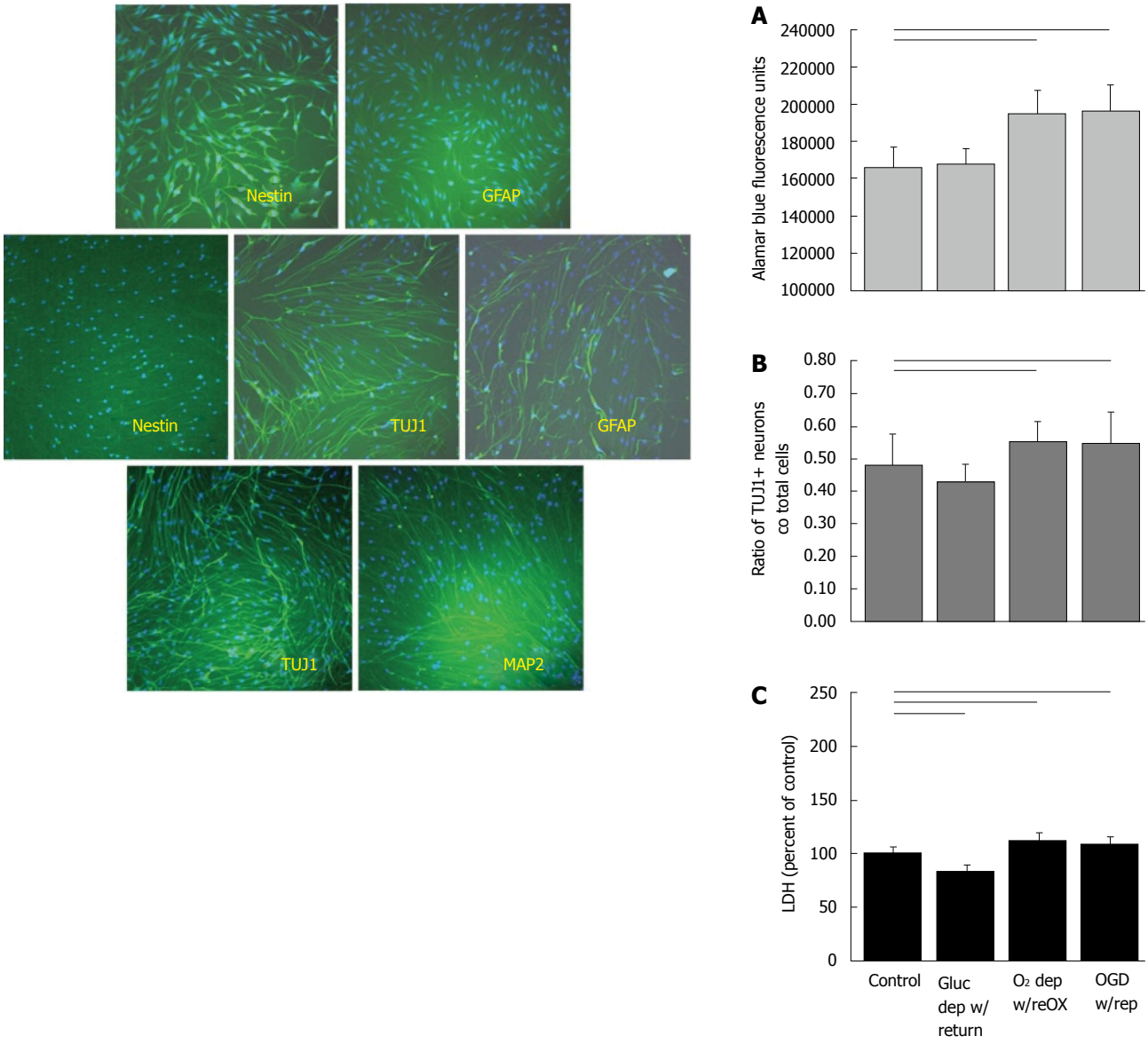


Figure 2 Cultures of arctic ground squirrel neuronal progenitor cells do not begin to express MAP2ab, characteristic of mature neurons, until 21 d *in vitro*. Cultures of AGS NPC express nestin, a marker of NSC, but not GFAP, a marker for astrocytes at 7 d *in vitro* (DIV) (top left). By 14 DIV cultures express TUJ1 a marker of neuronal progenitor cells that are committed to a neuronal fate, and GFAP a marker of progenitor cells commitment to an astrocytic fate. Nestin is no longer expressed at 14 DIV (middle left). At 21 DIV cultures express TUJ1 and begin to express MAP2ab (bottom left). AGS NPCs at 14 DIV increase in viability and appear to proliferate when deprived of oxygen or oxygen and glucose (right). When AGS NPC were deprived of oxygen or oxygen and glucose for 48 h followed by 24 h of resource replacement, cultures showed an increase in viability (A) and an increase in the proportion of neuronal progenitors (B) despite signs of cell death (C). Horizontal lines $P < 0.05$; ANOVA followed by a Holm-Sidak test. Mean ± SD. Only differences relative to control are shown. AGS: Arctic ground squirrel; NPC: Neuronal progenitor cell; OGD: Oxygen glucose deprivation.

human NPCs and neurons (Figure 7). While the viability of human cultures, indicated from alamarBlue® fluorescence, decreased after 6 h of exposure to 50 μmol/L H_2O_2 ($P < 0.05$, Kruskal-Wallis, ANOVA on ranks, $n = 20$), the viability of AGS cultures decreased after just 2 h of exposure ($P < 0.05$, Kruskal-Wallis, ANOVA on ranks, $n = 25$). Differences in percent glia in cultures can directly affect neuroprotective capacity. In looking at the percent of GFAP+ glial cells in each culture, differentiated AGS NPCs and differentiated human NPCs cultures, the number of GFAP positive glial cells in the AGS cultures (DIV12) was considerably lower than the percentage of GFAP+ glial cells in the differentiated human cultures (6.2% ± 3.7% *vs* 37.0% ± 1.9%, respectively, $n = 6$-12 wells).

DISCUSSION

AGS offer a unique and robust model of resistance to brain injury following global cerebral ischemia *in vivo* and OGD *in vitro*[3,4,6,7]. This resistance may be in part, intrinsic to AGS neurons and thus persist in culture. To study AGS neurons in culture we isolated AGS NSCs that were then differentiated into NPCs prior to insult with oxygen and/or glucose deprivation. Here we show that proliferation and differentiation of NSCs obtained

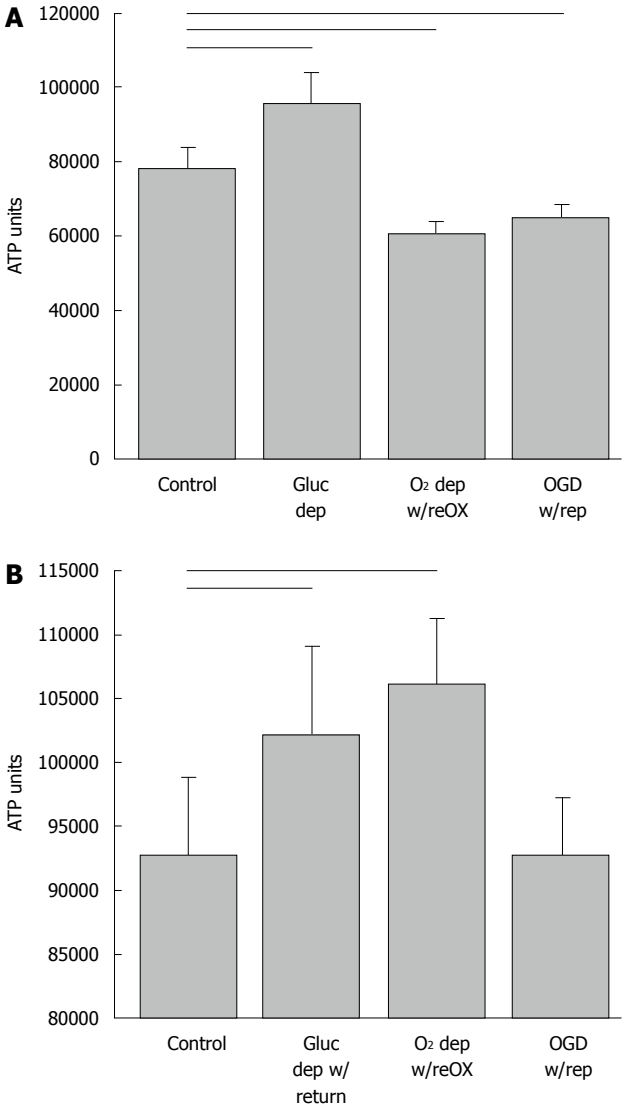


Figure 3 ATP (shown as relative luminescence units) declines in arctic ground squirrel neuronal progenitor cells during oxygen and glucose deprivation. NPCs at 14 d *in vitro* deprived for 48 h followed by 24 h resource replacement lost ATP when deprived of oxygen or oxygen and glucose (A). ATP recovered within 24 h of reoxygenation or reperfusion (modeled by return of oxygen and glucose) (B). Horizontal lines $P < 0.05$; ANOVA followed by a Holm-Sidak test. Mean + SD. Only differences relative to control are shown. OGD: Oxygen and glucose deprivation; NPCs: Neuronal progenitor cells.

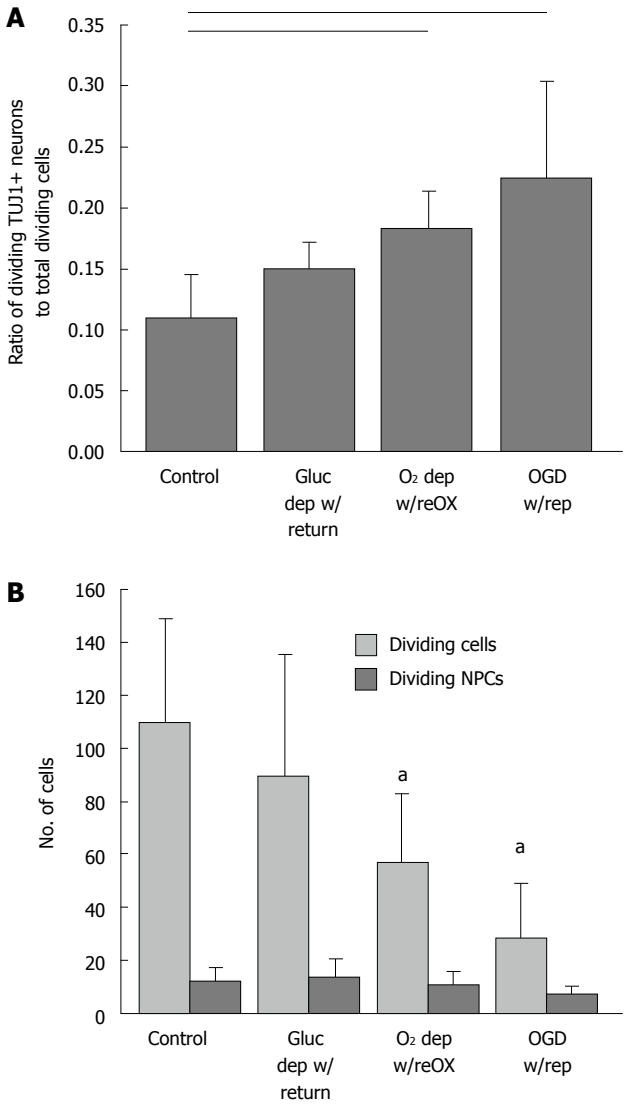


Figure 4 Arctic ground squirrel neuronal progenitor cells proliferate in response to injury faster than other cell lineages. AGS NPCs at 14 d *in vitro* deprived of oxygen or oxygen and glucose for 48 h followed by 24 h of reoxygenation or reperfusion (modeled by return of oxygen and glucose). The proportion of dividing NPCs (TUJ1+ and Ki67+/Ki67+) (A) increased because the number of dividing NPCs (TUJ1+ and Ki67+) did not change while the number of dividing cells (Ki67+) decreased (B). Horizontal lines $P < 0.05$; $^{a}P < 0.05$ compared to control; ANOVA followed by a Holm-Sidak test. Mean ± SD. Only differences relative to control are shown. OGD: Oxygen glucose deprivation; NPCs: Neuronal progenitor cells; AGS: Arctic ground squirrel.

from adult AGS hippocampus follows a characteristic progression of lineage-specific markers described for NSCs from other species[8]. We show that at 7 DIV AGS cultures express nestin but not GFAP, a characteristic of NSCs. These cells commit to a neuronal (TUJ1 positive) or astrocytic (GFAP positive) fate by 14 DIV. AGS NPCs continue to mature if cultured under conditions that favor survival and by 3 wk begin to express MAP2ab a marker of mature, terminally differentiated neurons. Interestingly, the human cultures at only 14 DIV already contain a large fraction of MAP2ab-ir cells showing that AGS NPCs mature more slowly than hNPCs in culture.

Glucose deprivation had little influence on viability or cell death and did not stimulate proliferation of NPCs. Similarly the effects of OGD were similar to the effects of hypoxia without glucose deprivation. We have shown previously that cultures of AGS NPCs passaged and differentiated in much the same manner as described here are vulnerable to iodoacetate, a chemical inhibitor of glycolysis, and less vulnerable to inhibition of oxidative metabolism[19]. Lack of effect of glucose deprivation, in the present study may be due to astrocytic stores of glycogen and the capacity for these cell cultures to survive on anaerobic glycolysis. These results are consistent with other studies showing that NSCs/NPCs cultured under low oxygen consume more glucose, produce energy by glycolysis, and upregulate genes involved in glycolysis[20]. Because glycogen is

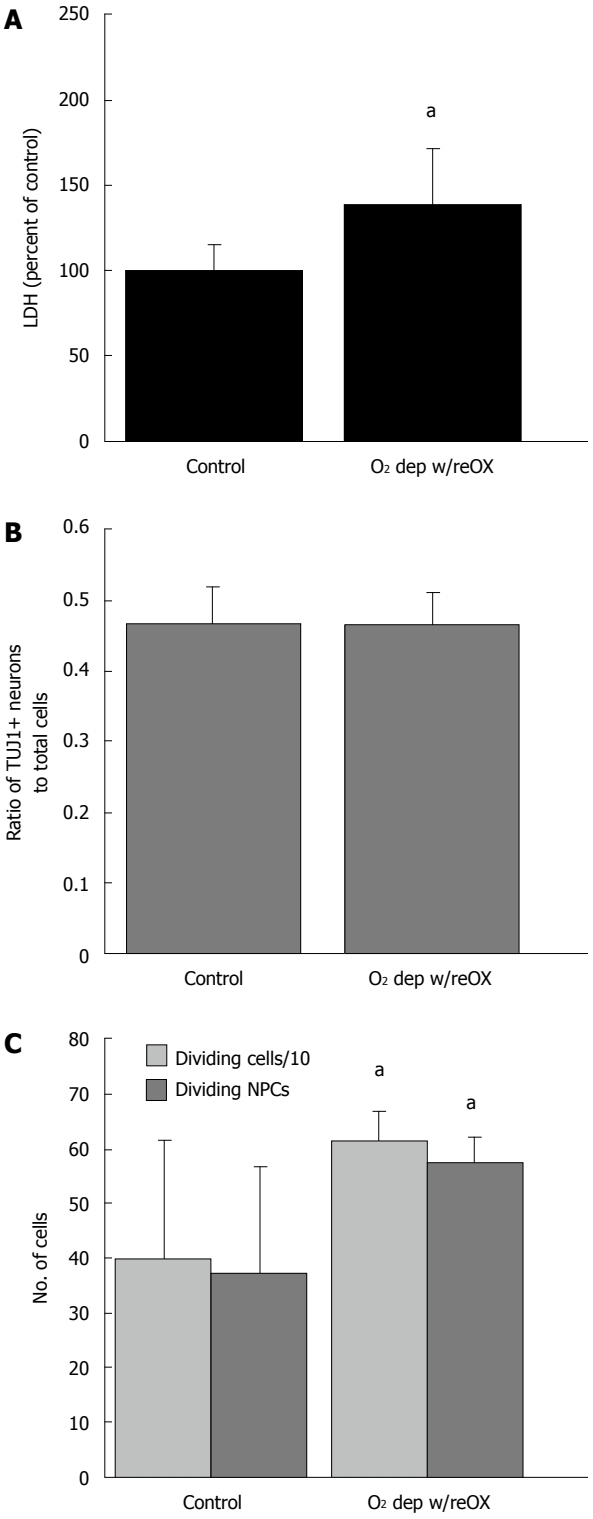


Figure 5 Arctic ground squirrel neuronal progenitor cell cultured for 21 d *in vitro* are more vulnerable to oxygen deprivation than cells cultured for 14 d *in vitro*. Longer time in culture lowers resistance to oxygen deprivation /reoxygenation indicated by an increase in LDH (A) not seen after 14 d *in vitro* (DIV). However, after 24 h of O_2 deprivation, followed by 24 h of reoxygenation the proportion of neuronal to nonneuronal progenitors did not change (B) while proliferation of NPCs increased (C). Data are from AGS NPCs cultured for 21 DIV that were deprived of oxygen followed by 24 h of reoxygenation. $^aP < 0.05$ compared to control, *t*-test. LDH: Lactate dehydrogenase; NPCs: Neuronal progenitor cells; AGS: Arctic ground squirrel.

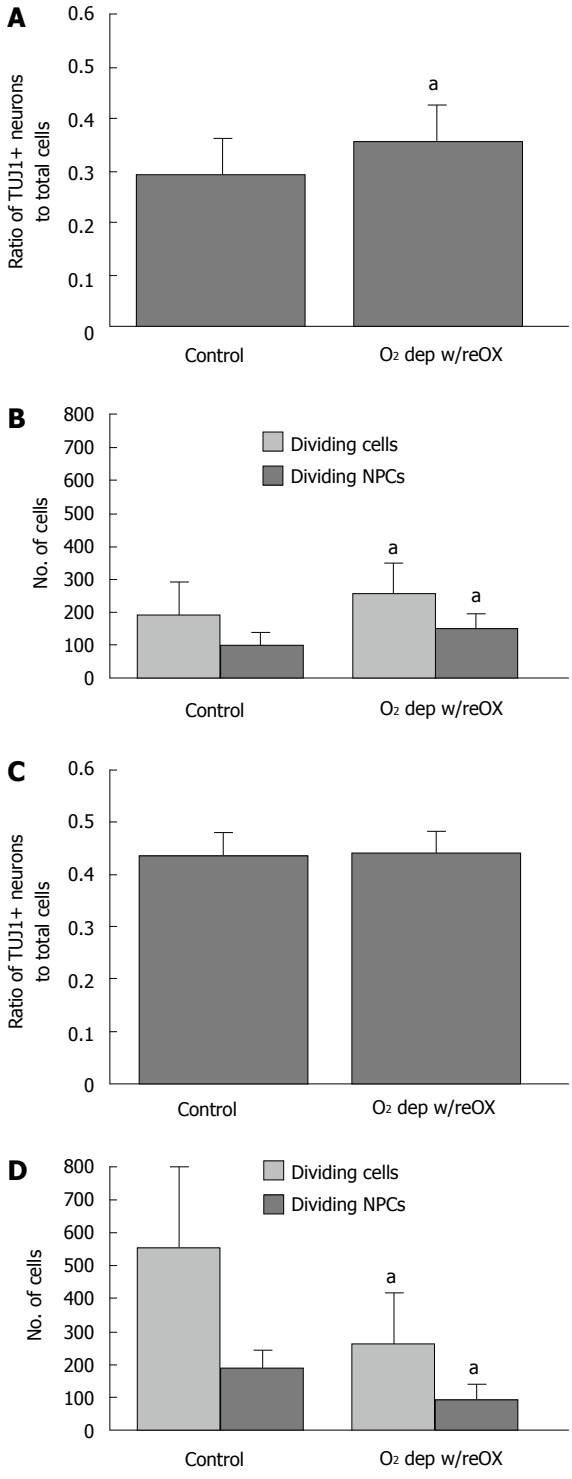


Figure 6 By 48 and 72 h after re-oxygenation the ratio of neuronal progenitor cells to total cells increases slightly or remains the same. AGS NPCs cultured for 21 d *in vitro* were deprived of oxygen followed by 48 h (A, B) or 72 h (C, D) of re-oxygenation. After 48 h of reoxygenation, the proportion of NPCs increased relative to total cells because the number of dividing NPCs increased (B). After 72 h the proportion of NPCs was sustained (C) because division of NPCs declined less than other cell lineages (D). $^aP < 0.05$ compared to control, *t*-test. Mean ± SD. AGS: Arctic ground squirrel; NPCs: Neuronal progenitor cells.

stored primarily in astrocytes, higher proportions of astrocytes to neurons in AGS NPCs cultures could contribute to lower levels of cell death compared to human NPCs. However, astrocytes do not appear to

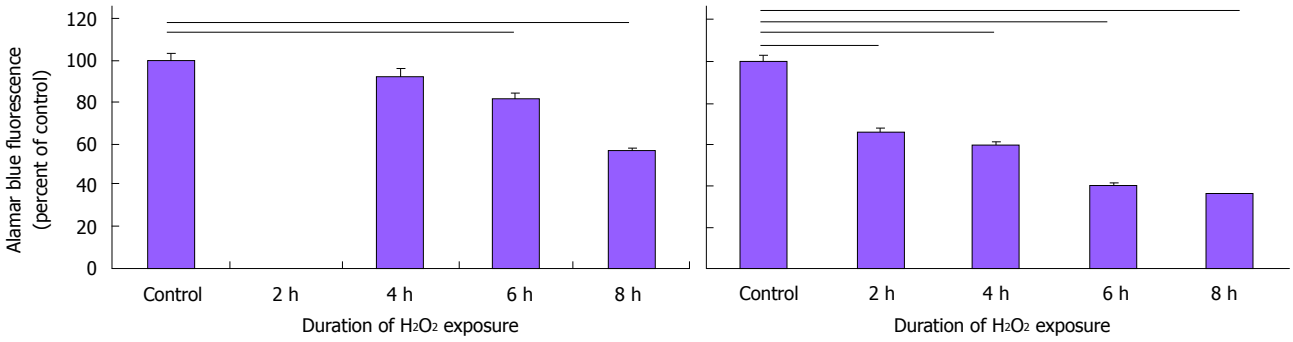


Figure 7 Arctic ground squirrel neuronal progenitor cells are vulnerable to oxidative stress. Cells, 14 d *in vitro*, were exposed to 50 μmol/L H_2O_2. After 2 to 8 h of exposure cellular respiration, a measure of cellular viability, was measured using alamarBlue®. The viability of human cultures (A) decreased after 6 h exposure while the viability of AGS NPCs (B) decreased after 2 h of exposure to H_2O_2. AGS: Arctic ground squirrel; NPCs: Neuronal progenitor cells.

contribute to neuroprotection in AGS NPCs since the percentage of GFAP+ astrocytes are considerably lower in AGS NPCs cultures than in human NPCs cultures. Although the degree of hypoxia experienced in culture is difficult to compare directly to hypoxia sensed by neurons *in vivo* the partial pressure of O_2 in our hypoxic chambers was 0.7% of atmospheric pressure or lower. This low level of O_2 is well below normal physiological levels[21].

Interpretation of a direct comparison with human NPCs is limited for several reasons. The percentage of MAP2ab-ir cells may influence tolerance to OGD since neurons are not anaerobic[22]. The human cultures at 14 DIV contained MAP2ab positive neurons, whereas the AGS cultures did not. Perhaps the increase in LDH with hypoxic conditions of AGS cultures at 21 DIV reflects the greater number of mature neurons lost. In order to increase the number of mature MAP2ab-ir cells we extended the culture period, but only in the presence of AGS neuron beneficial media, NeuraLife™. There was no discernible difference in TUJ1+ cell numbers early in the culture period, but as with MAP2ab-ir cells, the number of TUJ1+ cells (neurons and progenitors) was also greater at longer culture period, specifically 21 DIV. While the influence of relative proportions of astrocytes, NPCs and mature neurons to the response to oxygen and glucose deprivation may be inferred from these results it is important to not over-interpret the comparison between human and AGS cultures. hNPCs and AGS NSCs were derived from 2 different anatomical sources, the adult hippocampus for AGS and the first trimester fetal brains for the human line. Second, the stage of development differed between the two lines and time points studied. Humans are, however, a species known to be vulnerable to cerebral ischemia/reperfusion injury and for this reason hNPCs were included as a positive control.

Neurogenesis following cerebral ischemia *in vivo* has not been monitored in AGS or in any other hibernating species, to our knowledge. However, immature cells have been captured in a mitotic state in the adult dentate gyrus of a closely related species of ground squirrel where cooling during hibernation slowed and facilitated observations of mitosis[23]. AGS and other species of ground squirrels regenerate synapses and AGS show enhanced cognitive function after arousal from hibernation[24,25] when hemoglobin-oxygen saturation levels fall to as low as 57%[16]. Whether the tendency for AGS NPCs to resist terminal differentiation and to proliferate in response to a low oxygen environment contributes to this species' ability to tolerate or recover from global cerebral ischemia/reperfusion *in vivo* remains to be determined.

Cerebral ischemia associated with stroke, cardiac arrest, and traumatic brain injury leads to death or disability due in large part to progressive pathology for which effective therapeutic interventions are lacking. Neuroprotective therapeutics, have consistently failed in clinical trials to improve outcome following stroke, cardiac arrest and TBI despite positive preclinical results[26,27]. The reasons for clinical failure of neuroprotectants could be many fold[28], yet the possibility remains that without an actual reversal of ischemic injury the outcome will always be negative or not sufficient. Neurorestorative therapies for stroke[29] may provide improved outcomes over neuroprotectants. Recent evidence suggests that injury-induced proliferation of NSCs may provide a means to enhance recovery and decrease morbidity after stroke[9,30,31], although aberrant neurogenesis following stroke may contribute to aberrant functional outcomes[32]. Better understanding of mechanisms sufficient to promote survival and proliferation of AGS NPCs has the potential to unveil mechanisms that will also promote proliferation of human NPCs following brain injury. AGS NPCs offer a platform to investigate mechanisms of ischemia tolerance to inform development of novel therapies that target signal transduction pathways that contribute to ischemia tolerance and neurogenesis. Methods to promote neurogenesis after brain injury are expected to complement traditional neuroprotective therapies[33,34].

COMMENTS

Background

Many people have lost loved ones to cardiac arrest or stroke. Death is most

typically due to brain injury caused by a disruption in blood supply. Patients who survive are often burdened with severe neurological disabilities. Hibernating species resist brain injury following disruption of blood flow that would be lethal in other mammalian species, including humans. Unique physiology of these animals contributes to this resistance, but, it was unknown if cellular processes independent of environmental influences played a role in this tolerance. Here for the first time, we show aspects of cellular physiology in arctic ground squirrel (AGS) neurons that could contribute to ischemia tolerance. These observations now establish a foundation to study mechanisms unique to the AGS that may lead to the development of novel and efficacious treatments to reduce brain injury following cardiac arrest and stroke in humans and reduce effects of further neurodegenerative events.

Research frontiers

Opportunities to promote neurogenesis and neuron regeneration are a new area, ripe for drug development. While much is now known about how hypoxia influences proliferation and differentiation of neuronal stem cells, little work has been directed at understanding the longevity and survival of neuronal progenitor cells (NPCs), *i.e.*, young neurons, largely committed to a neuronal fate that retain the capacity to divide. Using the AGS as a unique model of ischemia tolerance we found that AGS NPCs persist longer in culture than human NPCs and divide in response to hypoxia. The mechanisms behind this phenomenon can now be studied and drugs developed to mimic these mechanisms for the treatment of cardiac arrest and stroke.

Innovations and breakthroughs

Characterization of unique properties of NPCs derived from the ischemia-resistant AGS is an innovative approach to understanding mechanisms of cellular response to hypoxia that will inform future drug development.

Applications

Better understanding of mechanisms sufficient to promote survival and proliferation of AGS NPCs has the potential to unveil mechanisms that will also promote proliferation of human NPCs following brain injury. AGS NPCs offer a platform to investigate mechanisms of ischemia tolerance to inform development of novel therapies that target signal transduction pathways that contribute to ischemia tolerance and neurogenesis. Methods to promote neurogenesis after brain injury are expected to complement traditional neuroprotective-targeted therapeutic approaches.

Terminology

NPCs*:* A NPCs is like a neural stem cell in that both have the ability to divide. A NPCs, however, has already begun to differentiate into a neuron and is more committed to a neuronal fate than a neural stem cell. NPCs are found in the adult, mammalian central nervous system, in two distinct brain regions, where they are thought to function in repair.

Peer-review

This study characterized an interesting oxygen and glucose deprivation resistance in a model of AGS derived NPCs. This study demonstrated an acute response of AGS NPCs to oxygen and glucose deprivation in a number of parameters, including proliferation, ATP, survival *etc*. The key to this study is the use of human NPCs under similar conditions as a comparison. The current data did clearly demonstrate a resistance of AGS NPCs cells, but also showed that the AGS NPCs are more vulnerable to H_2O_2, as compared to human NPCs. The study presented an interesting possibility that the AGS may carriy a different genetic background or be primed with differential epigenetic coding that bestows the differential resistance or response to environmental factors, *e.g.*, oxygen or glucose. This interpretation of the differential response was interpreted that AGS offer a unique and robust model of resistance to brain injury following global cerebral ischemia *in vivo*.

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ORIGINAL ARTICLE

Basic Study

High levels of homocysteine downregulate apolipoprotein E expression *via* nuclear factor kappa B

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Abstract

AIM: To investigate the effect of high homocysteine (Hcy) levels on apolipoprotein E (apoE) expression and the signaling pathways involved in this gene regulation.

METHODS: Reverse transcriptase polymerase chain reaction (RT-PCR) and Western blot were used to assess apoE expression in cells treated with various concentrations (50-500 μmol/L) of Hcy. Calcium phosphate-transient transfections were performed in HEK-293 and RAW 264.7 cells to evaluate the effect of Hcy on apoE regulatory elements [promoter and distal multienhancer 2 (ME2)]. To this aim, plasmids containing the proximal apoE promoter [(-500/+73)apoE construct] alone or in the presence of ME2 [ME2/(-500/+73)apoE construct] to drive the expression of the reporter luciferase gene were used. Co-transfection experiments were carried out to investigate the downstream effectors of Hcy-mediated regulation of apoE promoter by using specific inhibitors or a dominant negative form of IKβ. In other co-transfections, the luciferase reporter was under the control of synthetic promoters containing multiple specific binding sites for nuclear factor kappa B (NF-κB), activator protein-1 (AP-1) or nuclear factor of activated T cells (NFAT). Chromatin immunoprecipitation (ChIP)

assay was accomplished to detect the binding of NF-κB p65 subunit to the apoE promoter in HEK-293 treated with 500 μmol/L Hcy. As control, cells were incubated with similar concentration of cysteine. NF-κB p65 proteins bound to DNA were immunoprecipitated with anti-p65 antibodies and DNA was identified by PCR using primers amplifying the region -100/+4 of the apoE gene.

RESULTS: RT-PCR revealed that high levels of Hcy (250-750 μmol/L) induced a 2-3 fold decrease in apoE mRNA levels in HEK-293 cells, while apoE gene expression was not significantly affected by treatment with lower concentrations of Hcy (100 μmol/L). Immunoblotting data provided additional evidence for the negative role of Hcy in apoE expression. Hcy decreased apoE promoter activity, in the presence or absence of ME2, in a dose dependent manner, in both RAW 264.7 and HEK-293 cells, as revealed by transient transfection experiments. The downstream effectors of the signaling pathways of Hcy were also investigated. The inhibitory effect of Hcy on the apoE promoter activity was counteracted by MAPK/ERK kinase 1/2 (MEK1/2) inhibitor U0126, suggesting that MEK1/2 is involved in the downregulation of apoE promoter activity by Hcy. Our data demonstrated that Hcy-induced inhibition of apoE took place through activation of NF-κB. Moreover, we demonstrated that Hcy activated a synthetic promoter containing three NF-κB binding sites, but did not affect promoters containing AP-1 or NFAT binding sites. ChIP experiments revealed that NF-κB p65 subunit is recruited to the apoE promoter following Hcy treatment of cells.

CONCLUSION: Hcy-induced stress negatively modulates apoE expression *via* MEK1/2 and NF-κB activation. The decreased apoE expression in peripheral tissues may aggravate atherosclerosis, neurodegenerative diseases and renal dysfunctions.

Key words: Apolipoprotein E; Homocysteine; Nuclear factor kappa B; Gene regulation; MAPK/ERK kinase

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Core tip: This original manuscript investigates the effect of high homocysteine (Hcy) levels on apoE expression, and the signaling pathways involved in this gene regulation. Our novel findings show that high doses of Hcy decrease apolipoprotein E (apoE) expression. We revealed that this regulation involves nuclear factor kappa B activation, *via* MAPK/ERK kinase. The Hcy-mediated decrease of apoE expression in peripheral tissues may aggravate atherosclerosis, neurodegenerative diseases and renal dysfunctions. Thus, the current manuscript may be of interest for scientists working in the field of cardiovascular disease and related inflammatory disorders.

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INTRODUCTION

Cardiovascular disease, the main cause of death in Western world, is contributed by both genetic and environmental risk factors. Among these, hyperhomocysteinemia (HHcy) has received extensive attention since it has been hypothesized to be an independent risk factor for atherosclerosis[1]. However, the first report of the association between genetically induced high plasma level of homocysteine (Hcy) and premature arterial lesions belongs to McCully *et al*[2]. Hcy is a key intermediate in the metabolic pathway of the sulfur-containing amino acids leading from exogenous essential methionine to cysteine. Whether a cause or a marker for vascular pathology, HHcy remains strongly associated with alterations such as endothelial dysfunction, smooth muscle cells proliferation, increased inflammation, plaque instability[3]. Many of these alterations act in a concerted fashion toward an atherogenic phenotype. Thus, HHcy induces upregulation of PDGF production by endothelial cells, which in turn promotes proliferation and migration of co-cultured vascular smooth muscle cells[4]. Hcy accelerates senescence and reduces the endothelial progenitor cells proliferation through telomerase inactivation and Akt dephosphorylation[5].

Overall, the effects of elevated Hcy are multifactorial, affecting both the blood vessel structure and the coagulation cascade. Not only the arterial vasculature is challenged by increased Hcy, but also the brain, the bone, the kidney, and blood cells. Neurodegenerative diseases such as Alzheimer's[6] and Parkinson's[7] are associated with HHcy. Elderly individuals with high plasma Hcy are susceptible to hip fractures, exhibiting reduced bone mineral density, alterations of collagen stability due to diminished cross-linking and impaired bone blood flow[8].

Hcy may exert its action through several mechanisms, such as production of reactive oxygen species[9], which may also lead to decreased nitric oxide availability[10], endoplasmic reticulum stress[11], gene regulation[12-14], epigenetic changes such as altered DNA methylation patterns[15], altered protein modification (*e.g.,* N-homocysteinylation of fibrinogen)[16].

Apolipoprotein E (apoE) is an anti-atherogenic 35 kDa glycoprotein associated with all classes of lipoproteins, and it is synthesized by the liver and different peripheral sources, such as macrophages, adipocytes, astrocytes, kidney cells[17]. ApoE plays an important role in lipid metabolism, atherogenesis, neurodegenerative disorders, inflammation, *etc*[17-19]. ApoE gene regulatory mechanisms discovered so far have been recently reviewed[20]. Nevertheless, no reports exist so far regarding the gene regulation of apoE under HHcy stress. It is

only known from a recent report that at posttranslational level, HHcy exerts an inhibitory effect on *in vivo* and *in vitro* dimerization of apoE directly *via* the thiol group[21]. Herein, we investigated how increased Hcy levels affected apoE expression and the mechanisms involved in this modulation. The results obtained in the present study demonstrated that Hcy inhibits apoE expression and that this negative effect is mediated *via* nuclear factor kappa B (NF-κB) activation.

MATERIALS AND METHODS

Chemicals

Homocysteine was from Sigma-Aldrich (St. Louis, MO, United States). DMEM and fetal calf serum were obtained from EuroClone (Milano, Italy). M-MLV reverse transcriptase, oligo(dT), GoTaq DNA polymerase, Luciferase Assay System, U0126, SP600125 and N-p-Tosyl-L-phenylalanine chloromethyl ketone (TPCK) inhibitors were obtained from Promega Corp. (Madison, WI). Primers and TRIzol reagent were from Invitrogen Life Technologies (Carlsbad, CA). Anti-apoE and anti-actin antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). All other reagents were purchased from Sigma-Aldrich (St. Louis, MO, United States).

Plasmids

The construct (-500/+73)apoE-luc containing the apoE proximal promoter as well as the construct ME2/(-500/+73)apoE in which ME2 was cloned in front of the proximal apoE promoter in pGL3 basic vector were previously described[22]. The expression vectors for IKKβ, IKβ dominant negative (DN) and fusion heterodimer p65/p50, as well as the $(NF\text{-}\kappa B)_3$-luc construct containing three NF-κB binding sites, and the $(AP\text{-}1)_7$-luc construct containing seven AP-1 binding sites, were kindly supplied by Prof. D. Kardassis (University of Crete Medical School, Greece).

Cell culture

RAW 264.7 and HEK-293 cells (both from ATCC) were cultured in DMEM supplemented with 10% fetal calf serum and antibiotics.

Reverse transcription-polymerase chain reaction

Total cellular RNA was extracted using TRIzol reagent according to the manufacturer's instructions. After reverse transcription, polymerase chain reaction (PCR) was performed using the following specific primers: Human apoE forward, 5′-CCAGCGGAGGTGAAGGAC; human apoE reverse, 5′-CGCTTCTGCAG GTCATCG; human β-actin forward 5′-CAACCGCGAGAAGATGACCC; human β-actin reverse, 5′-GGAAGGAAGGCTGGAAGAGTGC. The fragments obtained by Reverse Transcription-Polymerase chain reaction (RT-PCR) were of 584 bp for human apoE, and 458 bp for β-actin. The mRNA levels of β-actin were used to normalize the *apoE* gene expression.

Transient transfections

RAW 264.7 and HEK-293 cells were transiently transfected by $Ca_3(PO_4)_2$ co-precipitation method[23] with the above mentioned plasmids, containing promoters driving the luciferase reporter. Luciferase assay was performed using the Luciferase Assay Kit from Promega. Promoter activity was normalized to the activity of the co-transfected β-galactosidase, determined using o-nitrophenyl β-D-galactopyranoside as a substrate.

Immunoblotting

Cells were incubated with various concentrations of homocysteine for 24 h, and then cell lysates were subjected to SDS-PAGE on a 12% polyacrylamide gel and transferred onto nitrocellulose. The blots were probed with anti-apoE or anti-β-actin antibodies, followed by horseradish peroxidase-conjugated secondary antibodies. The protein bands were detected using Super Signal West Pico chemiluminescent substrate (Pierce).

Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) experiments were performed using chromatin prepared from HEK-293 cells treated with 500 μmol/L homocysteine or cysteine for 24 h. Briefly, the cells were fixed with p-formaldehyde and the chromatin was sheared by sonication; p65 proteins bound to DNA were immunoprecipitated with anti-p65 antibodies and DNA was identified by PCR using primers amplifying the region -100/+4 of the apoE gene with the following primers: Forward 5′-ACCTCGTGACTGGGGGCTG, and reverse 5′-GCTGGGGCTGAGTAGGAC. The PCR products were analyzed by agarose gel electrophoresis.

Statistical analysis

Results are means ± SD of triplicate experiments. One-way analysis of variance was performed using OriginPro 7.5. Differences with $P < 0.05$ were considered statistically significant.

RESULTS

ApoE expression is downregulated by homocysteine in HEK-293 cells

We investigated the modulation of apoE expression at mRNA and protein levels in HEK-293 cells, in response to various concentrations of homocysteine. The apoE gene expression in HEK-293 cells exposed to different concentrations of homocysteine (50-750 μmol/L) for 24 h was determined by RT-PCR using primers specific for apoE, and the results were normalized to actin expression. Our experiments showed that homocysteine concentrations of 250-750 μmol/L induced a 2-3 fold decrease of apoE mRNA levels in HEK-293 cells (Figure 1A), while lower concentrations of homocysteine (100 μmol/L) had no significant effect on apoE gene expression ($P > 0.05$) as compared to the control.

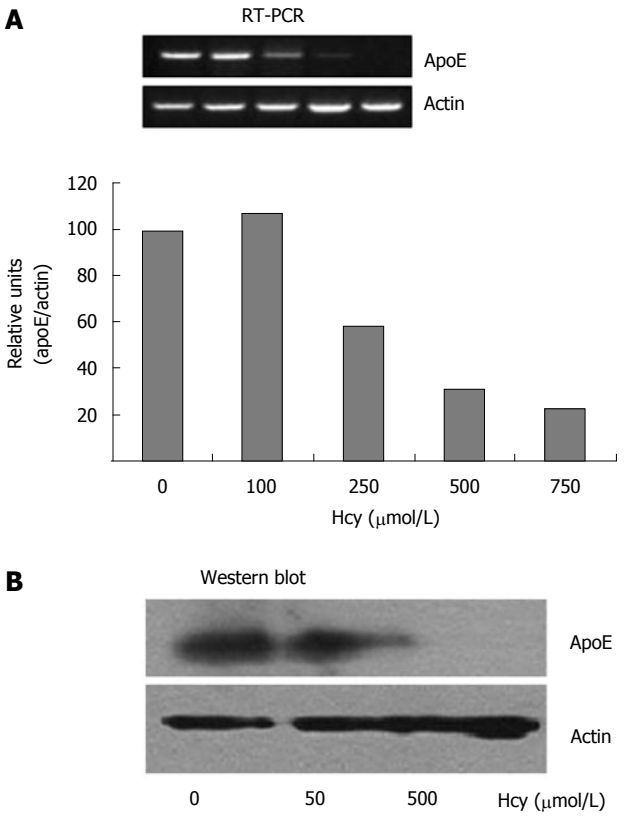


Figure 1 High homocysteine doses downregulate apoE expression in HEK-293 cells. ApoE expression in HEK-293 cells exposed for 24 h to increasing concentrations of homocysteine (50-750 μmol/L) was assessed by RT-PCR and immunoblotting. A: High homocysteine concentrations (250-750 μmol/L) significantly decreased apoE mRNA levels in HEK-293 cells; in contrast, lower doses of homocysteine (100 μmol/L), did not affect the expression level as compared to the control cells. The expression of β-actin was used for normalization; B: Treatment of HEK-293 cells with 500 μmol/L homocysteine, but not with 50 μmol/L homocysteine, dramatically reduced apoE protein expression. RT-PCR: Reverse transcriptase polymerase chain reaction; ApoE: Apolipoprotein E.

Next, we investigated the influence of Hcy treatment on apoE protein expression. HEK-293 cells were exposed to homocysteine (50-500 μmol/L) for 24 h and the endogenous levels of apoE protein were determined by immunoblotting using anti-apoE antibodies. Actin level was used as a control for the total protein load. Our data showed that apoE protein expression was not affected by 50 μmol/L Hcy (as compared to control cells), while it was dramatically reduced in cells exposed to 500 μmol/L Hcy (Figure 1B).

ApoE gene regulatory elements are affected by high homocysteine levels

The ability of homocysteine to modulate the transcriptional activity of apoE promoter was investigated by transient transfection experiments in HEK-293 and RAW 264.7 cells. For this purpose, cells were transiently transfected with plasmids containing apoE proximal promoter [(-500/+73)apoE construct] or plasmids in which multienhancer ME2 was cloned in front of the proximal apoE promoter [ME2/(-500/+73)apoE construct]. After 18 h, the transfected cells were treated with various concentrations of homocysteine (50-500 μmol/L) and the next day, the reporter gene activity was determined by luciferase assay according to the manufacturer's instructions (Promega). As presented in Figure 2, the treatment of cells with 500 μmol/L Hcy significantly decreased ($P < 0.0025$) the apoE promoter activity, in the presence (Figure 2B and D) or in the absence of ME2 (Figure 2A and C), in a dose dependent manner, in both RAW 264.7 (Figure 2A and B) and HEK-293 cells (Figure 2C and D). Treatment of RAW 264.7 or HEK-293 cells with low concentrations of Hcy (50 μmol/L) did not significantly affect ($P > 0.05$) the activity of apoE promoter either in the absence (Figure 2A and C) or in the presence of ME2 (Figure 2B and D).

Taken together, these experiments clearly indicated that high concentrations of homocysteine exerted a negative effect on apoE expression. Therefore, we next sought to investigate the mechanism underlying this inhibitory effect and to determine the signaling pathways possibly involved.

Molecular mechanism of action of high Hcy levels on apoE gene regulation

To test whether MAPK signaling pathway is involved in the Hcy-mediated downregulation of apoE promoter activity, HEK-293 cells were transiently transfected with the construct (-500/+73)apoE-luc in the presence or in the absence of MEK1 expression vectors or MEK inhibitor U0126 (10 μmol/L). The results of these experiments showed that MEK1 overexpression, as well as the treatment of cells with Hcy, significantly decreased ($P < 0.05$) the activity of apoE proximal promoter, as compared to the control (Figure 3, lane Hcy and lane MEK1). Notably, MEK inhibitor U0126 counteracted the inhibitory effect of Hcy on the apoE promoter activity (Figure 3, lane Hcy + U0126). Therefore, our data suggest that MEK1/2 is involved in Hcy-mediated downregulation of apoE promoter activity.

To determine whether Hcy modulates the apoE promoter activity through NF-κB signaling, cells transiently transfected with (-500/+73)apoE–luc construct were treated with Hcy simultaneously with NF-κB pathway inhibition. For this, either TPCK or overexpression of a DN form of IKβ was used. As shown in Figure 4, TPCK abrogated the inhibitory effect of Hcy on apoE promoter activity, similarly with the overexpression of IKβ DN. Moreover, the activity of apoE proximal promoter was significantly reduced ($P < 0.05$) by IKKβ overexpression, to a similar extent as Hcy treatment. Together, these data demonstrate the involvement of the NF-κB signaling pathway in the regulation of apoE promoter activity by homocysteine.

To evaluate the ability of homocysteine to activate NF-κB, HEK-293 cells were transiently transfected with $(NF\text{-}\kappa B)_3$-luc construct containing three NF-κB binding sites. The transfected cells were treated with various concentrations of homocysteine (50-500 μmol/L) for

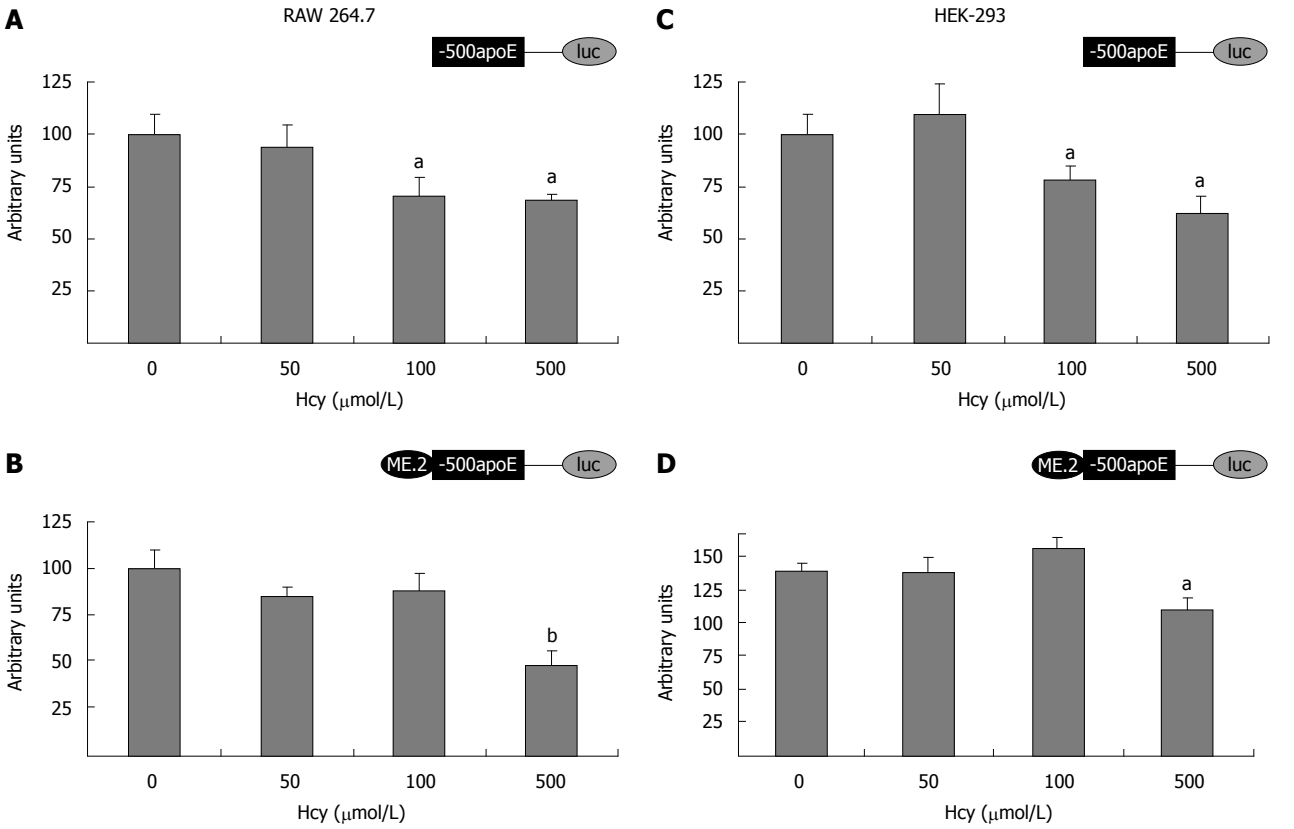


Figure 2 Homocysteine inhibits the activity of apolipoprotein E proximal promoter in either absence or presence of ME2 in RAW 264.7 macrophages and HEK-293 cells. Transient transfection experiments were performed using (-500/+73) apoE proximal promoter (A, C) or ME2/(-500/+73) multienhancer/apoE promoter constructs (B, D) driving the luciferase reporter and the transfected cells were incubated with 50-500 μmol/L homocysteine for 24 h. Then, luciferase activity was assessed and normalized to the activity of the co-transfected β-galactosidase. Treatment of RAW 264.7 (A, B) and HEK-293 cells (C, D) with 500 μmol/L Hcy significantly decreased ($^aP < 0.05$ or $^bP < 0.01$) apoE promoter activity, in the presence (B, D) or in the absence of ME2 (A, C). Low concentrations of Hcy (50 μmol/L) did not considerably decrease ($P > 0.05$) the activity of apoE promoter either in the absence (A, C) or in the presence of ME2 (B, D). MEK1/2: MAPK/ERK kinase; Hcy: Homocysteine; ApoE: Apolipoprotein E.

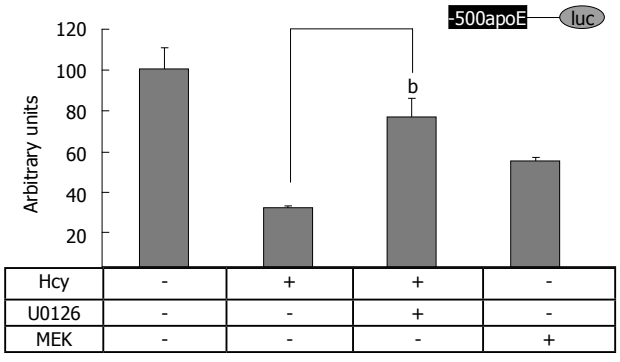


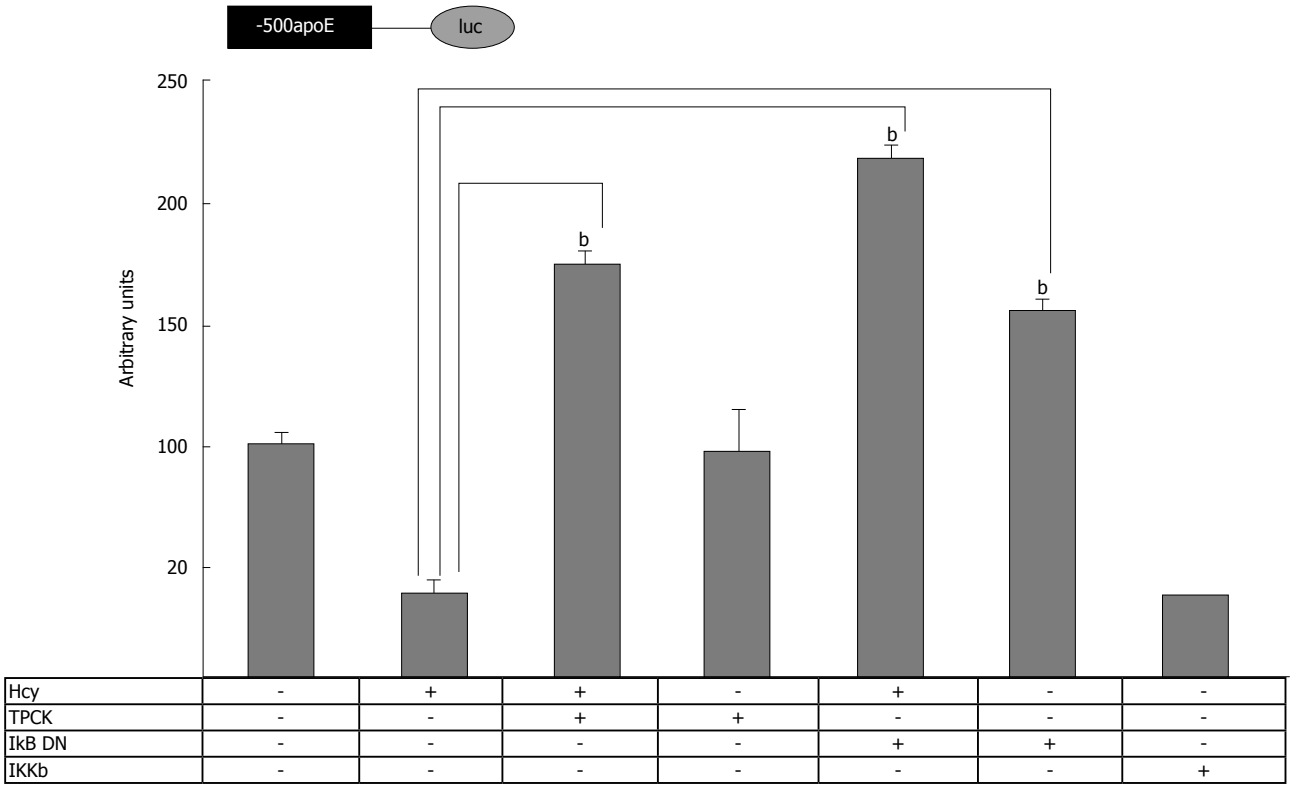
Figure 3 MEK1/2 is a downstream effector of Hcy action on apolipoprotein E promoter in HEK-293 cells. Transient transfections in HEK-293 cells were performed using plasmids containing apoE proximal promoter (-500/+73 region) in the presence or in the absence of homocysteine alone or in combination with U0126 (a MEK inhibitor), or MEK1 expression vectors. Hcy treatment as well as MEK1 overexpression significantly decreased ($P < 0.05$) the activity of (-500/+73) apoE-luc construct, as compared to the control. The decreased activity of the reporter luciferase gene driven by the apoE proximal promoter (-500/+73) in the presence of 500 μmol/L homocysteine was partially restored by 10 μmol/L U0126 ($^bP < 0.01$). MEK1/2: MAPK/ERK kinase; Hcy: Homocysteine; ApoE: Apolipoprotein E.

24 h and then, the reporter gene activity was determined. Our data showed that cell treatment with high concentrations of homocysteine (250 and 500 μmol/L) significantly increased ($P < 0.05$) the activity of the promoter containing the three NF-κB binding sites, while low concentrations of homocysteine (50 μmol/L) did not considerably affect ($P > 0.05$) its activity (Figure 5A). As a positive control for NF-κB activity, a synthetic fusion p65/p50 heterodimer was co-transfected. As expected, the activity of the $(NF\text{-}\kappa B)_3$-luc construct was greatly enhanced by p65/p50 overexpression (Figure 5B).

We subsequently tested whether homocysteine is able to modulate the activity of AP-1 complex or NFAT transcription factor. To this aim, HEK-293 cells were transiently transfected with $(AP\text{-}1)_7$-luc construct (containing seven AP-1 binding sites) or $(NFAT)_3$-luc construct (having three NFAT binding sites), and exposed to different Hcy concentrations (50-500 μmol/L). None of the Hcy concentrations tested affected the activity of AP-1 or NFAT constructs ($P > 0.05$).

Taken together, these results demonstrate that homocysteine downregulates the apoE promoter *via* NF-κB activation; however, AP-1 or NFAT transcription factors do not mediate Hcy effect on apoE transcription.

Considering our findings that revealed the involvement of NF-κB signaling pathway in Hcy-mediated



Hcy	-	+	+	-	+	-	-
TPCK	-	-	+	+	-	-	-
IkB DN	-	-	-	-	+	+	-
IKKb	-	-	-	-	-	-	+

Figure 4 Involvement of nuclear factor kappa B signaling pathway in homocysteine-mediated inhibition of apolipoprotein E promoter activity. HEK-293 cells were transiently transfected with plasmids containing apoE proximal promoter in the presence of homocysteine, TPCK or expression vectors for IKβ dominant negative (IKβ DN) or IKKβ. The activity of the luciferase reporter, normalized to the co-transfected β-galactosidase, was significantly reduced ($P < 0.05$) by IKKβ overexpression, similarly to Hcy treatment. TPCK (NF-κB inhibitor) as well as IKβ DN overexpression abrogated the inhibitory effect of homocysteine on apoE promoter activity, $^{b}P < 0.001$. NF-κB: Nuclear factor kappa B; TPCK: N-p-Tosyl-L-phenylalanine chloromethyl ketone; Hcy: Homocysteine; IKKβ: IκB kinase β; ApoE: Apolipoprotein E.

modulation of apoE promoter activity, we hypothesized that this downregulatory effect is exerted through NF-κB binding to the apoE promoter. Our previous data demonstrated that NF-κB p50 subunit binds to the apoE promoter[24,25]. Therefore, we tested whether NF-κB p65 subunit binds to the apoE promoter using the ChIP assay performed in HEK-293 cells treated with 500 μmol/L Hcy or cysteine (Cys). Chromatin was immunoprecipitated with anti-p65 antibodies and analyzed by PCR using primers to amplify the region -100/+4 of apoE gene. As shown in Figure 6, the results of ChIP experiments indicated that Hcy induced the recruitment of NF-κB p65 subunit to apoE promoter (Figure 6, lane "p65" Hcy). No binding of p65 proteins was observed to the apoE promoter following Cys treatment or in untreated cells (Figure 6, lanes "p65" Cys and Ctr, respectively). PCR using the input as template and primers for the apoE promoter resulted in the expected bands presented in Figure 6 (lanes "Input" Ctr, Cys and Hcy, respectively).

In summary, the results of our study clearly demonstrate that homocysteine downregulates apoE expression *via* MEK1/2 mediated activation of NF-κB. In addition, our results indicate that the activation of AP-1 or NFAT transcription factors is not involved in Hcy inhibition of apoE. Based on these findings, we propose a mechanism of homocysteine-mediated inhibition of apoE gene transcription, schematically illustrated in Figure 7.

DISCUSSION

Homocysteine (Hcy), a nonprotein amino acid resulted upon methionine demethylation is found in plasma in concentrations ranging from 3 to 15 μmol/L. In mild and intermediate HHCy, Hcy plasma levels are found in the domain of 16-100 μmol/L, while in severe HHCy, Hcy values are higher than 100 μmol/L. HHCy is an independent risk factor for cardio- and cerebro-vascular diseases, including atherosclerosis, as recently reviewed in[26], but has also implications in different disorders such as hepatic steatosis[27] and neurological disorders[28]. In addition, it was demonstrated that HHCy may aggravate kidney dysfunction[29] and is associated with impaired renal function in male patients with gout[30].

Our data demonstrate that high levels of Hcy decrease apoE expression in human embryonic kidney (HEK-293) cells (Figure 1A and B). The absence or the decreased apoE levels in different peripheral tissues leads to different pathologies. Kidney biopsies of apoE-deficient mice revealed increased mesangial cell proliferation and matrix formation, key features of the pathogenesis of renal diseases independently of hyperlipidemia[31,32]. In the atherosclerotic plaque, the macrophage-derived apoE plays important roles in the cholesterol efflux, in addition to its antioxidant and anti-inflammatory functions as reviewed in[33]. Thus, the decreased apoE expression in macrophages leads to

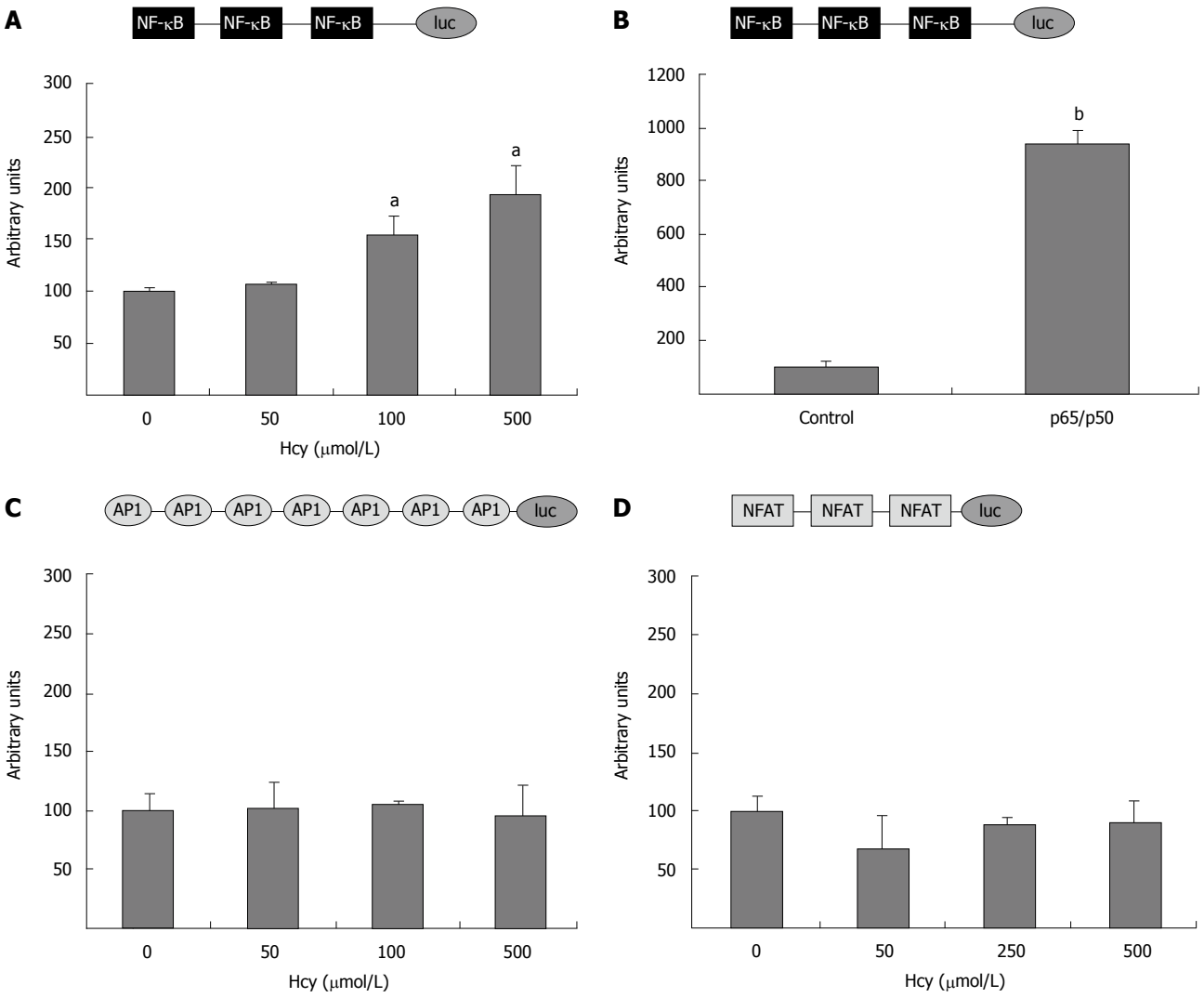


Figure 5 Homocysteine activates nuclear factor kappa B, but has no effect on activator protein-1 or nuclear factor of activated T cells activity. HEK-293 cells were transiently transfected with $(NF\text{-}\kappa B)_3$-luc construct (A), $(AP\text{-}1)_7$-luc (C), or $(NFAT)_3$-luc (D) and exposed to homocysteine (50-500 μmol/L). For positive control, the cells were co-transfected with $(NF\text{-}\kappa B)_3$-luc and the vector for the heterodimer p65/50. The activity of the luciferase reporter driven by the corresponding promoter was normalized to the co-transfected β-galactosidase. Treatment with high concentrations of homocysteine (250 and 500 μmol/L) significantly increased ($^aP < 0.05$) the activity of the promoter containing the three NF-κB binding sites, while no effect of Hcy ($P > 0.05$) was observed on promoters containing binding sites for AP-1 (C) or NFAT (D). The activity of the $(NF\text{-}\kappa B)_3$-luc construct was greatly enhanced by p65/p50 overexpression, used as positive control ($^bP < 0.001$). NF-κB: Nuclear factor kappa B; AP-1: Activator protein-1; NFAT: Nuclear factor of activated T cells; Hcy: Homocysteine.

exacerbation of atherosclerosis.

Our results show that Hcy represses the endogenous apoE gene in a dose dependent manner. A significant effect on apoE expression was noticed only for Hcy doses higher than 100 μmol/L. This is relevant considering that these concentrations are similar to the severe HHCy. These doses were in the same range with those used by other authors in experiments showing the Hcy-mediated modulation of heme oxygenase-1 expression in hepatocytes[34], matrix metalloproteinase-2 in cultured rat vascular smooth muscle cells[35] or vascular smooth muscle cells proliferation[36].

To be able to exert its downstream regulatory effects, Hcy is transported into the cells as shown for primary vascular endothelial cells[37] and is transcytosed through the endothelium to reach the adjacent tissues and cells[38].

Homocysteine acts *via* NMDA receptors and renal NMDA and Group 1 metabotropic glutamate receptors have been associated with HHcy-induced glomerulosclerosis[39]. A question arises regarding the mechanism through which HCy can exert its effects in a cell line like HEK 293, which is apparently void of NMDA receptors and has been used as such to functionally characterize the activity of various receptor subtypes overexpressed in this cell line[40]. However, Hcy can exert its effects not only directly by receptor-mediated cell targeting, but also by indirect actions, as through binding to serum proteins, mainly albumin. Up to 70% of the total HCy is in its protein-bound form and there is a strong correlation between albuminemia and total HCy content[41]. Furthermore, homocysteine was found to be among the strongest protein–bound uremic toxins, which leads to reduced removal upon conventional

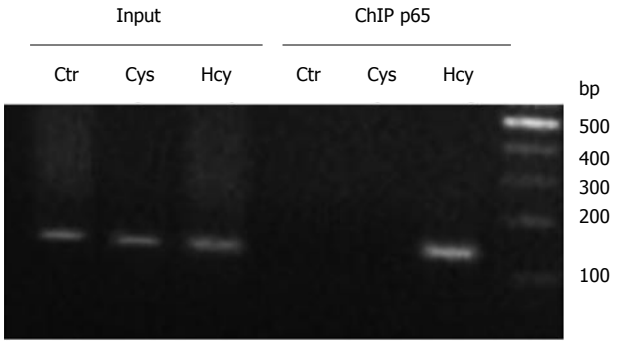


Figure 6 Nuclear factor kappa B p65 subunit is recruited to apolipoprotein E promoter following homocysteine treatment. Chromatin immunoprecipitation experiments were performed in HEK-293 cells treated with 500 μmol/L Hcy or Cys. Treatment with Hcy induced the recruitment of NF-κB p65 subunit to apoE promoter (lane p65 Hcy), while no binding of p65 proteins was observed on the apoE promoter following Cys treatment or in untreated cells (lanes p65 Cys and Ctr, respectively). PCR using the input as template and primers for the apoE promoter resulted in the expected bands (lanes Input Ctr, Cys and Hcy, respectively). NF-κB: Nuclear factor kappa B; ApoE: Apolipoprotein E; Hcy: Homocysteine; Cys: Cysteine.

hemodialysis and thus poor prognosis in atherosclerosis-associated renal complications. Thus, an indirect action could explain the high, non-physiological concentrations at which effects were noticed in our experiments, as well as in reports of other groups. Additionally, it was shown that Hcy could act as a ligand[42,43] or a competitive inhibitor[44,45] for various amino acid transport systems in both laboratory animals and humans, not only in renal cells, but also in other cell types. Therefore, despite the lack of NMDA receptors, it could be envisioned that Hcy may exert its effects in HEK-293 cells by certain non-receptor, indirect actions.

We studied the regulatory mechanisms of Hcy-induced apoE modulation. For this, we used plasmids containing the luciferase gene under apoE regulatory elements, the proximal promoter and the distal enhancer (multienhancer 2), in transient transfection experiments. The data showed that Hcy-mediated regulation involved the repression of the promoter activity (Figure 2). This Hcy-induced downregulation was observed also for other apolipoproteins such as apoAI[12]. The synergic decrease of the anti-atherogenic apolipoproteins apoAI and apoE is significant for atherosclerosis aggravation under hyperhomocysteinemic stress. The involvement of MEK1/2 in Hcy induced apoE regulation was also demonstrated (Figure 3). Similarly, MEK1/2 was shown to be involved in Hcy-induced regulation of MMP-9 in microvascular endothelial cells[46].

At the level of transcription factors, many downstream targets for Hcy were revealed to be implicated in the gene modulation of various proteins: PPARα for apoAI[12], CREB for Herp protein[47], Nrf2 for heme oxygenase-1[13]. Our study demonstrated by transient transfections and ChIP assay, that NF-κB is a downstream target of Hcy, involved in *apoE* gene regulation (Figures 4 and 6, respectively). In addition, our data showed that NF-κB binding sites included in a synthetic promoter

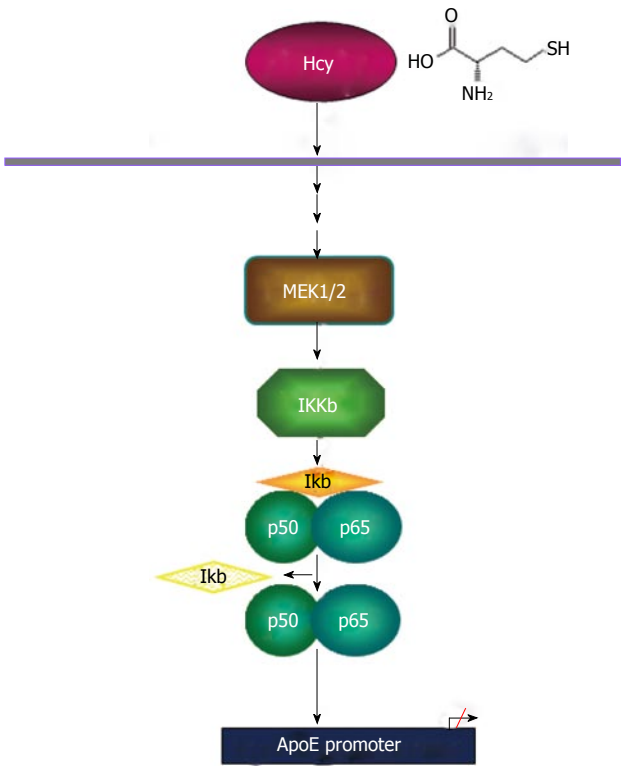


Figure 7 Schematic model of homocysteine-mediated apolipoprotein E gene downregulation. Homocysteine downregulates apoE expression *via* MEK1/2 activation, IKKβ and p65/50 subunits of NF-κB. NF-κB: Nuclear factor kappa B; apoE: Apolipoprotein E; IKKβ: IκB kinase β.

are activated by Hcy (Figure 5A). Recently, the research group of Wang demonstrated the induction in monocytes of inflammatory factors by Hcy, among which NF-κB[48]. Unexpectedly, Hcy did not activate AP-1 (Figure 5C), through MEK1/2, as we detected for LPS-induced gene modulation[24]. However, our findings are in agreement with the data concerning the involvement of NF-κB, but not AP-1, as downstream effectors of high doses of homocysteine[49]. Moreover, we also showed that NFAT pathway is not implicated in Hcy signaling (Figure 5D) in contrast with other Hcy-induced proatherogenic events modulated by this transcription factor known for its participation in pathological cardiac remodeling and vascular lesion formation[50,51].

In conclusion, we report that high level of homocysteine, through MEK1/2, activates NF-κB transcription factors that act on the apoE promoter mediating the repression of *apoE* gene expression. The importance of these findings is given by the fact that the negative effect of high Hcy concentrations on apoE expression in peripheral tissues may aggravate atherosclerosis, neurodegenerative diseases and renal dysfunctions.

COMMENTS

Background

Homocysteine (Hcy) is an independent risk factor for the cardiovascular disease and associated pathologies such as neurodegenerative diseases and renal failure. Apolipoprotein E (apoE) is an anti-atherogenic apolipoprotein

synthesized by the liver and different peripheral sources, such as macrophages, adipocytes, astrocytes, kidney cells, with an important role in lipid metabolism, atherogenesis, and inflammation. ApoE deficient mice are susceptible to atherosclerosis. Data about Hcy effects on apoE modulation are sparse, and it would be of interest to establish a possible connection between the two participants with antagonistic behavior in atherogenesis.

Research frontiers

Previous work from our and others' laboratories, demonstrated the down-regulation of apoE in macrophages under inflammatory conditions, *via* activation of the transcription factor nuclear factor kappa B (NF-κB), leading to a decreased cholesterol efflux in the atherosclerotic lesion and a consequent aggravation of the atherosclerotic phenotype.

Innovations and breakthroughs

So far, the only report connecting Hcy to apoE showed that at the post-translational level, hyperhomocysteinemia (HHCy) exerted an inhibitory effect on *in vivo* and *in vitro* dimerization of apoE directly *via* the thiol group, but no data were available on the transcriptional regulation of apoE under Hcy stress. Herein, the authors investigated the effect of increased Hcy levels on apoE expression and the mechanisms involved in this modulation. The results obtained in the present study demonstrate that Hcy inhibited apoE expression and that this negative effect is mediated *via* the activation of the pro-inflammatory transcription factor NF-κB.

Applications

The authors reported that high levels of homocysteine repress *apoE* gene expression, *via* MEK/NF-κB signaling pathway that act on apoE promoter in HEK-293 cells. This downregulation of apoE expression in peripheral tissues may be one way through high Hcy concentrations aggravate atherosclerosis, neurodegenerative diseases and renal dysfunctions and provides a link between atherogenesis, inflammation and Hcy stress.

Terminology

HHCy, characterized by an abnormally high level of plasma Hcy, is a risk factor for cardiovascular diseases.

Peer-review

In this manuscript, the authors investigated how HHcy downregulates ApoE, thus potentially contributing to the development of atherosclerosis. This is an interesting study where the experiments are reasonably well planned and executed. They collected data showing Hcy regulation of the apoE promoter and expression using cell culture model. They observed that high Hcy dose downregulated ApoE mRNA and protein in cultured RAW 264.7 and HEK293 cells and further found that activity of the ApoE proximal promoter (-500/+73)-luciferase reporter is regulated by Hcy in transient transfected cell lines. Their data potentially provide insight of Hcy related atherosclerosis and thus contribute to our understanding of atherosclerosis development.

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ORIGINAL ARTICLE

Basic Study

Epigenetic alteration to activate Bmp2-Smad signaling in Raf-induced senescence

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Abstract

AIM: To investigate epigenomic and gene expression alterations during cellular senescence induced by oncogenic *Raf*.

METHODS: Cellular senescence was induced into mouse embryonic fibroblasts (MEFs) by infecting retrovirus to express oncogenic *Raf* (RafV600E). RNA was collected from RafV600E cells as well as MEFs without infection and MEFs with mock infection, and a genome-wide gene expression analysis was performed using microarray. The epigenomic status for active H3K4me3 and repressive H3K27me3 histone marks was analyzed by chromatin

immunoprecipitation-sequencing for RafV600E cells on day 7 and for MEFs without infection. These data for *Raf*-induced senescence were compared with data for *Ras*-induced senescence that were obtained in our previous study. Gene knockdown and overexpression were done by retrovirus infection.

RESULTS: Although the expression of some genes including secreted factors was specifically altered in either *Ras*- or *Raf*-induced senescence, many genes showed similar alteration pattern in *Raf*- and *Ras*-induced senescence. A total of 841 commonly upregulated 841 genes and 573 commonly downregulated genes showed a significant enrichment of genes related to signal and secreted proteins, suggesting the importance of alterations in secreted factors. *Bmp2*, a secreted protein to activate Bmp2-Smad signaling, was highly upregulated with gain of H3K4me3 and loss of H3K27me3 during *Raf*-induced senescence, as previously detected in *Ras*-induced senescence, and the knockdown of *Bmp2* by shRNA lead to escape from *Raf*-induced senescence. Bmp2-Smad inhibitor *Smad6* was strongly repressed with H3K4me3 loss in *Raf*-induced senescence, as detected in *Ras*-induced senescence, and senescence was also bypassed by Smad6 induction in *Raf*-activated cells. Different from *Ras*-induced senescence, however, gain of H3K27me3 did not occur in the *Smad6* promoter region during *Raf*-induced senescence. When comparing genome-wide alteration between *Ras*- and *Raf*-induced senescence, genes showing loss of H3K27me3 during senescence significantly overlapped; genes showing H3K4me3 gain, or those showing H3K4me3 loss, also well-overlapped between *Ras*- and *Raf*-induced senescence. However, genes with gain of H3K27me3 overlapped significantly rarely, compared with those with H3K27me3 loss, with H3K4me3 gain, or with H3K4me3 loss.

CONCLUSION: Although epigenetic alterations are partly different, *Bmp2* upregulation and *Smad6* repression occur and contribute to *Raf*-induced senescence, as detected in *Ras*-induced senescence.

Key words: Senescence; Epigenome; *Raf*; H3K27me3; Histone; *Ras*

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Core tip: By examining gene expressions and active and repressive histone marks on genome-wide scale, many genes were found to show common expression changes during *Ras*- and *Raf*-induced senescence, including genes related to secreted factors and cell cycle. Upregulation of *Bmp2* and downregulation of *Smad6* were detected and played a role in *Raf*-induced senescence as well as in *Ras*-induced senescence. Epigenetic alterations to regulate gene expression were also similar, including gain and loss of active histone mark H3K4me3 and loss of H3K27me3. But epigenetic changes are different in part, *e.g.*, in gain of H3K27me3 in *Smad6* promoter region.

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INTRODUCTION

Cellular senescence is a phenomenon of permanent growth arrest after the limited cell division of primary culture cells[1]. Similar to replicative senescence of cells, the premature form of cellular senescence can be induced by activated oncogenes, oxidase stress, telomere shortening, and DNA damage[2,3]. It is assumed that premature senescence may function as barrier mechanism against cancer progression[4-7]; in fact, many premature senescent cells have been seen in premalignant tumors[8-10].

Similar to replicative senescence, premature senescence is identified by biomarkers of senescence, *e.g.*, senescence-associated β-galactosidase (SA-β-gal). In addition to oncogenic *Ras*, premature senescence can also be induced by other oncogenes, *e.g.*, *Braf*, *AKT*, *E2F1*, *cyclin E*, *Mos*, and *Cdc6*, and by the inactivation of tumor-suppressor genes including *PTEN* and *NF1*[11]. Some oncogenes induce senescence through DNA damage responses[12], or by hyper-replication of DNA caused by sustained oncogenic signals[13,14].

Oncogene-induced senescence and replicative senescence activate tumor suppressor cascades, including p16Ink4a-Rb and p19Arf (p14ARF in human)-TP53 signaling pathways[15,16]. Though the roles of RB and TP53 signals in senescence are undisputable, other factors are also known to be involved.

Senescent cells are observed to express a vast number of secreted proteins, *e.g.,* transforming growth factor-β, insulin, Wnt, and interleukin signaling cascades protein. This phenotype was known as "senescence-associated secretory phenotype (SASP)" or "senescence-messaging secretome"[17,18]. The SASP includes many inflammatory cytokines and chemokines[17], providing a strong link between senescence and inflammation. SASP activation is largely mediated by the transcription factors nuclear factor -kB and CCAAT/enhancer-binding protein beta[19-21], and comprises a range of chemokines (chemoattractants and macrophage inflammatory proteins), proinflammatory cytokines (IL-1a, IL-1b, IL-6, and IL-8), growth factors (hepatocyte growth factor, TGFb, granulocyte-macrophage colony-stimulating factor)[22,23] and matrix-remodeling enzymes[19,20,24].

Epigenetic mechanisms are also involved in senescence. When human fibroblasts leads to cellular senescence, heterochromatic regions gather to form

senescence-associated heterochromatic foci (SAHF), in which genomic regions with histone H3K9 trimethylation condense and are surrounded by histone H3K27 trimethylation (H3K27me3). These heterochromatin foci are colocalized with heterochromatin protein HP1, macroH2A and HMGA[25]. The p16 and ARF locus is regulated by Polycomb repressive complexes (PRCs), which repress transcription in proliferating cells. The H3K27me3 mark at the locus was lost during oncogene-induced senescence, which lead to expression of *p16*^{INK4A} and *ARF* in senescence, and these molecular alterations were also observed in replicative senescence[26-29]. The histone demethylase for H3K27, Jmjd3, was reported to be critical for senescence, and knockdown of *Jmjd3* sustained repression of *p16* by H3K27me3, leading to escape from senescence[30,31].

We previously analyzed epigenetic and gene expression changes in *Ras*-induced senescence of mouse embryonic fibroblasts (MEFs) on genome-wide scale[22]. We showed that Bmp2-Smad signaling is important in *Ras*-induced senescence, and is regulated by coordinated dynamic alterations of the H3K4me3 and H3K27me3 marks. We further examined the downstream target genes of the signal genome-widely, and showed that dynamic epigenomic alteration disrupts negative feedback loop of the signal, and that continuously activated downstream targets contribute to growth arrest. These epigenetic alterations associated with Bmp2-Smad1 signal were found to occur specifically in *Ras*-activated cells, not in control cells with mock retrovirus infection[22]. In contrast, the epigenetic alteration at the *Ink4a-Arf* locus was commonly observed in *Ras*-activated cells and mock cells, *i.e.,* both in cells under *Ras*-induced senescence and cells under replicative senescence[22]. It was suggested that there might be common epigenetic mechanisms in premature senescence and replicative senescence, and also different epigenetic regulation on specific signals between the two senescence programs. Within such molecular alterations specifically observed during premature senescence, there might be some common alterations and some different alterations in premature senescence induced by different stresses, but such analysis to compare different types of premature senescence has not been conducted in detail.

Here we analyzed genome-wide transcription and epigenetic changes during *Raf*-induced senescence by microarray and ChIP-seq. We found that the majority of genes that were altered during *Ras*- and *Raf*-induced senescence were similar, and that activation of *Bmp2* and repression of *Smad6* were also involved in *Raf*-induced senescence. However, epigenetic alterations might be somewhat different between *Ras*- and *Raf*-induced senescence, *e.g.,* in gain of H3K27me3 mark at the *Smad6* locus.

MATERIALS AND METHODS

Cells and viral infection

All of the experiments were approved by from the Ethics Committee for Animal Research Studies at the University of Tokyo. The animal protocol was designed to minimize any pain or discomfort to mice. The mice were acclimatized to laboratory conditions for two weeks prior to experiments, and were euthanized by carbon dioxide inhalation for tissue collection. MEF cells were established using 13.5-embryonic-day embryos of C57/B6 mice[23]. Embryos were minced with the head and red organs removed, and digested twice with trypsin/EDTA for 15 min at 37 ℃. The cells were seeded onto 10-cm dishes and cultured in Dulbecco's modified Eagle's medium containing 100 UI/mL penicillin and 100 g/mL streptomycin sulfate, supplemented with 10% (V/V) fetal bovine serum at 37 ℃ in 50 mL/L CO_2. When cells were passed twice, the MEF cells (MEFp2) underwent retrovirus infection for 48 h. Then the cells were seeded at a density of 1×10^5 cells/6-cm dish (day 0), and total RNA was collected using TRIzol (Invitrogen, Carlsbad, CA), from MEFp2 and from virus-infected MEFs at the indicated time points.

Retroviral vectors

To induce senescence in MEF, we cloned cDNA for wild type *BRAF* (RafWT) and mutated *BRAF* (RafV600E) into a CMV promoter driven expression vector pMX-neo that contains the neomycin-resistance gene (Cell Biolabs, San Diego, CA). Using FuGENE 6 Transfection Reagent (Roche, Germany), an empty pMX-neo vector (Mock), and vectors containing RafWT and RafV600E were transfected in plat-E packaging cells (kindly gifted from T. Kitamura, the Institute of Medical Science, the University of Tokyo, Tokyo, Japan) to prepare retroviruses. The infected cells were exposed to 700 μg/mL of G418 (Invitrogen) from days 0-10 for selection. It was confirmed that MEF cells without infection completely died under these conditions by day 5. When mock-infected cells were cultured with and without G418, they did not show a difference in cellular growth, indicating that the multiplicity of infection is nearly 100%. *Smad6* cDNA with an N-terminal 6x Myc tag was cloned into pMX-puro vector in our previous study, and retroviruses were prepared using plat-E cells as above. The infected cells were exposed to 1 μg/mL of puromycin from days 0-3 for selection.

shRNA retrovirus

Using pSIREN-RetroQ Vector (Clontech, CA), a retrovirus vector to express small hairpin RNA (shRNA) against *Bmp2* was constructed as previously reported[22]. The oligonucleotide sequences targeting *Bmp2* is: GATCCG GGACACCAGGTTAGTGAAT TTCAAGAGA ATTCACTAACCTGGTGTCC CTTTTTTCTCGAGG, with the 19-mer target sense and antisense sequences underlined. Viral packaging for shRNA retrovirus vectors against *Bmp2* (shBmp2) was also performed using plat-E cells and FuGENE 6 as above. To MEFp2, RafV600E and shRNA viruses were simultaneously infected for 48 h. The infected cells were exposed to 1 μg/mL of puromycin during days 0-3 for selection.

Expression array analysis

For genome-wide gene expression analysis, GeneChip Mouse Genome 430 2.0 Array (Affymetrix) was used. Data were collected by an Affymetrix GeneChip Scanner 3000 (Affymetrix). To obtain the signal value (GeneChip score) for each probe, the GeneChip data were analyzed using Affymetrix GeneChip Operating Software v1.3 by MAS5 algorithms. The average signal in an array was made equal to 100 for global normalization. Expression array data for *Raf*-induced senescence were collected, and array data for *Ras*-induced senescence was previously submitted to GEO (#GSE18125).

Gene annotation enrichment analysis

Gene annotation enrichment was analyzed for Gene Ontology (GO) (biological process, cellular component, and molecular function) and for Functional Categories (Swiss Prot PIR database keywords, and Uniprot Sequence Feature) using the Functional Annotation tool at DAVID Bioinformatics Resources (http://david.abcc.ncifcrf.gov/).

Chromatin immunoprecipitation

MEFp2 and infected cells at day 7 were cross-linked with 1% formaldehyde for 10 min, and chromatin immunoprecipitation (ChIP) using anti-H3K4me3 (Abcam ab8580, rabbit polyclonal) and H3K27me3 (Upstate 07-142, rabbit polyclonal) was performed. Briefly, cells were crosslinked with 1% formalin for 10 min, and the crosslinked cells were sonicated for fragmentation. The fragmented samples were incubated with antibodies bound to protein A-sepharose beads at 4 ℃ overnight. The beads were then washed eight times and eluted with elution buffer. The eluates were treated with pronase at 42 ℃ for 2 h and then underwent incubation at 65 ℃ overnight to reverse the crosslinks. The immunoprecipitated DNA was purified by the phenol/chloroform procedure and precipitated with LiCl and 70% ethanol.

ChIP-sequencing

Samples for ChIP-sequencing were prepared according to the manufacturer's instructions by Illumina, and sequencing was performed using Solexa Genome Analyzer Ⅱ. By using the Illumina pipeline software v1.4, 36-bp single-end sequence reads were mapped to the reference mouse genome NCBI Build #36 (UCSC mm8). The numbers of uniquely mapped reads were 10845082 (H3K4me3), 11519151 (H3K27me3), and 5688804 (Input) for MEFp2, and were 26339875 (H3K4me3), 27239707 (H3K27me3), and 6126206 (Input) for RafV600E cells. ChIP-seq data for *Ras*-induced senescence was previously submitted to GEO (#GSE18125).

To determine the distribution of immunoprecipitated DNA fragments in a certain region, we set a window in genomic DNA, within which we counted a number of uniquely mapped Solexa reads, as previously performed[22]. A window size of 300 bp was used in ChIP-seq of H3K4me3, and that of 500 bp for H3K27me3, because H3K4me3 was distributed in narrow DNA regions and 300 bp was wide enough for a window, but H3K27me3 is rather broad modification than H3K4me3, so a wider window, 500 bp, was necessary to detect read count. To express the modification status for the center position of the window, the number of mapped reads per 1000000 reads within a window was counted and calculated. Within ± 2 kb from the TSS of each gene, the maximum number of mapped reads per 1000000 reads in a window was adopted as the modification status of each gene. When the number was > 3.5 and < 3 for H3K4me3, the gene was regarded as H3K4me3(+) and (-), respectively. When the number was > 1.5 and < 1 for H3K27me3, the gene was regarded as H3K27me3(+) and (-), respectively.

SA-β-gal analysis

MEFp2 and infected cells on days 3, 5, 7 and 10 underwent SA-β-gal staining[32]. Cells were washed with PBS twice, and incubated in fixation buffer [2% (w/v) formaldehyde, 0.2% (w/v) glutaraldehyde in PBS] for 5 min. The cells were washed with PBS twice again, then incubated in the SA-β-gal staining solution (40 mmol/L citric acid/sodium phosphate, pH 6.0, 150 mmol/L NaCl, 2.0 mmol/L MgCl2, 5 mM K4[Fe(CN)6], 5 mmol/L K3[Fe(CN)6], 1 mg/mL X-gal) overnight.

Growth curve

The infected cells were counted on days 3, 5, 7, and 10 using the Countess cell counter (Invitrogen). A growth curve was drawn by calculating mean number of three dishes.

Quantitative real-time RT-PCR and ChIP-PCR

For RT-PCR, the total RNA was treated with DNase Ⅰ (Invitrogen), and cDNA was synthesized from 1 μg of total RNA using a Superscript Ⅲ kit (Invitrogen). Real-time RT-PCR was performed using SYBR Green and iCycler Thermal Cycler (Bio-Rad Laboratories). The number of molecules of *BRAF*, *Bmp2* and *Smad6* in a sample was measured by comparing with the standard samples containing 10^1 to 10^6 copies of the genes. The quantity of mRNA of each gene was normalized to that of *Ppib*, and the relative expression levels compared to those in RafV600E were shown. The experiment was performed three times and the mean and standard error were calculated and shown. The following PCR primers were used. *BRAF*: CACCATCTCC ATATCATTGA GACCA and CGAGATTTCA CTGTAGCTAG ACCAA. *Bmp2*: TATCATGCCT TTTACTGCCA and ATTCACAGAG TTCACCAGAG TC. *Smad6*: ATCCCCAAGC CAGACAGTCC and TCCTTGAGCC TCTTGAGCAG C. *Ppib*: ATGTGGTACG GAAGGTGGAG A and AGCTGCTTAG AGGGATGAGG.

For ChIP-PCR, real-time PCR using SYBR Green and iCycler Thermal Cycler (Bio-Rad Laboratories) was performed to amplify ChIP samples, and they were

quantified by comparing with the standard samples, *i.e.,* 20, 2, 0.2, and 0.02 ng/μL of sonicated genomic DNA of MEF. The following PCR primers were used. *Actb*: TGAGGTACTA GCCACGAGAG AG and ACACCCGCCA CCAGGTAAGC A. *Gcgr*: TGCTGTCATG TCTGGTGAGT G and GGAGCTGTCA GCACTTGTGT A. *Bmp2*: CTTGGCTGGA GACTTCTTGA ACT and TGGAGGCGGC AAGACTGGAT. *Smad6*: CTGGGGTTTG GAATGCCTAA and CTAAAAGCTA TGTACCGACT GAGG.

Statistical analysis

Unsupervised two-way hierarchical clustering was performed using the Cluster 3.0 software (Stanford University, http://bonsai.ims.u-tokyo.ac.jp/~mdehoon/software/cluster/software.htm#ctv). A heat map was drawn by Java TreeView software (Alok, http://jtreeview.sourceforge.net/). Fisher's exact test, Student's *t*-test, Kolmogorov-Smirnov test, phi coefficient analysis, and log linear analysis were performed using R software (http://www.R-project.org/).

RESULTS

Gene expression analysis

MEF cells after two passages (MEFp2) were infected with retrovirus of wild type *Raf* (RafWT) or oncogenic *Raf* (RafV600E), or mock retrovirus, and cultured through day 10 (Figure 1). While mock cells showed cellular growth similar to that of MEF cells without infection and RafWT cells showed slightly but significantly faster growth than those, RafV600E cells hardly showed cellular growth (Figure 1B).

SA-β-gal staining was performed on RafV600E cells on days 3, 5, 7, and 10, and the number of SA-β-gal(+) cells was counted and compared with those for MEF cells without retrovirus infection, mock cells and RafWT cells. RafV600E cells showed a marked increase in number of SA-β-gal(+) cells after day 5, but the number of mock cells and RafWT cells did not increase until day 10 (Figure 1C and D). When cells were infected with retrovirus of oncogenic *Ras* (RasG12V) and wild type *Ras* (RasWT) as previous study[22], RasG12V cells showed a marked increase of SA-β-gal(+) cells after day 7, while RasWT cells did not until day 10 (Figure 1D).

Gene expression analysis was performed on genome-wide scale using expression array, and gene expression changes in *Raf*-induced senescence were compared with those in *Ras*-induced senescence (Figure 2). A two-way hierarchical clustering analysis was performed for genes showing > 5-fold increase or < 0.2-fold decrease in any samples of *Ras*-induced senescence and *Raf*-induced senescence compared with MEFp2. The major cluster of upregulated genes contained three subgroups: Genes upregulated by *Ras* and *Raf* commonly, by *Ras* specifically, and by *Raf* specifically. Though it is less clear, the cluster of downregulated genes also showed three similar subgroups: Genes downregulated by *Ras* and *Raf* commonly, by *Ras* specifically, and by *Raf* specifically.

We previously reported that epigenetic activation of *Bmp2* and repression of *Smad6* is important in *Ras*-induced senescence. *Bmp2* was included in the subgroup of genes upregulated by *Ras* and *Raf* commonly, and *Smad6* was included among those that were downregulated by *Ras* and *Raf* commonly.

Comparison of genes upregulated/downregulated in Ras-/Raf-induced senescence

We performed GO enrichment analysis for 841 genes showing > 5-fold increase in RasV12 cells and RafV600E cells commonly (Figure 3A). Genes associated with signal/secreted proteins ($P = 3.8 \times 10^{-30}$), extracellular regions ($P = 5.3 \times 10^{-19}$), and cell differentiation/development ($P = 6.1 \times 10^{-9}$) were upregulated, *e.g., Bmp2* and *Igfbp3*, supporting the alteration of secreted factor expression during senescence, and these factors might play a common role in *Ras*- and *Raf*-induced senescence. The 573 genes showing < 0.2-fold decrease commonly RasV12 cells and RafV600E cells, showed genes related to cell cycle ($P = 1.3 \times 10^{-33}$) such as *Cdc6*, in good agreement with growth arrest (Figure 3B). Genes related to secreted protein ($P = 2.0 \times 10^{-12}$) and extracellular region ($P = 5.4 \times 10^{-13}$), such as *Cxcl5* and *Wnt4*, were also enriched, suggesting that the dynamic activation and repression of some secreted factors occur commonly during *Ras*- and *Raf*-induced senescence. The GO-term "protein binding" also showed significant enrichment, and *Smad6* was included in this term.

Genes altered in Ras-/Raf-induced senescence and also in mock cells

Among these genes altered commonly during *Ras*- and *Raf*-induced senescence, some genes showed similar alteration patterns in mock cells (Figure 4). There were 348 genes showing > 5-fold upregulation commonly in mock cells, RasG12V cells and RafV600E cells, compared with MEF. Genes associated with signal/secreted proteins proteins ($P = 6.7 \times 10^{-12}$) or extracellular regions ($P = 3.3 \times 10^{-9}$), *e.g., Igfbp3* and *Cd5l*, were significantly enriched in these genes, suggesting the involvement of common secreted factors in both oncogene-induced senescence and replicative senescence (Figure 4A). Similarly, there were 80 genes, *e.g., Cdc6* and *Wnt4*, showing < 0.2-fold downregulation commonly in mock cells, RasG12V cells and RafV600E cells, compared with MEF (Figure 4B).

Genes altered in Ras-/Raf-induced senescence but not in mock cells

In some genes, in contrast, the expression alterations commonly observed in *Ras-/Raf*-induced senescence did not occur in mock cells (Figure 5). There were 259 genes showing > 5-fold upregulation commonly in RasG12V cells and RafV600E cells, but not in mock cells. Genes associated with signal/secreted proteins ($P = 1.8 \times 10^{-10}$) or extracellular regions ($P = 7.0 \times 10^{-5}$), *e.g., Bmp2*, were also significantly enriched in these

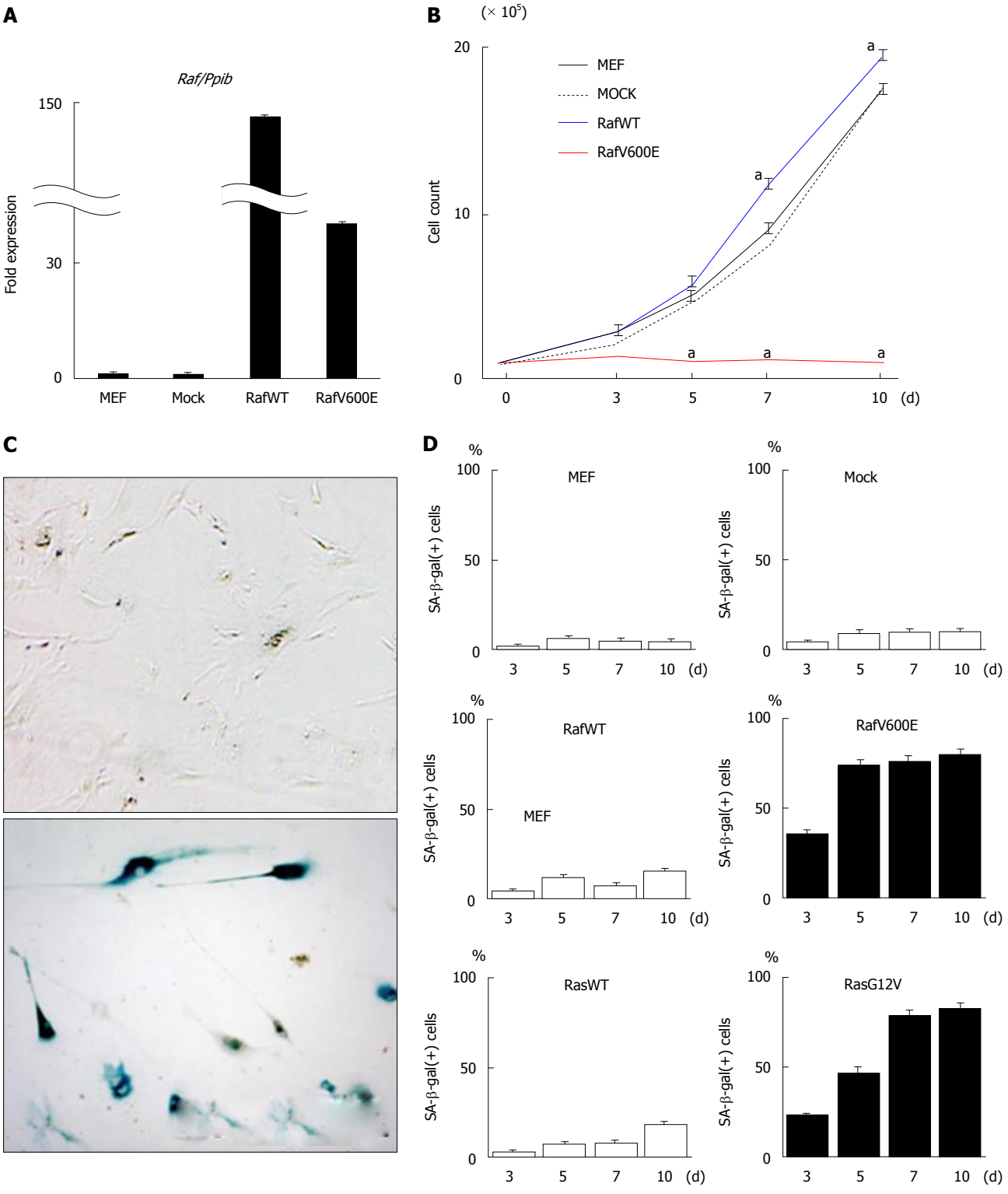


Figure 1 Induction of cellular senescence in mouse embryonic fibroblast by oncogenic *Raf*. A: Induction of *Raf* by retroviral infection. Retrovirus was infected to MEFs on day -2 to induce the expression of wild type *Raf* (RafWT) or oncogenic *Raf* (RafV00E). RNA was collected on day 7, and the expression level of BRAF was analyzed by real-time RT-PCR to confirm expression induction; B: Growth curve. Retrovirus was infected into MEFs on day -2. After two days (day 0), the cell number was counted to draw the growth curve. Cells that were infected with mock retrovirus Mock cells (black dotted line) showed cellular growth similar to that of MEF cells without infection (black line), and RafWT cells (blue line) showed slightly but significantly faster growth than did MEF cells without infection. RafV600E cells hardly showed cellular growth (red line). $^{a}P < 0.05$ (t); C: SA-β-gal staining. Compared with MEF cells on day 7 (above), RafV600E cells on day 7 (bottom) showed positive staining of SA-β-gal; D: Count of SA-β-gal(+) cells. The number of SA-β-gal(+) cells was counted on days 3, 5, 7, and 10. MEF cells without virus infection rarely showed SA-β-gal(+) cells. While mock cells and RafWT cells did not show a marked increase in the number of SA-β-gal(+) cells, RafV600E cells showed a marked increase in the number of SA-β-gal(+) cells after day 5 (73.3% ± 2.9% on day 5). When cells were infected with retrovirus of oncogenic *Ras* (RasG12V) and wild type *Ras* (RasWT) as previous study[22], RasG12V cells showed marked increase of SA-β-gal(+) cells after day 7 (showed 78.3% ± 4.5% on day 7), while RasWT cells did not until day 10. The mean and standard error in three repeated experiments were shown. MEF: Mouse embryonic fibroblast.

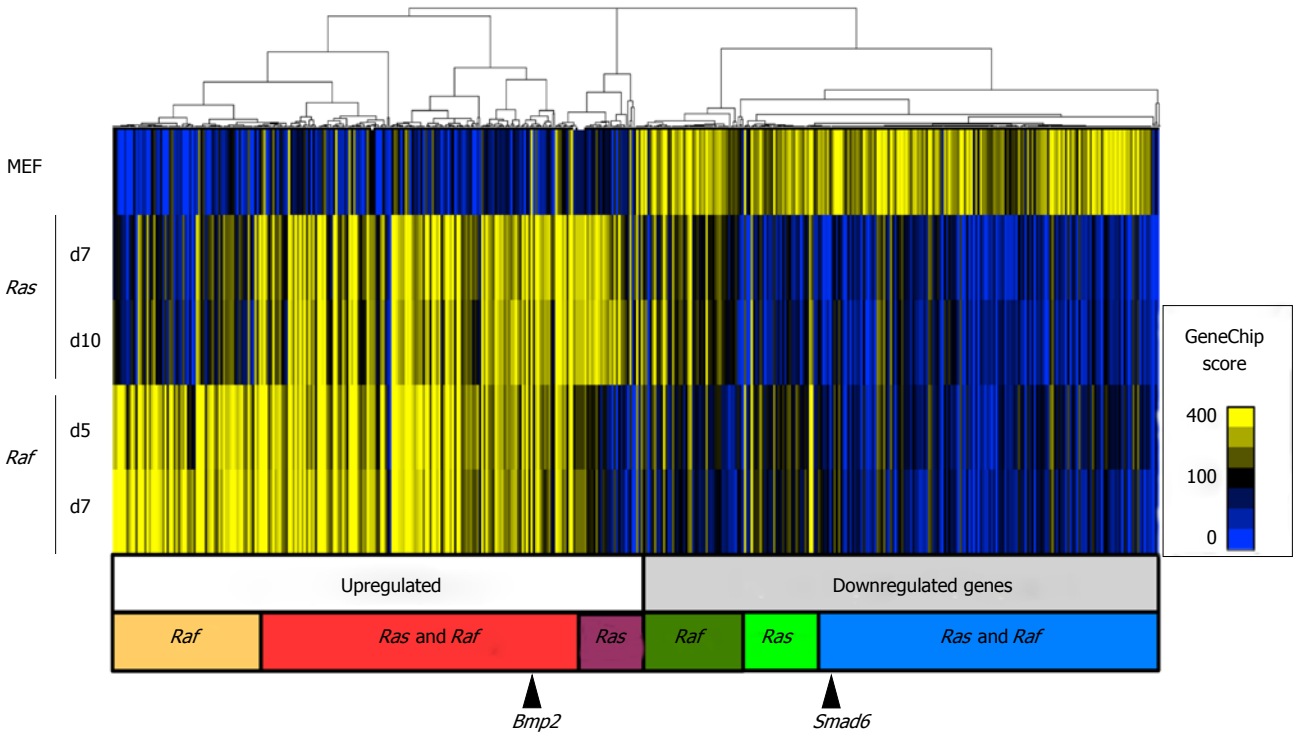


Figure 2 Hierarchical clustering analysis of genes altering expression levels during senescence. Gene expression levels were analyzed for 15653 genes using microarray for MEF cells, and RafV600E cells on days 5 and 7. For senescence induced by oncogenic *Ras* (RasG12V), RasG12V cells on days 7 and 10 were analyzed in our previous study, and compared with *Raf*. A total of 729 probes showing an expression level higher than 100 in GeneChip score in any of the five samples, and showing either > 5-fold upregulation or > 0.2-fold downregulation in any RafV600E or RasG12V samples compared with MEF, were extracted for clustering analysis. The major cluster for upregulated genes consisted of three subclusters: Genes upregulated specifically in *Raf*-induced senescence, those commonly upregulated in *Ras*- and *Raf*-induced senescence, and those upregulated specifically in *Ras*-induced senescence. The other major cluster for downregulated genes also contained three subgroups of genes, albeit not very clear: Genes downregulated specifically in *Raf*-induced senescence or *Ras*-induced senescence, and those commonly downregulated in *Ras*- and *Raf*-induced senescence. *Bmp2* was included in commonly upregulated genes and *Smad6* was included in commonly downregulated genes (black arrows). MEF: Mouse embryonic fibroblast.

genes, suggesting involvement of some secreted factors specifically in oncogene-induced senescence, but not in replicative senescence (Figure 5A). Similarly there were 242 genes, *e.g., Smad6*, showing < 0.2-fold downregulation commonly in RasG12V cells and RafV600E cells, but not in mock cells. Genes associated with signal/secreted protein ($P = 2.3 \times 10^{-17}$) or extracellular region ($P = 1.3 \times 10^{-17}$) were also significantly enriched in these genes, *e.g., Cxcl5* (Figure 5B). Interestingly, these GO terms were not enriched in genes commonly downregulated in mock, RasG12V, and RafV600E cells (Figure 4B), suggesting that repression of secreted factors occur more dynamically during *Ras*- and *Raf*-induced senescence, than in replicative senescence.

Genes altered specifically either in Ras- or Raf-induced senescence

Some genes altered specifically in RasG12V cells, but not in mock cells and RafV600E cells, including 36 upregulated and 167 downregulated genes. The 167 downregulated genes showed a significant enrichment of genes associated with signal/secreted proteins, *e.g., Wnt2* (Figure 6A and B). Similarly, some genes altered specifically in RafV600E, but not in mock cells and RasG12V cells, including 63 upregulated and 170 downregulated genes. These genes showed significant enrichment of GO-terms such as "secreted" and "membrane", *e.g., Cd40* in upregulated genes and *Mcam* in downregulated genes (Figure 6C and D). These results suggested that some environment of secreted proteins might be specifically induced by *Ras*- or *Raf*-induced senescence.

Increase of Bmp2 and decrease of Smad6

We previously reported that the activation of Bmp2-Smad signaling by the harmonized epigenetic activation of *Bmp2* and repression of *Smad6* contributes to *Ras*-induced senescence[22]. In the present study, *Bmp2* and *Smad6* were found to be altered commonly in *Ras*- and *Raf*-induced senescence, but not in mock cells (Figure 5). In our recent targeted exon sequencing analysis in colorectal tumors, mutation of genes in BMP signaling, *e.g., BMPR2*, *BMP2*, and *SMAD4*, were significantlydetected in *BRAF*-mutation(+) colorectal cancer[33]. It was thus suggested that activation of Bmp2-Smad signalling might be also critical in Raf-induced senescence and disruption of the signaling may play a role in tumorigenesis of *BRAF*-mutation(+) colorectal cancer. To examine whether an increase of *Bmp2* and decrease of *Smad6* contributed to *Raf*-induced

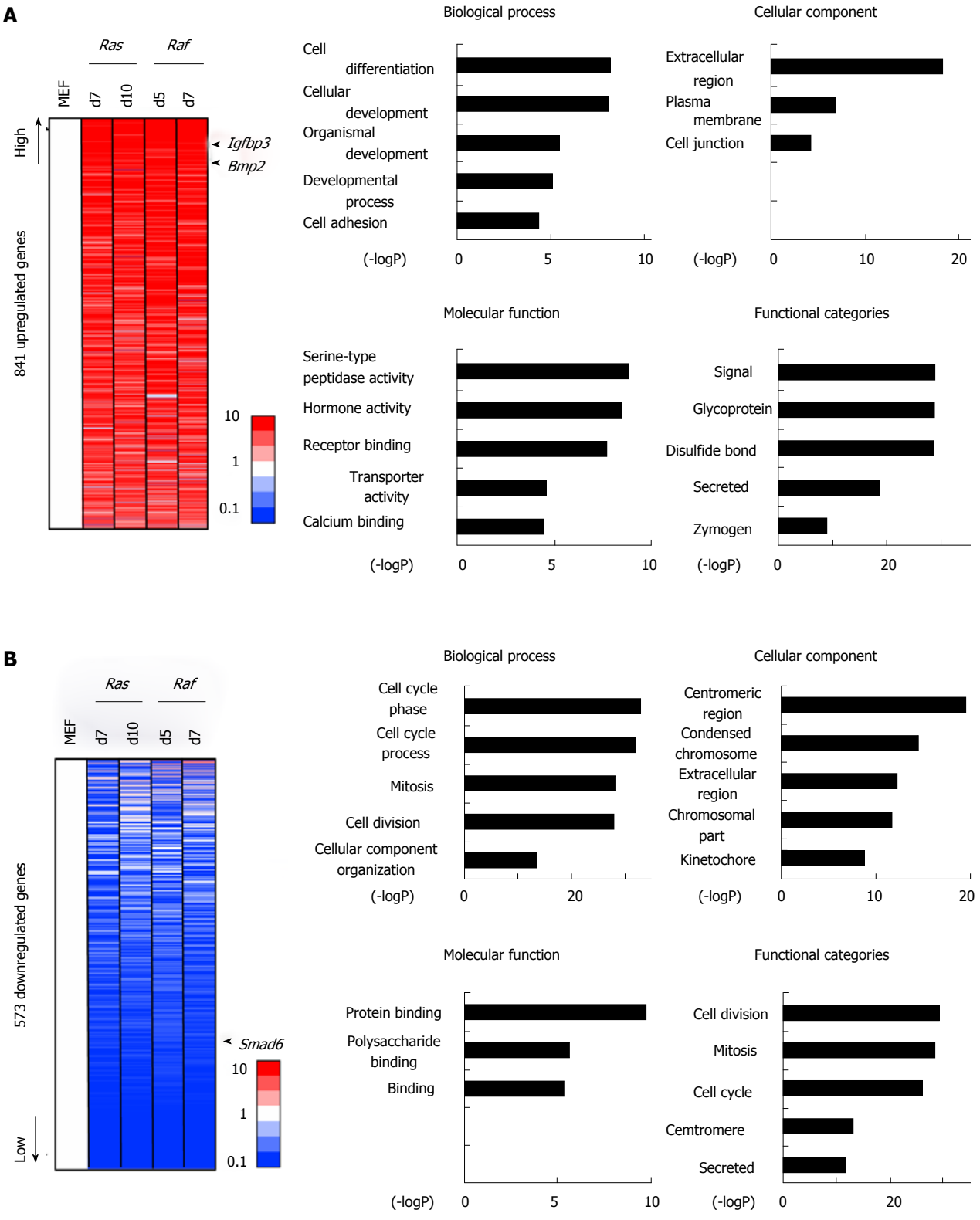


Figure 3 Gene annotation enrichment analysis for commonly altered genes. A: Commonly upregulated genes. A total of 841 genes showed > 5-fold upregulation commonly in either of RasG12V cells on days 7 (*Ras d7*) and 10 (*Ras d10*) and in either of RafV600E cells on days 5 (*Raf d5*) and 7 (*Raf d7*), compared with MEF. More upregulated genes were sorted upward. Gene annotation enrichment was analyzed for biological process, cellular component, molecular function, and functional categories. Genes associated with signal/secreted proteins ($P = 3.8 \times 10^{-30}$), extracellular regions ($P = 5.3 \times 10^{-19}$), and cell differentiation/development ($P = 6.1 \times 10^{-9}$), *e.g.*, *Bmp2* and *Igfbp3*, were upregulated; B: Commonly downregulated genes. A total of 573 genes showed < 0.2-fold downregulation commonly in either of the two RasG12V cells and in either of the two RafV600E cells, compared with MEF. More downregulated genes were sorted downward. Gene annotation enrichment analysis showed that genes related to cell cycle ($P = 1.3 \times 10^{-33}$) such as *Cdc6* were significantly enriched, in good agreement with growth arrest. Genes associated with secreted protein ($P = 2.0 \times 10^{-12}$) and extracellular region ($P = 5.4 \times 10^{-13}$) such as *Cxcl5* and *Wnt4* were also enriched, suggesting that the dynamic activation and repression of secreted factors occurred commonly during *Ras*- and *Raf*-induced senescence. The GO-term "protein binding" also showed significant enrichment, and *Smad6* was included in this term. MEF: Mouse embryonic fibroblast.

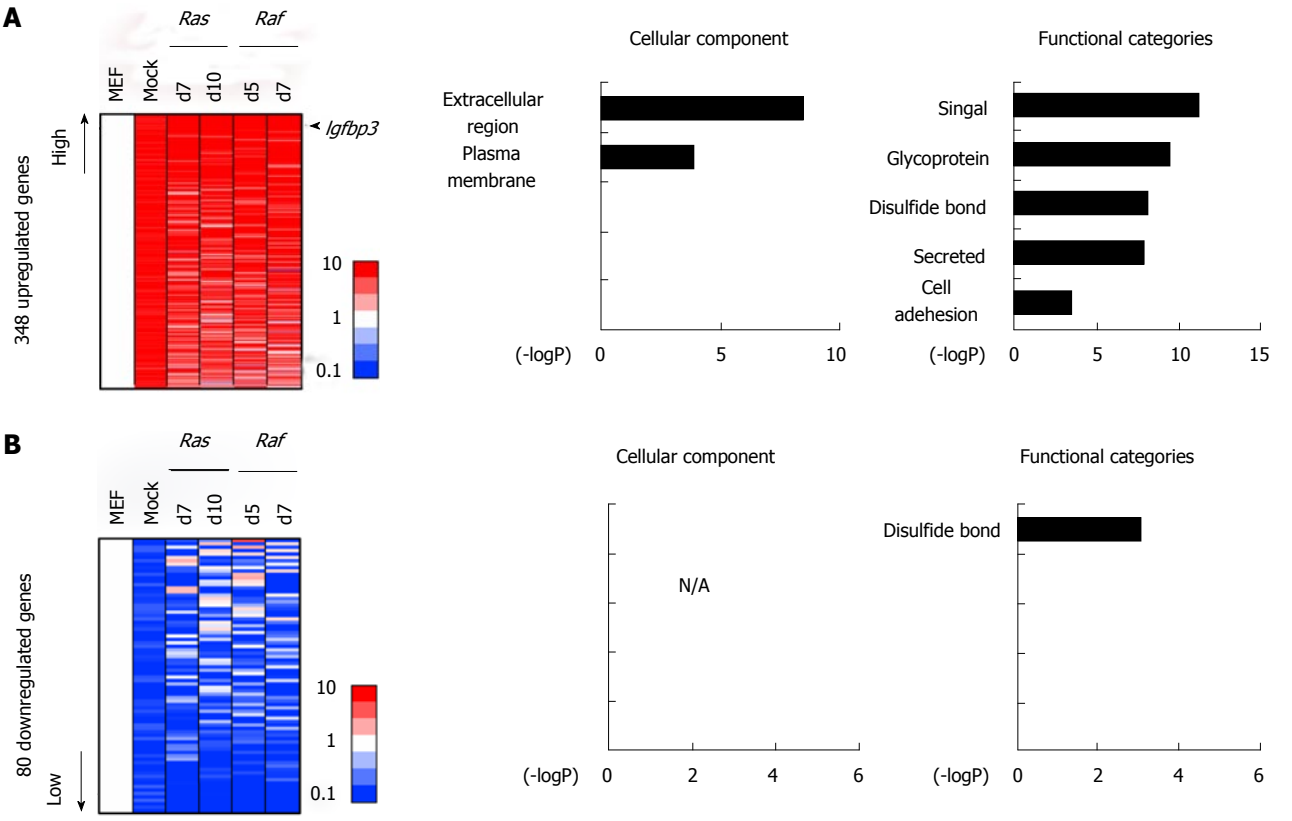


Figure 4 Genes altered commonly in *Ras*-/*Raf*-induced senescence and in mock cells. A: Commonly upregulated genes. There were 348 genes showing > 5-fold upregulation commonly in mock cells, in either of the two RasG12V cells and in either of the two RafV600E cells, compared with MEF. Genes associated with signal/secreted proteins proteins ($P = 6.7 \times 10^{-12}$) or extracellular regions ($P = 3.3 \times 10^{-9}$), *e.g.*, *Igfbp3* and *Cd5l*, were significantly enriched in these genes. B: Commonly downregulated genes. There were 80 genes, *e.g.*, *Cdc6* and *Wnt4*, showing < 0.2-fold downregulation commonly in mock cells, in either of two RasG12V cells and in either of two RafV600E cells, compared with MEF. Genes related to disulfide bond were significantly enriched. MEF: Mouse embryonic fibroblast.

senescence, a retrovirus to express shRNA against *Bmp2* (shBmp2) or retrovirus to express *Smad6* was infected together with RafV600E infection (Figure 7A). When *Bmp2* was knocked down, RafV600E cells showed escape from senescence with number of SA-β-gal(+) cells decreased (Figure 7B and C). *Smad6* expression also caused escape from senescence with number of SA-β-gal(+) cells decreased (Figure 7B and D). These indicated that increase of *Bmp2* and decrease of *Smad6* also play an important role in *Raf*-induced senescence, as well as *Ras*-induced senescence.

In *Ras*-induced senescence, while the *Bmp2* promoter region gained H3K4me3 mark and lost H3K27me3 mark, the *Smad6* promoter region gained H3K27me3 mark and lost H3K4me3 mark in opposite manner. To examine epigenomic alteration of these histone modifications in *Raf*-induced senescence, ChIP analysis for H3K4me3 and H3K27me3 was performed for RafV600E cells (Figure 8). In the *Bmp2* promoter, gain of H3K4me3 and loss of H3K27me3 were detected in *Raf*-induced senescence as well as in *Ras*-induced senescence. In the *Smad6* promoter, however, loss of H3K4me3 was detected in *Raf*-induced senescence while de novo H3K27me3 induction was not detected. These results indicated that decrease of *Smad6* involved loss of H3K4me3, but did not involve PRC2 recruitment to the promoter region, and suggested that epigenetic alteration might be somewhat different between *Ras*- and *Raf*-induced senescence.

Integrated analysis of epigenetic alteration and expression

To compare histone modification alterations between *Ras*- and *Raf*-induced senescence on genome-wide scale, we performed integrated analyses of epigenetic and expression alterations (Figure 9). Among the analyzed 20232 genes with epigenetic alteration, 15653 genes were also analyzed for expression on microarray, and for integrated analyses. For epigenetic status of H3K27me3, 483 genes showed > 1.5 reads per 1000000 reads in MEFp2, but decreased to < 1.0 reads per 1000000 reads in both or either of *Ras*- and *Raf*-induced senescence. Among these 483 genes losing H3K27me3 in senescence, 27 genes in *Ras*-induced senescence and 18 genes in *Raf*-induced senescence showed a simultaneous loss of H3K27me3 and gain of H3K4me3 (with increase from < 3.0 reads in MEFp2 to > 3.5 reads in senescence), and 9 genes, including *Bmp2*, showed simultaneous H3K27me3 loss and H3K4me3 gain in both senescence mechanisms. These genes showed significant enrichment in upregulated genes among the 483 genes ($P = 1 \times 10^{-5}$ in *Ras*-

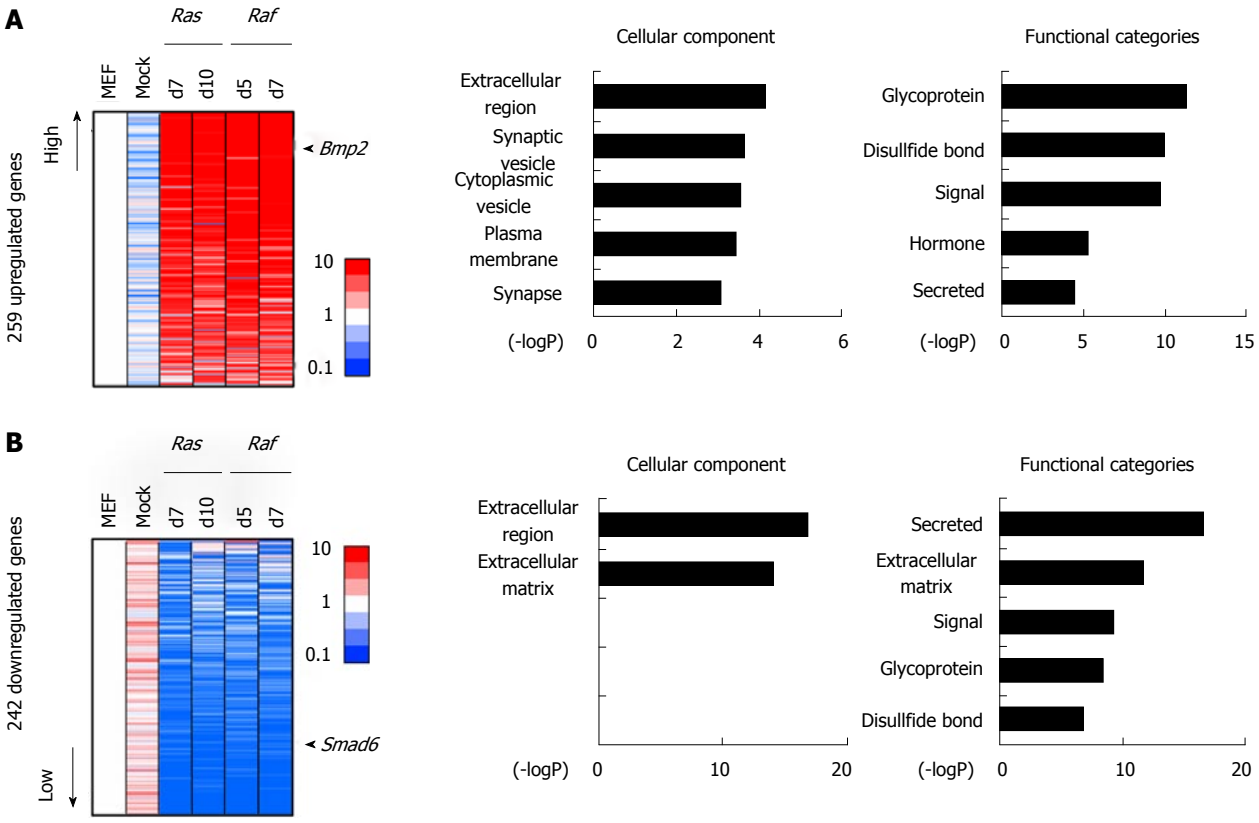


Figure 5 Genes altered commonly in *Ras*-/*Raf*-induced senescence but not in mock cells. A: Genes upregulated commonly, but not in mock cells. There were 259 genes showing > 5-fold upregulation commonly in either of the two RasG12V cells and in either of the two RafV600E cells, but not in mock cells. Genes associated with signal/secreted proteins ($P = 1.8 \times 10^{-10}$) or extracellular regions ($P = 7.0 \times 10^{-5}$), *e.g.*, *Bmp2*, were significantly enriched in these genes; B: Genes downregulated commonly, but not in mock cells. There were 242 genes, *e.g.*, *Smad6*, showing < 0.2-fold downregulation commonly in either of the two RasG12V cells and in either of the two RafV600E cells, but not in mock cells. Genes associated with signal/secreted protein ($P = 2.3 \times 10^{-17}$) or extracellular region ($P = 1.3 \times 10^{-17}$) were significantly enriched, *e.g.*, *Cxcl5*.

induced senescence, $P = 2 \times 10^{-9}$ in *Raf*-induced senescence, and $P = 2 \times 10^{-8}$ in both senescence, Kolmogorov-Smirnov test, Figure 9).

In contrast, 240 genes showed < 1.0 reads per 1000000 reads in MEFp2, but increased to > 1.5 reads per 1000000 reads in both or either *Ras*- and *Raf*-induced senescence (Figure 10A). Among these 240 genes acquiring *de novo* H3K27me3 in senescence, 10 genes in Ras-induced senescence (like *Smad6*) and 7 genes in Raf-induced senescence showed simultaneous gain of H3K27me3 and loss of H3K4me3, and these genes showed significant enrichment in downregulated genes among the 240 genes ($P = 4 \times 10^{-6}$ in *Ras*-induced senescence, $P = 0.02$ in *Raf*-induced senescence, Kolmogorov-Smirnov test, Figure 10A). However, the 10 genes and 7 genes did not overlap at all, including *Smad6*.

Regarding the overlap of H3K27me3 alterations, 171 genes with H3K27me3 loss in *Ras*-induced senescence and 417 genes with H3K27me3 loss in *Raf*-induced senescence showed significant overlap (105 genes, $P < 1 \times 10^{-15}$, phi coefficient = 0.386). However, 185 genes with H3K27me3 gain in *Ras*-induced senescence and 67 genes with H3K27me3 gain in *Raf*-induced senescence showed limited overlap (12 genes only, phi coefficient = 0.103), and the latter overlap in H3K27me3 gain was markedly rare compared with the former overlap in H3K27me3 loss ($P < 1 \times 10^{-15}$, log linear analysis).

Regarding the overlap of H3K4me3 alterations, genes with H3K4me3 gain in *Ras*- and *Raf* induced senescence showed a significant overlap (50 genes, $P < 1 \times 10^{-15}$, phi coefficient = 0.413), and also genes with H3K4me3 loss in *Ras*- and *Raf*-induced senescence showed a significant overlap (380 genes, $P < 1 \times 10^{-15}$, phi coefficient = 0.559) (Figure 10B). The overlap in H3K27me3 gain was again markedly rare, when compared with the overlap in H3K4me3 gain ($P = 2 \times 10^{-9}$, log linear analysis) or with that in H3K4me3 loss ($P < 1 \times 10^{-15}$, log linear analysis).

DISCUSSION

In this study, we examined epigenomic and gene expression alterations during *Raf*-induced senescence, comparing them with alterations during *Ras*-induced senescence. The expression of many genes was commonly altered between *Ras*- and *Raf*- induced senescence, including genes related to secreted proteins and cell cycle. *Bmp2* upregulation and *Smad6* downregulation were observed and played a role in *Raf*-induced

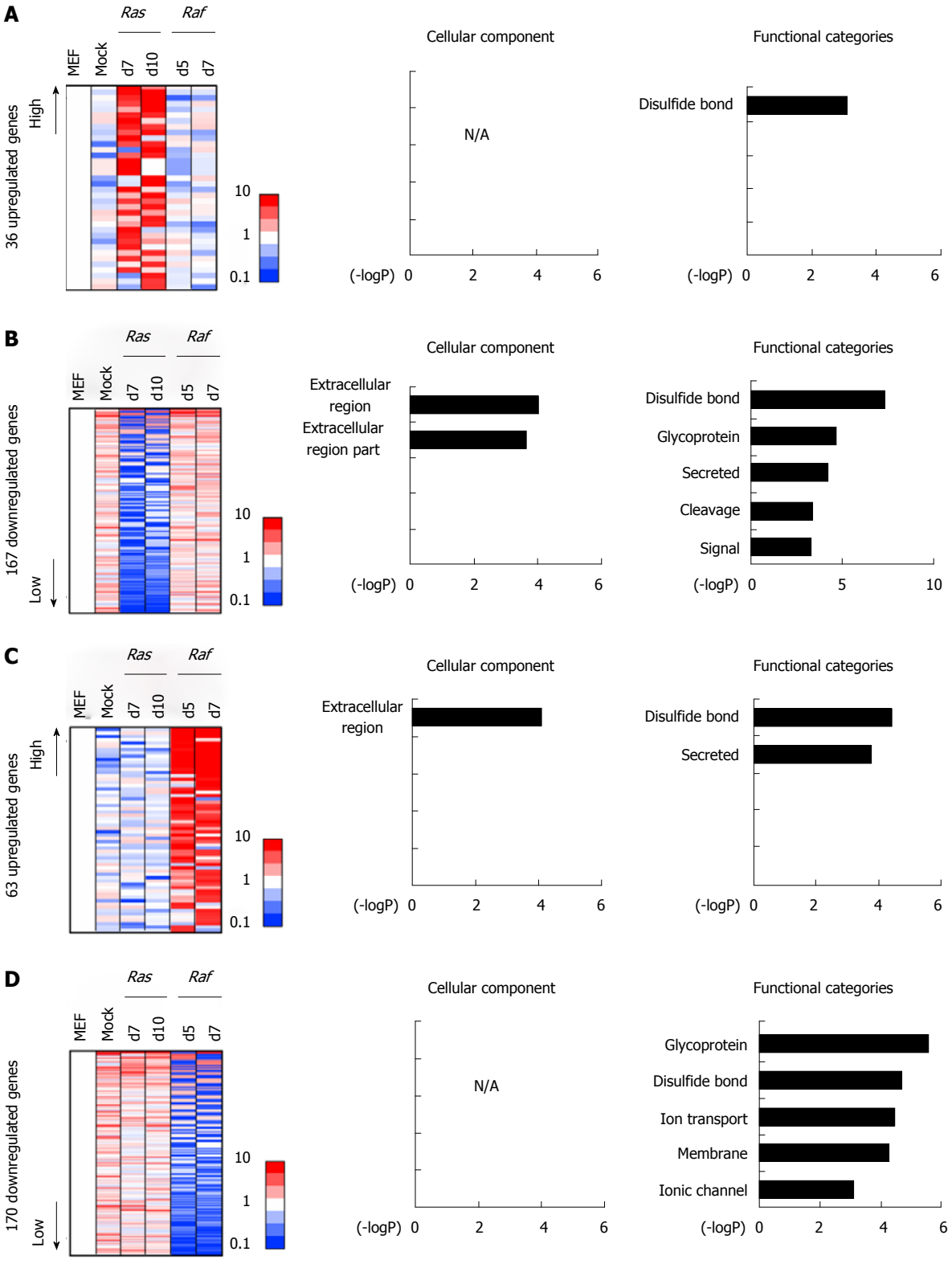


Figure 6 Gene annotation enrichment analysis for specifically altered genes. A: Genes specifically upregulated in *Ras*-induced senescence. A total of 36 genes showed > 5-fold expression in either of the two RasG12V cells, but showed < 1.5-fold expression in both RafV600E cells and in mock cells, compared with MEF cells. Gene annotation enrichment analysis did not reveal any significant enrichment of GO-terms other than "disulfide bond"; B: Genes specifically downregulated in *Ras*-induced senescence. A total of 167 genes showed < 0.2-fold expression in either of the two RasG12V cells, but showed > 0.65-fold expression in both RafV600E cells and in mock cells, compared with MEF cells. Gene annotation enrichment was analyzed, showing a significant enrichment of genes associated with signal/secreted factors, *e.g.*, *Wnt*; C: Genes specifically upregulated in *Raf*-induced senescence. A total of 63 genes showed > 5-fold expression in either of the two RafV600E cells, but showed < 1.5-fold expression in both RasG12V cells and in mock cells, compared with MEF cells. Gene annotation enrichment analysis showed significant enrichment of genes associated with secreted proteins, *e.g.*, *Cd40*; D: Genes specifically downregulated in *Raf*-induced senescence. A total of 170 genes showed < 0.2-fold expression in either of the two RafV600E cells, but showed > 0.65-fold expression in both RasG12V cells and in mock cells, compared with MEF cells. Gene annotation enrichment analysis showed a significant enrichment of genes related to membrane, *e.g.*, *Mcam*. MEF: Mouse embryonic fibroblast.

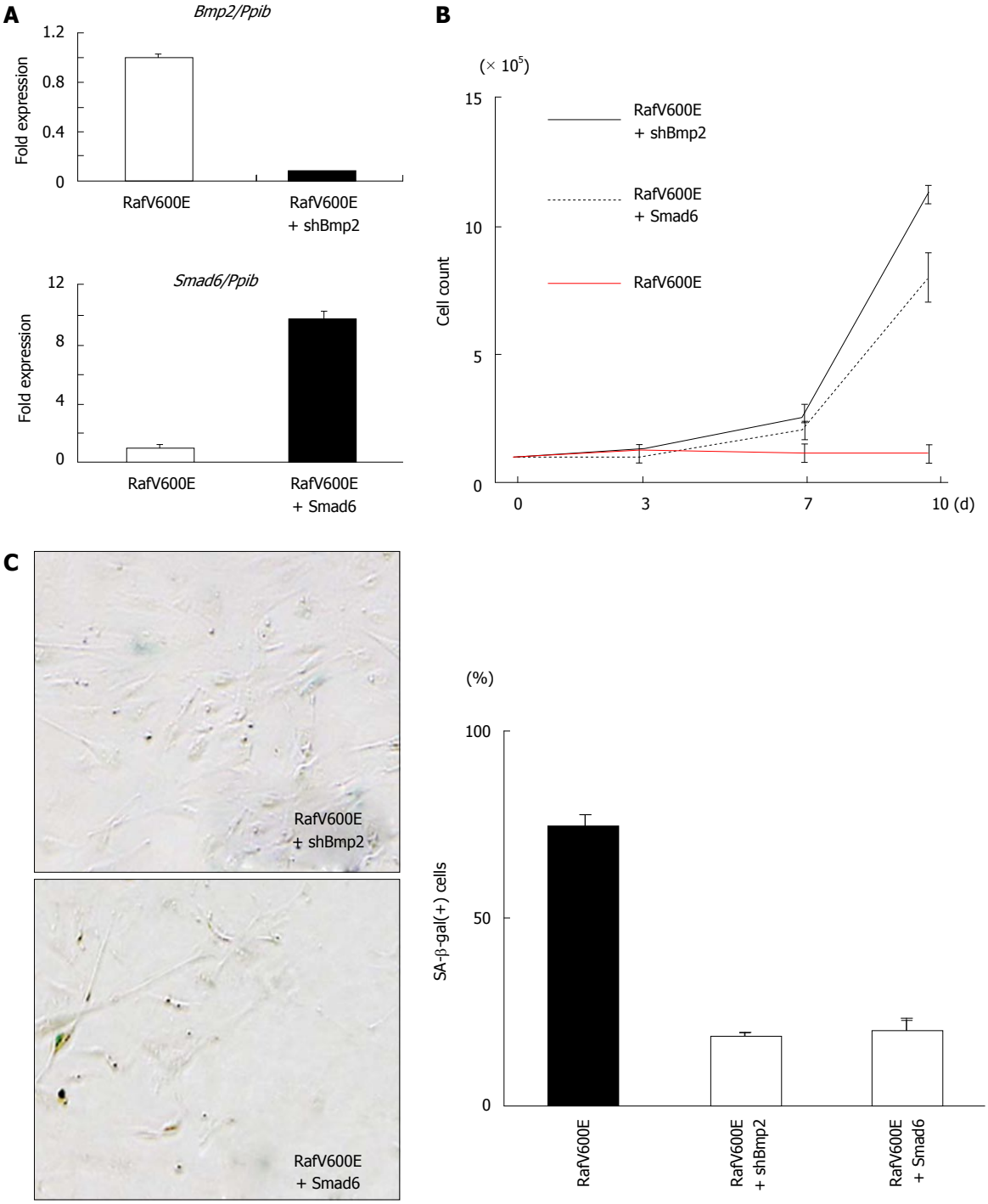


Figure 7 Knockdown of *Bmp2* and overexpression of *Smad6* inhibited growth arrest by *Raf*. A: Real-time-PCR for *Bmp2* and *Smad6*, normalized to *Ppib*. Fold expression levels compared to RafV600E cells are shown. Knockdown of *Bmp2* expression by shRNA and induction of *Smad6* expression by retrovirus infection were confirmed. Mean and standard error in triplicated samples are shown; B: Growth curve of RafV600E + shBmp2 and RafV600E + *Smad6* cells. While RafV600E cells showed growth arrest, *Bmp2*-knocked-down RafV600E cells (RafV600E + shBmp2, black line) and *Smad6*-overexpressed RafV600E cells (RafV600E + Smad6, black dotted line) showed continual growth and escape from *Raf*-induced senescence; C: Count of SA-β-gal(+) cells. Compared with RafV600E cells, *Bmp2*-knocked-down RafV600E cells (RafV600E + shBmp2) and *Smad6*-overexpressed RafV600E cells (RafV600E + Smad6) rarely showed SA-β-gal(+) cells. Mean and standard error in three repeated experiments are shown. SA-β-gal: Senescence-associated β-galactosidase.

senescence as well as *Ras*-induced senescence. But there were clusters of genes showing expression altered specifically in either *Ras*- or *Raf*-induced senescence, and epigenetic alteration might also be somewhat different, *e.g.*, in gain of H3K27me3.

Ras- and *Raf*-induced senescence share several features. SAHF formation was observed in the cellular senescence of human fibroblasts, by both *Ras*- and *Raf*-activation[34,35]. Growth arrest induced by *Ras* and *Raf* is accompanied by accumulation of TP53 and p16, and was reported to be phenotypically indistinguishable from replicative senescence[2,36-38]. Regarding SASP,

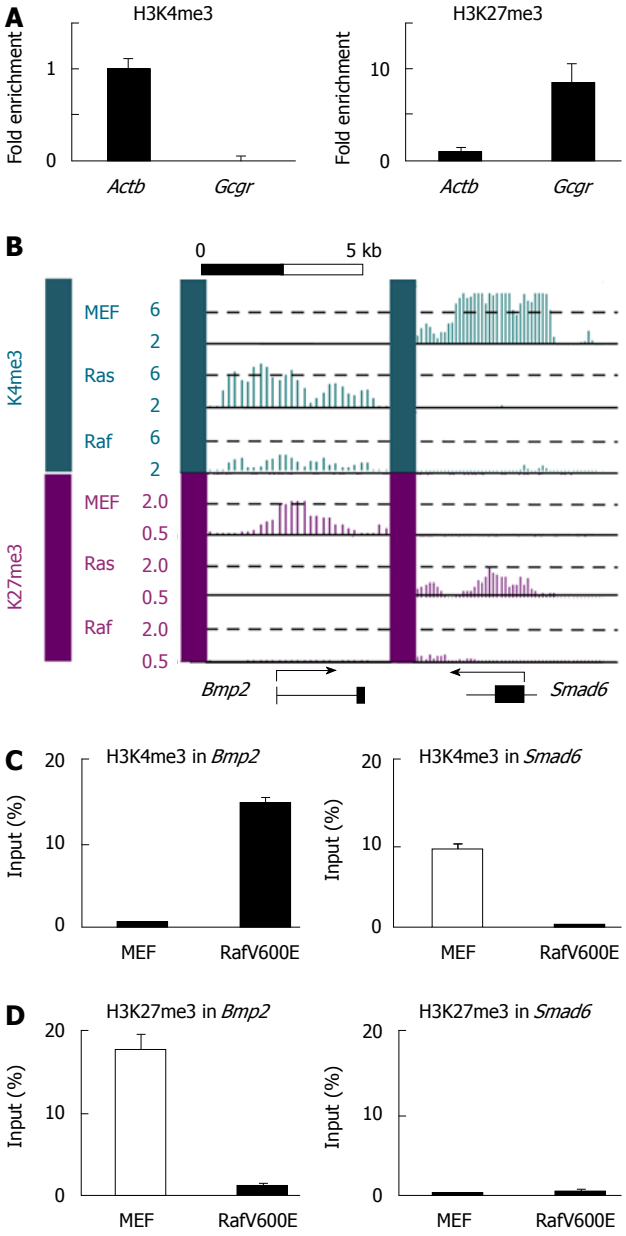


Figure 8 Epigenetic alteration of the *Bmp2* and *Smad6* locus in *Raf*-induced senescence. A: Real-time ChIP-PCR for H3K4me3 and H3K27me3 in RafV600E cells on day 7. Relative enrichment compared with *Actb* was shown, and it was confirmed that ChIP was performed properly. *Actb* was positive control region for H3K4me3 and *Gcgr* was positive control for H3K27me3; B: H3K4me3 and HK27me3 mapped by ChIP-sequencing. Y-axis, the number of mapped reads per 1000000 reads, within a window (300bp for H3K4me3 and 500bp for H3K27me3). The *Bmp2* locus showed H3K27me3 mark, but no H3K4me3 mark, in MEF cells. Loss of H3K27me3 and gain of H3K4me3 were detected commonly in *Ras*- and *Raf*-induced senescence. The *Smad6* locus showed H3K4me3 mark, but no H3K27me3 mark, in MEF cells. Loss of H3K4me3 was detected commonly in *Ras*- and *Raf*-induced senescence, but gain of H3K27me3 was detected specifically in *Ras*-induced senescence; C: Validation of the epigenetic status of the *Bmp2* locus by ChIP-PCR. Loss of H3K27me3 and gain of H3K4me3 in RafV600E cells were confirmed. We repeated ChIP assay twice, and obtained the similar results by ChIP-PCR using those ChIP products; D: Validation of the epigenetic status of the *Smad6* locus by ChIP-PCR. Loss of H3K4me3 and no gain of H3K27me3 in RafV600E cells were confirmed. We repeated ChIP assay twice, and obtained the similar results by ChIP-PCR using those ChIP products. MEF: Mouse embryonic fibroblast.

while cytokines *e.g.,* IL-6 and IL-8 are upregulated and secreted by senescent cells in a paracrine fashion in *Ras*-induced senescence[24], IL-6 and IL-8 are also secreted and play a role in *Raf*-induced senescence[19]. In *Ras*- and *Raf*-induced senescence in primary melanocytes, pigment epithelium-derived factor and Microphthalmia-associated transcription factor were commonly downregulated[39].

In this study, genes related to secreted factors, including *Igfbp3* and *Cd5l*, were commonly upregulated in *Ras*- and *Raf*-induced senescence, and also in mock cells (Figure 4A). In ageing cells, *Igfbp3* and *Cd5l* were reported to be upregulated and contribute to cellular senescence. Genes downregulated commonly in *Ras*- and *Raf*-induced senescence and in mock cells (Figure 4B) also included interesting genes. Cdc6 suppresses $p16^{INK4a}$ function, and the knockdown of Cdc6 induces senescence in cancer cells. Downregulation of *Wnt4* expression and activation of repressor pathways to suppress β-catenin dependent signaling were reported to play a role in the initiation of age-related senescence of thymic epithelial cells[40]. Alteration of these genes may play a role in both *Ras*- and *Raf*-induced senescence.

Some genes showed expression alteration commonly in *Ras*- and *Raf*-induced senescence, but not in mock cells. Sox2 regulates Atg10 expression and induces cellular senescence through autophagy phenomenon[41]. Upregulation of *Bmp2* and downregulation of *Smad6* were found in *Ras*-induced senescence in our previous study, and will be discussed in detail below. Alteration of these factors may play a role in both *Ras*- and *Raf*-induced senescence, but not in replicative senescence.

Although there are not so many genes, genes showing expression changes specifically in either *Ras*- or *Raf*-induced senescence also include interesting genes. *Wnt2* and *Hey1* were downregulated specifically in *Ras*-induced senescence, but not in *Raf*-induced senescence or mock cells (Figure 6). *Wnt2* is repressed in *Ras*-induced senescence, and the knockdown of *Wnt2* increases amount of SAHF and HIRA foci[42]. Targeting *Maml1* in melanoma cells was reported to suppress the downstream transcriptional repressor *Hey1*, resulting in senescence differentiation of melanoma cells, and increased melanin production[43].

Cd40 was upregulated, and *Mcam* and *Nfia* were downregulated specifically in *Raf*-induced senescence, but not in *Ras*-induced senescence or mock cells (Figure 6). *Cd40* was upregulated in senescence by histone deacetylase inhibitors[44]. The knockdown of *Mcam* in human mesenchymal stromal cells caused positive SA-β-gal staining[45]. *Nfia* promoted proliferation of glioma cells, and knockdown of *Nfia* by shRNA inhibited their proliferation[46]. *Raf*-specific alteration of these genes may play a specific role in *Raf*-induced senescence.

Among the genes showing expression alteration commonly or specifically in *Ras*- and *Raf*-induced senescence, we focused on an increase of *Bmp2* and decrease of *Smad6*. These alterations were commonly observed in *Ras*- and *Raf*-induced senescence, but not

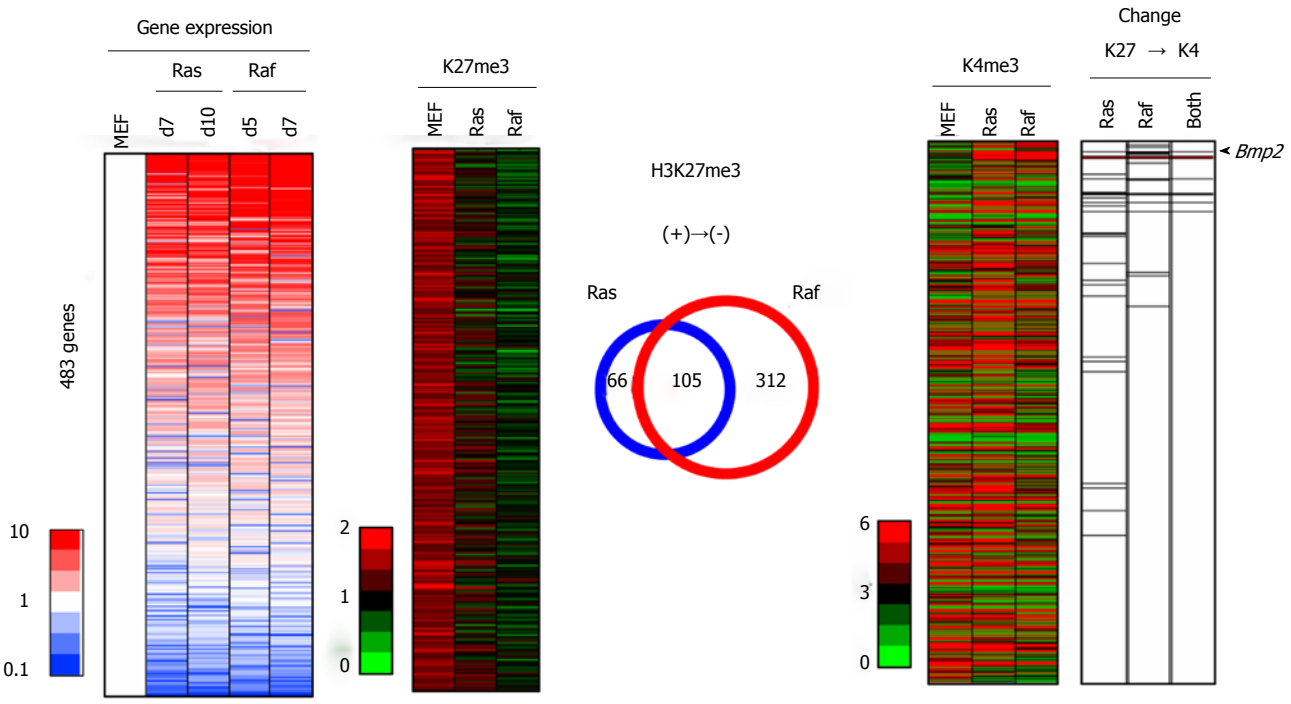


Figure 9 Integrated analysis of epigenetic and expression changes for genes losing H3K27me3. A total of 483 genes showed > 1.5 reads per 1000000 reads in MEF, but decreased to < 1.0 in both or either *Ras*- and *Raf*-induced senescence, and were sorted by the fold expression change between MEF and mean of RasG12V cells and RafV600E cells (left). Among these 483 genes losing H3K27me3 in senescence, 27 genes in *Ras*-induced senescence and 18 genes in *Raf*-induced senescence showed loss of H3K27me3 and gain of H3K4me3 (with increase from < 3.0 reads in MEF to > 3.5 reads in senescence) simultaneously, and 9 genes showed a simultaneous H3K27me3 loss and H3K4me3 gain in both *Ras*- and *Raf*-induced senescence (right). These genes showed a significant enrichment in upregulated genes among the 483 genes ($P = 1 \times 10^{-5}$ in *Ras*-induced senescence, $P = 2 \times 10^{-9}$ in *Raf*-induced senescence, and $P = 2 \times 10^{-8}$ in both senescence, Kolmogorov-Smirnov test), and included *Bmp2* (arrow head). Regarding the overlap of H3K27me3 alterations (center), 171 genes with H3K27me3 loss in Ras-induced senescence and 417 genes with H3K27me3 loss in *Raf*-induced senescence overlapped well (105 genes, $P < 1 \times 10^{-15}$, phi coefficient = 0.386).

in mock cells. In our previous study, the coordinated epigenetic regulation of these changes was found to be critical in *Ras*-induced senescence[22]. In this study, knockdown of *Bmp2* by shRNA and induction of *Smad6* in *Raf*-activated cells showed escape from senescence, indicating that increase of *Bmp2* and decrease of *Smad6* are also important in *Raf*-induced senescence as well as *Ras*-induced senescence. In colorectal cancer, disruption of Bmp2-Smad signaling has been suggested to be important. While the expression of BMP2 was observed in mature colonic epithelium, promoting differentiation and inhibiting proliferation, the expression of BMP2 was lost in adenoma of familial adenomatous polyposis[47]. In sporadic colorectal cancer, BMPR2, BMPR1A, and SMAD4 were frequently inactivated, correlating with loss of Smad1 phosphorylation[48]. The germline mutation of genes in BMP signaling causes Juvenile polyposis syndrome, a hereditary disease with increased risk of colorectal cancer[49,50]. Recently, we performed targeted exon sequencing analysis in colorectal tumors, and *BRAF*-mutation(+) high-methylation colorectal cancer showed the frequent mutation of genes in BMP signaling, *e.g.*, *BMPR2*, *BMP2*, and *SMAD4*[33]. Disruption of Bmp2-Smad signaling by mutation may play an important role in escaping from *Raf*-induced senescence and establish *Raf*-mutation(+) cancer.

The epigenetic alterations to regulate these upregulated and downregulated genes were mostly similar. Gain and loss of H3K4me3, and loss of H3K27me3 showed a significant overlap between *Ras*- and *Raf*-induced senescence. These epigenetic changes in *Bmp2* and *Smad6* promoter regions were commonly observed in *Ras*- and *Raf*-induced senescence. However, gain of H3K27me3 was quite different between *Ras*- and *Raf*-induced senescence on genome-wide scale. For *Smad6*, it was repressed by loss of H3K4me3 and gain of H3K27me3 in *Ras*-induced senescence, but by only loss of H3K4me3 without gain of H3K27me3 in *Raf*-induced senescence.

Although the mechanism to program epigenetic regulations is largely unknown, non-coding RNA might be involved[51,52]. PRC2 is known to be recruited to target genes in *trans* through an association with HOTAIR, a non-coding RNA at the *HOXC* locus[53]. Antisense non-coding RNA in the INK4 locus, ANRIL, binds to PRC1 and PRC2, and represses genes within INK4b/ARF/INK4a locus in *cis*, but ANRIL was downregulated when *Ras* was activated and these genes were upregulated[54,55]. It might be interesting to analyze whether PRC is recruited to the *Smad6* or *Bmp2* region by any non-coding RNAs in *cis* or *trans*, and the similarities and differences in epigenetic alterations in these gene regions during *Ras*- and *Raf*-induced senescence is caused by an expression

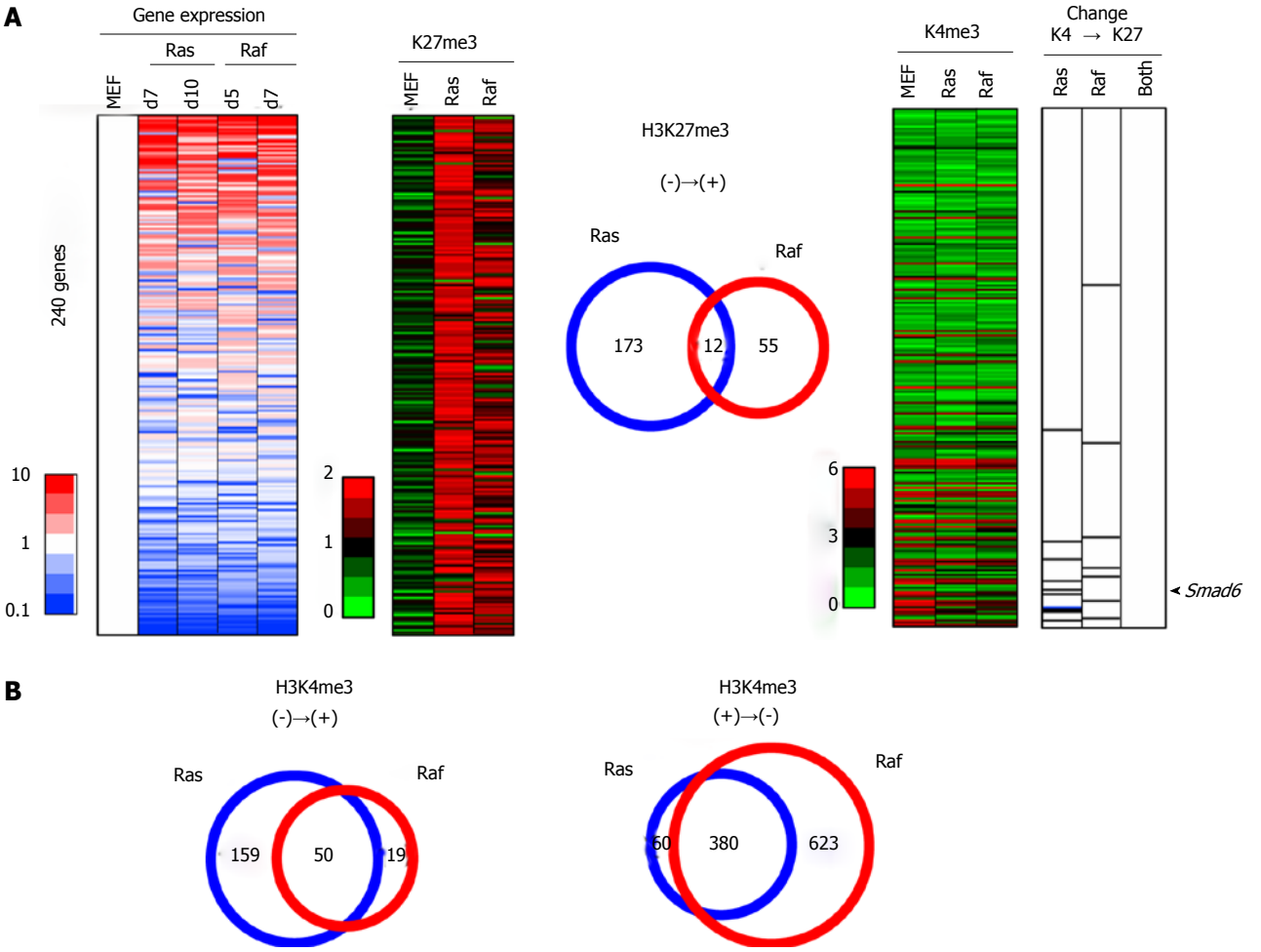


Figure 10 Integrated analysis of epigenetic and expression changes for genes acquiring H3K27me3. A: A total of 240 genes showed < 1.0 reads per 1000000 reads in MEF, but increased to > 1.5 in both or either of *Ras*- and *Raf*-induced senescence, and were sorted by the fold expression change between MEF and mean of RasG12V cells and RafV600E cells (left). Among these 240 genes acquiring de novo H3K27me3 in senescence, 10 genes in *Ras*-induced senescence and 7 genes in Raf-induced senescence showed a simultaneous gain of H3K27me3 and loss of H3K4me3 (right). These genes showed a significant enrichment in downregulated genes among the 240 genes ($P = 4 \times 10^{-6}$ in *Ras*-induced senescence, and $P = 0.02$ in *Raf*-induced senescence, Kolmogorov-Smirnov test). However, these 10 genes, *e.g.*, *Smad6* (arrow head), and 7 genes did not overlap at all. Regarding the overlap of H3K27me3 alterations (center), 185 genes with H3K27me3 gain in *Ras*-induced senescence and 67 genes with H3K27me3 gain in *Raf*-induced senescence showed limited overlap (12 genes only, phi coefficient = 0.103), and this overlap in H3K27me3 gain was markedly rare compared with the overlap in H3K27me3 loss (Figure 9) ($P < 1 \times 10^{-15}$, log linear analysis); B: Overlap of H3K4me3 alterations. Genes with H3K4me3 gain in *Ras*- and *Raf* induced senescence overlapped well (50 genes, $P < 1 \times 10^{-15}$, phi coefficient = 0.413) (left), and also genes with H3K4me3 loss in *Ras*- and *Raf*-induced senescence overlapped well (380 genes, $P < 1 \times 10^{-15}$, phi coefficient = 0.559) (right). The overlap in H3K27me3 gain (Figure 10A) was again markedly rare, compared with the overlap in H3K4me3 gain ($P = 2 \times 10^{-9}$, log linear analysis) or with that in H3K4me3 loss ($P < 1 \times 10^{-15}$, log linear analysis).

alteration of non-coding RNAs.

While the epigenetic regulation of the *Smad6* locus was somewhat different between *Ras*- and *Raf*-induced senescence, some other genes showing expression changes specifically in either of *Ras*- and *Raf*-induced senescence, were accompanied by different epigenomic alterations. *Nfia* was downregulated specifically in *Raf*-induced senescence, with H3K4me3 lost specifically in *Raf*-induced senescence. The difference in epigenetic alteration may cause difference in gene expression patterns, at least in part, and may result in difference in genesis for senescence.

In summary, while gene expression changes are mostly similar between *Ras*- and *Raf*-induced senescence, the upregulation of *Bmp2* and repression of *Smad6* were also important in *Raf*-induced senescence, and epigenetic alterations might be somewhat different, *e.g.,* in gain of H3K27me3 marks.

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COMMENTS

Background

When an oncogene is activated, cells lead to cellular senescence, *i.e.*, irreversible growth arrest as a barrier against malignant transformation. In the authors previous studies, the authors performed a comprehensive analysis of aberrant DNA methylation in colorectal cancer, and reported three subtypes of

colorectal cancer with distinct DNA methylation epigenotypes. Different patterns of aberrant methylation accumulation significantly correlated with distinct oncogene mutations, suggesting that the accumulation of aberrant methylation might be a requested phenotype for oncogenic transformation by inactivation of critical factors for oncogene-induced senescence. To gain insight into critical gene inactivation in oncogene-mutation(+) cancer, the authors previously analyzed epigenomic and gene expression alterations in *Ras*-induced senescence in normal cells, and found a critical role of Bmp2-Smad signaling in *Ras*-induced senescence and its regulation by coordinated epigenetic alteration.

Research frontiers

The author aimed to investigate critical genes in *Raf*-induced senescence, and any difference between *Ras*- and *Raf*-induced senescence, to gain insight into critical gene inactivation in *Ras*-mutation(+) and *Raf*-mutation(+) colorectal cancer.

Innovations and breakthroughs

This study demonstrates that while some genes including those related to secreted factors showed expression changes specifically in *Ras*- or *Raf*-induced senescence, expression of many genes was commonly altered in *Ras*- and *Raf*-induced senescence. These changes include an increase of *Bmp2* and decrease of *Smad6*, and play a role in *Raf*-induced senescence as well as *Ras*-induced senescence. Epigenetic changes are also similar, but somewhat different, *e.g.,* gain of H3K27me3 in the *Smad6* promoter region.

Applications

The authors hypothesize that critical gene expression changes in oncogene-induced senescence should be disrupted in oncogene-mutation(+) cancer, to escape from growth arrest. DNA methylation and target exon sequencing are under analysis for colorectal cancer. An integrated analysis of these data will reveal different genesis of colorectal cancer with mutation of different oncogene.

Terminology

Comprehensive genomic and epigenomic data can stratify cancer into several subtypes. Colorectal cancer is stratified into *BRAF*-mutation(+) cancer, *KRAS*-mutation(+) cancer, and oncogene-mutation(-) cancer. These subgroups show different tumor morphologies, and accompany different genetic and epigenetic alterations, suggesting that these subgroups of cancer may arise through different tumorigenic mechanisms.

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In this study, the authors examined the expression changes induced by oncogenic RAF. Previously, the authors have conducted similar study on oncogenic Ras.

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REVIEW

High mobility group protein 1: A collaborator in nucleosome dynamics and estrogen-responsive gene expression

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Abstract

High mobility group protein 1 (HMGB1) is a multifunctional protein that interacts with DNA and chromatin to influence the regulation of transcription, DNA replication and repair and recombination. We show that HMGB1 alters the structure and stability of the canonical nucleosome (N) in a nonenzymatic, adenosine triphosphate-independent manner. As a result, the canonical nucleosome is converted to two stable, physically distinct nucleosome conformers. Although estrogen receptor (ER) does not bind to its consensus estrogen response element within a nucleosome, HMGB1 restructures the nucleosome to facilitate strong ER binding. The isolated HMGB1-restructured nucleosomes (N′ and N″) remain stable and exhibit a number of characteristics that are distinctly different from the canonical nucleosome. These findings complement previous studies that showed (1) HMGB1 stimulates *in vivo* transcriptional activation at estrogen response elements and (2) knock down of HMGB1 expression by siRNA precipitously reduced transcriptional activation. The findings indicate that a major facet of the mechanism of HMGB1 action involves a restructuring of aspects of the nucleosome that appear to relax structural constraints within the nucleosome. The findings are extended to reveal the differences between ER and the other steroid hormone receptors. A working proposal outlines mechanisms that highlight the multiple facets that HMGB1 may utilize in restructuring the nucleosome.

Key words: Nucleosome dynamics; Estrogen receptor; High mobility group protein 1; Conformational dynamics; Energy landscape

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Core tip: Response elements or target sites for transcription factors in DNA are often found veiled within a nucleosome in a chromatin milieu and in many cases are not accessible. Although the nucleosome/chromatin network is generally repressive to transcription, there are now a number of enzymatic strategies that have now been recognized that remodel the nucleosome to facilitate transcription factor access. We recently showed that estrogen receptor does not bind to a canonical nucleosome. However, we have discovered that high mobility group protein 1 (HMGB1) restructures the nucleosome in a nonenzymatic manner to facilitate

strong estrogen receptor binding. This review will provide background for this work and outline our findings, characterize the HMGB1-restructured nucleosomes and propose a working model to account for the HMGB1 restructuring activity.

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INTRODUCTION

Life is dynamic and ever changing by its very nature. We sense immediate changes in our environment and react to the challenges to best deal with them with the resources available to us. And so it is also within the trillions of individual cells that make up our bodies and the communication network that coordinates these efforts. As diverse extracellular conditions and challenges fluctuate endlessly, the cells sense and integrate these signals to react to and regulate the expression of the appropriate genes to address these diverse conditions. Herein lies the heart of cellular phenotypic plasticity[1-5].

Hormones released from an endocrine gland provide an excellent example of molecular signals that can impinge on the dynamic character within the cell and result in extraordinary changes in cellular activities. The lipophilic steroid hormones are a subgroup of the nuclear hormone superfamily that includes estrogens, androgens, progestins, glucocorticoids, and mineralocorticoids[6].

The action by estrogen on its ligand activated receptor, the estrogen receptor (ER), and the role of high mobility group protein 1 (HMGB1) in the subsequent transcriptional activation is the focus of this minireview. ER plays a dominant role in a number of different tissues and in a host of physiological and pathophysiological activities that include sexual development, atherosclerosis, osteoporosis, dementia and cancer, to mention just a few of its major influences. The effects of estrogen can be derived from two avenues of action: (1) non-genomic, associated with signal transduction networks and is independent of transcription; and (2) genomic, in which the action occurs on the genome at the transcriptional level[7-9].

BASIC VIEW OF EUKARYOTIC TRANSCRIPTION

Eukaryotic transcription of protein-coding genes is carried out by RNA polymerase Ⅱ (RNA pol Ⅱ) in multiple steps. A sequence-specific activator or transcription factor (TF) binds to its response element (upstream promoter or enhancer elements). Upon TF binding, coregulators are recruited to the site and this complex must then communicate with the pre-initiation complex (PIC) at the proximal promoter. The complex at this site is made up of RNA pol Ⅱ, the Mediator complex and a collection of general transcription factors that include TFIIA, TFIIB, TFIID [TATA binding protein (TBP) and associated TAFs], TFIIE, TFIIF and TFIIH to initiate transcription[10]. The enormous Mediator complex appears to be a global participant in this communication network, with its most distinguishing characteristic being the central "hub" in regulating most, if not all RNA pol Ⅱ transcription. Mediator communicates between a spectrum of different sequence-specific transcription factors (bound at upstream promoters or enhancer elements) and the PIC. Thus, it is not surprising that its subunit composition is variable since it must display conformational, structural and functional flexibility, all of which is consistent with the demands that it function for different genes, different physiological conditions and in different cell types[11-13].

However, this process is more complex in the context of the cell since the DNA is complexed within a nucleosome/chromatin network and many of the response elements are inaccessible to TFs as a result.

CHROMATIN BASICS

Chromatin presents a formidable barrier for all manipulations on the genetic material - be it DNA replication, DNA repair, transcription or genetic recombination. The first level of DNA packaging is the nucleosome in which 147 bps of DNA makes approximately 1 ¾ left-handed superhelical turns around the outside surface of an octamer of core histone proteins, which includes two copies each of four highly conserved core histones - H3, H4, H2A and H2B. The histone octamer binds the DNA at 14 superhelical locations, making greater than 120 direct contacts with the histone interactions. These contacts are exclusively in the minor groove with the phosphate groups of the DNA backbone, with few, if any, contacts to the base[14,15]. The DNA structure differs considerably from the classical B form DNA in that it is highly bent, with regions of DNA that are over- or under-wound, which may affect the successful binding of many transcription factors to their cognate sites in nucleosomal DNA[16-20]. Adjacent nucleosome cores are interconnected by an additional length of DNA (about 20-80 bps of linker DNA) that are less associated with the histone octamer, which together make up the complete repeating nucleosome structural unit.

The N-terminal tails of the core histones, which make up about 30% of the proteins, extend out from the core nucleosome and are considered unstructured and mobile since they have not been resolved in the crystal structure of the nucleosome[21,22]. The positively charged residues in the tails have been implicated in interacting with DNA, are subject to post-

translational modifications, the status of which can influence the higher-order chromatin structure and clearly participate in regulating gene expression[23]. Of the many post-translational modifications and their role in the epigenetic marking system, acetylation of the tails appears important here since, at least some of its effects, may have parallels and implication on a potential role for HMGB1 in altering histone-DNA interactions[24]. Nucleosomes can then cluster together to form a secondary level of chromatin structure, the 30 nm fiber, that is a tight compaction of nucleosomes and is stabilized by the core histone tails and the linker histone H1, which binds to linker DNA. Studies show that removal of the core histone tails inhibits compaction and opens up chromatin structure, even in the presence of the linker histone H1[25].

The 30 nm fiber can be further condensed into a more highly compacted state and is generally inaccessible to active processes on DNA[26,27].

NUCLEOSOME DYNAMICS AND TRANSCRIPTIONAL ACTIVITY

The nucleosome, even though an enormous structure (250 kDa), is inherently a stable structure due to the constraints from multiple weak histone-DNA interactions. Not withstanding this fundamental stability, the structure of the nucleosome is continuously manipulated to accommodate changes for "local" gene expression and other DNA transactions. For our purposes, because selective gene expression within the cell is both temporal and dynamic, the structure and/or the position of the nucleosome within the chromatin milieu are likewise dynamic.

Key elements in the control of gene expression are regulating the access and binding affinity of transcription factors to their response elements and the successful assembly of multifunctional complexes to link the activation event to the transcriptional machinery at the promoter. Response elements in the genome can be regarded as interacting on the surface of the nucleosome with the histone octamer, in which the sequences are either accessible or inaccessible, or alternatively, they may reside in a nucleosome-free region[16]. Notably, if the sites are inaccessible, eukaryotes have evolved a number of strategies to regulate its dynamic nature in selected genomic stretches to facilitate factors to gain access to them, with the focus here on the action of two major types of complexes and their activities[23,28].

The first class of proteins includes a collection of histone modifying enzymes that post-translationally recognize and reversibly modify specific residues in the histone tails that extend on the outside surface of the nucleosome. These enzymes covalently add or remove small groups (referred to as writing, reading and erasing the message) including acetyl, phosphate and other groups. One subset of such enzymes is the histone acetyltransferase that adds acetyl groups to selected lysines in histones H3 and H4, with such modified nucleosomes being generally correlated with transcriptional activation. On the other hand, the removal of these acetyl groups by histone deacetylases is correlated with transcriptionally inactive regions of chromatin. The distinctive combination of these directed chromatin "marks" leads a key role in defining the transcriptional activity of a gene since these modifications on specific nucleosomes help direct the transcriptional activity or program at selected genes in a "sea" of thousands of other genes within the vast genome. The nature of these progressive histone modifications has been called the "histone code hypothesis". These "marks" are thought to not only influence the dynamic nature of the nucleosome, but also act as epitopes for the recruitment of additional regulatory proteins[24,29-31]. These inheritable changes in gene function, epigenetic markers, are not associated with alterations in the gene sequence. So, even at this first level of nucleosome structure, there is clearly a heterogeneous family of nucleosome states[32].

The second class of complexes affecting nucleosome dynamics is the ATP-dependent chromatin remodeling complexes (CRCs). They represent multisubunit complexes that contain a core ATPase catalytic subunit that is an energetic engine for altering the nature of the nucleosome. Depending on which of the five families of CRCs, this activity can either alter the position of the nucleosome or lead to nucleosome instability by a number of routes. In some cases, regions may require the collaborative effect of more than one chromatin remodelers to regulate TF accessibility[33-35]. It has become increasingly evident that different remodeling complexes generate different remodeled substrates that depend on the DNA-dependent ATPase present in the complex and perhaps the model system used to study the activities[36-38].

In addition to these enzymatic CRCs, different multisubunit complexes found in human and *S. cerevisiae* cells, referred to as facilitates chromatin transcription (FACT), interact with nucleosomes, reorganize them in an ATP-independent manner and therefore utilize a nonenzymatic mechanism of action that differs from the ATP-dependent CRCs. The FACT complex contains an HMG box subunit that is essential for activity and both *in vitro* and *in vivo* studies reinforce the hypothesis that FACT destabilizes nucleosomes without altering its position by disrupting interactions between DNA and the H2A/H2B dimers. This prevents histone eviction during this process and promotes nucleosome recovery afterwards[39-42].

ESTROGEN RECEPTOR

The ER (classified as NR3A1), like all the nuclear hormone receptors, is a ligand-activated transcription factor[43]. The NHRs have a common modular domain structure that includes the N-terminal transactivation domain, a well-characterized DNA binding domain

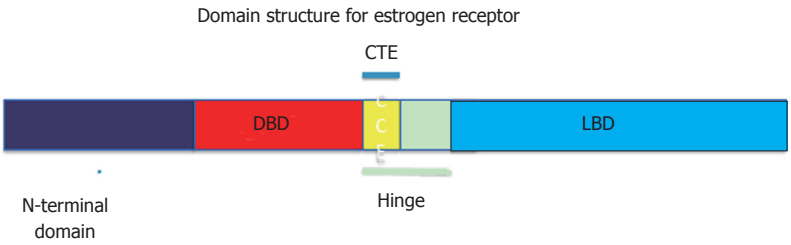


Figure 1 Domain structure for estrogen receptor. The structural and functional domains include the N-terminal domain, DBD, CTE, the flexible hinge region and LBD. DBD: DNA binding domain; CTE: C-terminal extension; LBD: Ligand binding domain.

(DBD), followed immediately by a stretch of amino acids referred to as the C-terminal extension (CTE), which extends into the hinge region, with the ligand binding domain at the C-terminal end, at which the hormone and other agonist and antagonists may bind. The CTE was shown to interact with HMGB1 and is thought to provide additional stability to the ER/consensus estrogen response element (cERE) interaction[44,45] (Figure 1).

Upon estrogen binding to ER within the cell, the receptor dimerizes to a functional homodimer and binds to the canonical 15 bp cERE (cERE = AGGTCAnnn-TGACCT). Each ER monomer unit uses its DBD to make direct contacts in the major groove to each of the 6 bp consensus ERE half-sites (cHERE) that are separated by 3 unspecified bps. The bound receptor then recruits coactivators that then communicate with the PIC to initiate the subsequent steps in the transcriptional process.

Although the ER dimer does not stably bind to a single cERE half-site, the presence of HMGB1 facilitates strong binding to a half-sites (cHERE), in addition to binding to direct repeats and everted repeats, which have been shown to functionally influence specific estrogen-responsive genes[46-48].

It has been shown that the rotational and translational positioning of a response element in nucleosomal DNA is crucial in determining the level at which the factor gains access to the site[17,49-51]. Within the human mammalian cell line, MCF-7, the chromatin structure of the estrogen-responsive pS2 promoter was mapped to single nucleotide resolution. This included the ERE (in nucleosome E) and the TATA box (nucleosome T), which are each rotationally phased and translationally positioned at the edge of the nucleosome. The rotational phasing showed that the major groove of the ERE was facing outward from the histone octamer and suggested that the ERE was probably accessible to ER binding since there was no nucleosome displacement, even upon maximum transcriptional activation[52].

HMGB1 (ALSO RECOGNIZED AS AMPHOTERIN) AND MELANOMA-ASSOCIATED FACTOR

HMGB1 appears to be multifunctional - a protein "for all seasons". It is involved in replication, DNA repair, transcription and recombination within the nucleus, while acting outside the cell as a cytokine and proinflammatory mediator[53-62]. As a result, HMGB1 is associated with many aspects of health and disease[63-66].

HMGB1 is a small (about 25 kDa), highly conserved, ubiquitous and abundant nuclear protein that binds in a non-sequence specific manner in the minor groove of DNA. It is considered an "architectural" protein because HMGB1 binding facilitates the assembly of a wide variety of nucleoprotein complexes as noted above[67].

From both a structural and functional perspective, HMGB1 is best described as having three modular domains. It contains tandem HMG boxes, the N-terminal A-box and the central B-box, both having 80-90 residues and are highly basic in nature. These boxes bind to B-form DNA to produce large bends as large as 90 degrees, but bind preferentially to bent, kinked or underwound DNA (Figure 2). The HMGB1 interactions and the bending interactions are dynamic and result in an increase in flexibility or flexure in the DNA that permits DNA to accommodate a broader spectrum of protein interaction in nucleoprotein structures[68,69]. In this regard, HMGB1 has been shown to enhance the binding of a number of basal and regulatory transcription factors to their cognate DNA binding sites, including the human TBP, p53, p73, HoxD9, Oct 1 and 2, nuclear factor kappa beta, sterol regulatory element-binding protein-1, c-Rel proteins, the Epstein Barr viral activator, ZEBRA, the steroid hormone receptors. In many cases, the interaction of HMGB1 with these "HMGB1-sensitive transcription factors" influences the activation of transcription[37,46-48,70-79].

The C-terminal domain of HMGB1 is composed completely of about 30 acidic residues that modulates the interactions with DNA and interacts directly with TBP, the core histones and plays a role in transcriptional activation[67,68,70,80-83]. Both *in vitro* and *in vivo* evidence shows that HMGB1 binds to the histone H3. The *in vitro* study showed that the interaction was between the C-terminal domain of HMGB1 and the H3 tail. It was proposed that this interaction may serve to localize HMGB1 binding near the nucleosome dyad, where the HMGB1 boxes would interact with the DNA to induce further bending and untwisting of the DNA, destabilizing the nucleosome and increasing accessibility[83-85]. In addition, HMGB1 was shown to increase the kinetics of

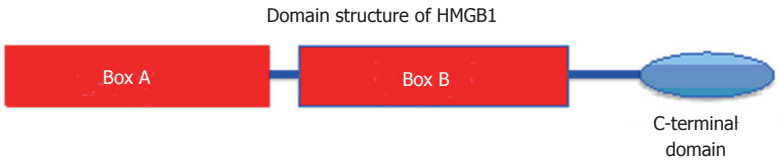


Figure 2 The structural and functional domains of high mobility group protein 1 protein. The tripartite structure includes the A and B boxes which are basic and the acidic C-terminal domain made up entirely of acidic (asp/glu) residues. HMGB1: High mobility group protein 1.

the ATP-dependent CRC, ACF/CHRAC, which facilitates nucleosome sliding. The acidic C-terminal domain of HMGB1 was shown to be required for this *in vitro* activity[86].

Numerous reports find that the binding of histone H1 and HMGB1 compete *in vitro* with one another at the entry point of the nucleosome or at linker DNA. Both proteins influence nucleosome dynamics, but in opposite ways. Histone H1 decreases nucleosome accessibility, while HMGB1 decrease compactness and is thought to increase accessibility[70,80,87].

The most detailed model for the steps in estrogen-responsive transcriptional activation that clearly implicates a role for HMGB1 comes from a study that focused on the pS2 promoter in MCF-7 cell. Initial ERE binding by the estrogen-bound ER leads to the recruitment of a TopoIIβ/PARP-1 complex [PARP-1 = poly(adenosine diphosphate-ribose) polymerase-1] to the promoter, which produces a transient, site specific ds DNA cleavage by TopoIIβ. This results in activation of PARP-1 enzymatic activity, which drives the binding of HMGB1/2, with the concomitant release of the previously bound histone H1 and a resulting change in the local chromatin conformation. Importantly, HMGB1 has been identified as an essential component in the transcriptional activation at the pS2 promoter. Further experiments suggested that this mechanism for transcriptional activation may well apply more generally and certainly to other nuclear hormone receptors, including the androgen receptor (AR), retinoic acid receptor (RAR) and thyroid receptor (T3R)[88,89].

Interestingly, for the first time, histone H1 and HMGB1 were found in the novel situation of simultaneously residing on the tumor necrosis factor alpha (TNF-α) promoter to produce endotoxin-mediated silencing of proinflammatory TNF-α activity[90].

Collectively, it is clear that in eukaryotic transcription, HMGB1 interacts with TBP and competes with TFIIA and TFIIB in the PIC at the promoter, enhances regulatory factors binding at their response elements, increases CRC (ACF/CHRAC) activity and stimulates transcriptional activation[36,46-48,67,68,70-83,88-92].

CURRENT FINDINGS: ER BINDING TO RESPONSE ELEMENTS IN DNA

ER binding to the cERE in DNA exhibited a Kd about 10 nmol/L, while the Kd for ER binding in the presence of 400 nmol/L HMGB1 was about 4 nmol/L, increasing ER binding by approximately 2-3-fold. On the other hand, ER does not bind to a single half-site (cHERE) of cERE. However, the presence of HMGB1 facilitates strong ER binding to a single half-site, with ER essentially having the same binding affinity as observed when ER binds to cERE in the absence of HMGB1. In addition, ER binds to a spectrum of noncanonical binding sites including direct repeats, everted repeats and inverted repeats of the cHERE with a variety of spacer DNA between the half-sites. The presence of HMGB1 further enhances ER binding[46,47].

ER BINDING AND TRANSCRIPTIONAL REPORTER ASSAYS FOR ERES WITHIN U2OS CELLS

It was of interest to evaluate the extent to which cERE and these *in vitro* noncanonical ER binding sites could actually serve as functional EREs and drive transcription within a cell. Using a subset of these binding sites described above - canonical cHERE and cERE, multiple tangent cHEREs and cEREs, direct repeats and everted repeats of the cHERE - a transient transfection assay involving a luciferase reporter assay was used to determine if they could drive transcriptional activity and the effect of exogenous HMGB1 had on the level of expression. The results indicated that ERE half-sites in multiple arrangements do drive luciferase expression and that HMGB1 enhances the level of expression. With the use of siRNA strategies, HMGB1 was shown to play an essential role in enhancing the level of transcription[48].

These results led to the focal question of this minireview. Is the effect of HMGB1 activity associated with restructuring of the nucleosome to facilitate ER/cERE binding[93]?

ER DOES NOT BIND TO THE OPTIMALLY OREINTED CERE IN NUCLEOSOMAL DNA

Figure 3 shows the schematic diagram for the 161 bp DNA used in the binding studies. Although the DNA binding site in a nucleosome could potentially take on many different orientations, the DNA was engineered to define the cERE in a specific rotational and translational position within a nucleosome for optimum binding access. The nucleosome is defined as translationally positioned if the nucleosome occupies a fixed position

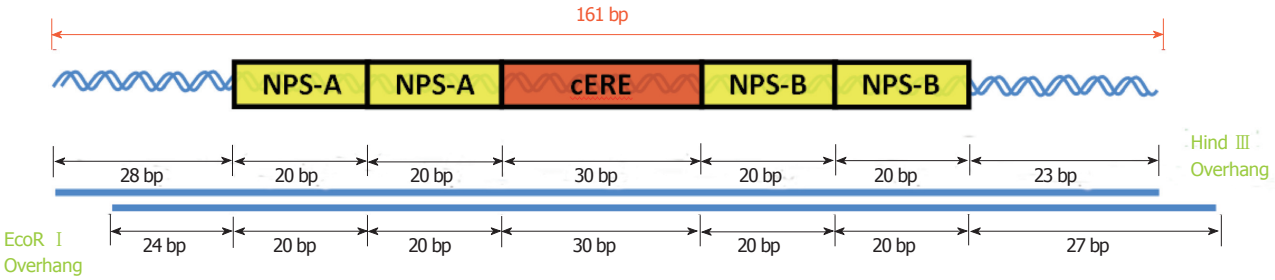


Figure 3 A schematic drawing of the 161 bp DNA containing four nucleosome positioning sequences (yellow boxes), that straddle the 30 bp consensus estrogen response element (red box) at the dyad axis. The 161 bp DNA was excised by EcoRI and HindIII digestion of the pGEM-Q2-2E2 plasmid. cERE: Consensus estrogen response element.

relative to the underlying DNA sequence. The DNA is rotationally phased as well when an orientation of a DNA sequence in the (DNA) helix is fixed relative to the histone octamer surface. The cERE resides in the center of the DNA and will be positioned at the dyad axis (red in Figure 3) within the nucleosome. With the incorporation of four nucleosome-positioning sequences, two on each side of the cERE (yellow in Figure 3), the cERE is rotationally phased so that the two binding sites, the major grooves in the cERE, for each of the monomers in the ER dimer, are sterically unobstructed and positioned outward from the histone octamer for optimum binding. The same DNA construct, with the replacement of the cERE with a cGRE (consensus glucocorticoid response element) and the designed nucleosome were previously used in analogous studies to investigate glucocorticoid receptors (GR) binding to cGRE. Using the same construct permits a direct comparison of the binding of ER/cERE with GR/cGRE under the same nucleosomal constraints[49].

The initial EMSA binding studies showed that, with this most ideal orientation of cERE, ER exhibited no detectable binding to the cERE up to about 80 nmol/L ER, with less than 20% binding up to about 200 nmol/L ER (Kd estimated about 300 nmol/L)[94]. This is in contrast to a Kd about 10 nmol/L for ER binding to DNA[46]. Therefore, although the cERE was rotationally phased for optimum binding at the dyad in the nucleosome, the cERE was inaccessible to ER and it binding affinity in nucleosomal DNA was reduced by about 30-fold from that on DNA[51].

PRELIMINARY FINDINGS: HMGB1 INFLUENCES ER BINDING TO CERE IN NUCLEOSOMAL DNA

ER binding was then tested with the nucleosome incubated at a number of HMGB1 levels (400, 800 and 1600 nmol/L). In each case, the EMSA showed that ER bound, with the EMSA band becoming increasing broad as the levels of HMGB1 was increased. This prompted us to explore the effect of HMGB1 on the character of the nucleosome under more exacting conditions.

CHARACTERIZATION OF THE HMGB1 RESTRUCTURED NUCLEOSOMES (N' and N'')

The canonical nucleosome was treated with 1600 nmol/L HMGB1 and then the nucleosomes fractionated on a 5%-20% sucrose gradient. The HMGB1-treated nucleosomes sedimented as a single well-defined band, in the same fraction as previously found for the (untreated) canonical nucleosomes. However, the EMSA of the contents of this band revealed two discrete bands that both had electrophoretic mobilities distinctly different (lower mobility) than that for the canonical nucleosome. The two nucleosome states, referred to as N' and N'', which were at comparable levels, were not further separated, so that experiments done on the HMGB1-restructured nucleosomes refer to these species. The EMSA results clearly indicated that HMGB1 treatment changed, or restructured, the nature of the canonical nucleosome to convert it into two stable, physically distinct non-canonical nucleosome conformers or structures.

Although the canonical nucleosomes were treated with 1600 nmol/L HMGB1, the sedimentation would be expected to reduce the level of HMGB1 in the isolated fraction. The determination of the level of HMGB1 in the (N'/N'') fraction indicated HMGB1 remained with these nucleosomes, but at a greatly reduced level - the level being approximately 2 HMGB1/nucleosome.

The presence of the four core histones in the restructured nucleosomes was verified by a supershift of the nucleosome EMSA band by each of the individual histone antibodies. However, antibodies to HMGB1 did not produce a corresponding supershift, indicating that HMGB1 was not a stable component of N'/N''. This is consistent with the finding that HMGB1 interactions are transient and HMGB1 functions generally by a "hit-and-run" mechanism on DNA and isolated nucleosomes[46].

An inherent characteristic of canonical nucleosomes is a DNase Ⅰ cutting pattern, in which the DNase Ⅰ cuts in the exposed minor groove, having a periodicity about every 10 bp. This periodicity is disrupted for nucleosome remodeling by the ATP-dependent human SWI/SNF complex, presumably as a result of translocation of the

DNA and altered rotational phasing, with the resulting pattern tending to look more like that of DNA[17,95]. In contrast, the DNase Ⅰ cutting pattern for N′/N″ exhibited the same 10 bp periodicity as observed on the canonical nucleosome, with the additional of a few extra weak bands. This suggested that the general nature of the DNA wrapping around the histone octamer was not changed significantly in the HMGB1-restructured nucleosomes and that the restructuring by HMGB1 was distinctly different than for the hSWI/SNF complex. The Exonuclease Ⅲ (Exo Ⅲ) digestion pattern was also essentially the same for N and N′/N″, indicating that there was no translocation of the DNA as a result of the restructuring. In addition, the invariance of the Exo Ⅲ pattern indicates that the presence of HMGB1, which has been suggested to bind at the linker DNA region, did not alter the accessibility of the ends of the DNA to this exonuclease.

To determine if the HMGB1 facilitated access to the isolated nucleosomal DNA, ER was reacted with N′/N″. ER bound strongly, with a Kd about 50 nmol/L and the DNase I footprint verified that ER bound to the cERE in a sequence-specific manner. This Kd value is 6-fold stronger than the estimated Kd value (about 300 nmol/L) for the canonical nucleosome and at about the same value as that for the canonical nucleosomes in the presence of 400 nmol/L HMGB1. In addition, ER binds to N′/N″ only five times weaker than on naked DNA. HMGB1 has no known enzymatic activity and the nucleosome restructuring activity was unchanged by the presence of ATP. Therefore, the effect of HMGB1 on the nucleosome is nonenzymatic, with the activity being independent of an ATPase activity and so must operate by an different mechanism than CRCs that require ATP hydrolysis to drive nucleosome remodeling. These findings led us to characterize any differences between the restructured nucleosomes (N′, N″) and the canonical nucleosomes (N) and how these differences might support possible mechanisms by which HMGB restructures the nucleosomes to facilitate ER access to, and binding to cERE.

Previous studies that used atomic force microscopy (AFM) to characterize the mononucleosomes that were remodeled by the SWI/SNF complex showed that the mononucleosomes were converted into altered dimers or a dinucleosome unit[95,96]. To determine if similar dinucleosomes were produced as a result of HMGB1 restructuring on mononucleosomes, analysis of the N′/N″ fraction by AFM showed only mononucleosomes, with no evidence of dinucleosomes. This further indicates that the HMGB1-restructured nucleosomes are distinctly different than canonical nucleosomes remodeled by hSWI/SNF.

One of the key characteristics of the HMGB1-restructured nucleosomes (N′/N″) is that they are uniquely stable for extended periods of time at -20 ℃ - for at least a few months. This is a unique and extremely important property and, as far as I know, is unlike the relative instability (about 30 min or less) for remodeled nucleosomes in which times of stability were reported[97-99].

Nucleosome stability as a function of temperature was examined first. It was shown that the canonical nucleosomes remained stable overnight at 37 ℃. Although the N′/N″ nucleosomes were stable over this same time period at 4 ℃, on incubation at 37 ℃, the N″ form was unstable and was converted into a mixture of N′ and N. The N′ form remained stable in an overnight incubation. Therefore the relative thermal stability of the nucleosome states is N about N′ > N″, with the restructured N′ having a thermal stability comparable to that of the canonical nucleosome.

The influence of electrostatic interactions on stability was tested by challenging the N″/N′ states with increasing levels of NaCl. At low levels of NaCl, about 0.25 mmol/L, N″ was unstable, converting into a mixture of N′/N, with increasing NaCl levels leading to the complete conversion of the N′ state into the canonical nucleosome at about 200 mmol/L. These data indicate that there is a differential stability in N′ and N″, again with N′ being more stable than the N″ state. This may occur as a result of the restructured nucleosome states having electrostatic interactions that are much more sensitive to NaCl than is the canonical nucleosome and/or that increasing levels of NaCl may weaken, or effectively eliminate HMGB1 interactions with the nucleosomes.

When challenged with low levels of competitor DNA, N′/N″ converted to N, with N″ making the transition at lower levels than did N′. Finally, above about a few hundred nmol/L DNA, only the canonical nucleosome was stable. These findings suggest that the competitor DNA acted as a "HMGB1-sink", interfering with its interaction with the nucleosomes and leading the conversion to the canonical state. Importantly (see later), all three nucleosome states were found to be stable simultaneously and present in equilibrium from about 25-50 mmol/L NaCl and about 12-50 nmol/L DNA.

It should be noted that in all cases, there was no disruption of the nucleosome as would have been indicated by the presence of a DNA band. We also noted that the supershift experiment indicated that all four different core histones were in the stable complex. The fact that the restructured nucleosomes reverted faithfully to the canonical state also indicates that the stoichiometry of the histones remained the same in all nucleosome states and that the "core" nucleosome forces remained intact and exhibited no significant irreversible disruption.

ER BINDS STRONGLY TO TAILLESS NUCLEOSOMES IN THE ABSENCE OF HMGB1

There were a number of findings that led us to investigate the possible role of the histone tails on

Table 1 Estrogen receptor binding affinity to consensus estrogen response element

Target	Kd (nmol/L)
N	Approximately 300
N/400 nmol/L HMGB1	52
(N′, N″)/10 nmol/L HMGB1	50
N tailless	45
N Tailless/400 nmol/L HMGB1	Approximately 30
Restructured tailless N	Approximately 30
DNA	10

HMGB1: High mobility group protein 1.

ER accessibility to the cERE and how the tails might impact the activity of HMGB1. It was observed that the HMGB1-restructured nucleosomes did not disrupt the DNase I cutting pattern that occurs every 10 bp. Similar to our finding, this same DNase I cutting pattern was also retained on nucleosomes that were made "tailless" by trypsin cleavage of the tails of the core histones. Likewise, the presence of the histone tails plays an important role in their thermal stability[100,101]. The basic residues in the tails are specific targets for post-translational modification, including reversible acetylation and phosphorylation that play key roles in regulatory aspects of transcription. For example, acetylation of the tail domains is important in recruiting coactivators to the nucleosome and influences the accessibility and transcriptional activity of nucleosomes[102-104].

The interaction of the acidic C-terminal domain of HMGB1 has likewise been proposed to interact with the histone tails and therefore possibly provide an additional mechanism to reduce their electrostatic interaction with the DNA[84,85]. As a result, tailless nucleosomes, in the absence of HMGB1, were prepared to determine their influence on ER binding affinity. ER binds strongly to the tailless nucleosomes, with a Kd value of about 45 nmol/L (Table 1). This Kd value is a comparable binding affinity for that reported for both (1) the canonical (tailed) nucleosomes in the presence of 400 nmol/L HMGB1 and with (2) N′/N″, and in marked contrast to the lack of ER binding to the canonical nucleosomes. This strongly supports the notion that the major effect of the HMGB1 interactions with the nucleosome is to disrupt one or more of the histone tail interactions with the nucleosomal DNA. The Kd value for the tailless nucleosome in 400 nmol/L HMGB1 or the (sucrose gradient) isolated restructured tailless nucleosome was about 30 nmol/L. This suggests that the effect of HMGB1 may extend somewhat beyond disrupting the DNA-tail interactions and is consistent with weakening the DNA-core histone interactions as well. Table 1 summarizes the Kd values for the various nucleosome states.

DISCUSSION

ER does not bind to a rotationally phased and translationally positioned cERE centered at the dyad axis in the canonical nucleosome. The cERE is rotationally phased such that the major groove of the cERE faces outward from the surface of the histone octamer to facilitate the most optimum orientation for the ER dimer to bind in nucleosomal DNA. A combination of experimental strategies collectively demonstrates that HMGB1 restructures the canonical nucleosome and that ER binds strongly to the HMGB1-restructured nucleosome states. This supports the contention that by HMGB1 changing the forces within the nucleosome, it drives the dynamic change to restructure a novel state of the nucleosome that is linked to the functional significance of ER binding.

SIMILARITIES OF THE CANONICAL AND HMGB1-RESTRUCTURED NUCLEOSOMES

The HMGB1-restructured nucleosomes (N′, N″), in many ways, are not unlike the canonical nucleosome (N). In all cases, the different nucleosome states exhibit the same hydrodynamic property, have the same stoichiometry of core histones and sediment in the same fraction in a sucrose gradient. This is not unexpected since the core histone tails have no significant effect on the frictional coefficient and the hydrodynamic properties of the core particle[101].

The lack of any significant alteration in the characteristic DNase I 10 bp pattern suggests that no major changes in the DNA interactions with the inner histone octamer occurs and that the wrapping of the DNA around the histone octamer (rotational phasing) experiences little or no change as a result of the presence of HMGB1. The fact that cleaving off the histone tail domains with trypsin also does not affect the DNase I 10 bp pattern in a "tailless" nucleosome and that ER binds strongly to the "tailless" nucleosomes in the absence of HMGB1 supports the view that HMGB1 interactions interfere with the tail-DNA interactions and have a significant contribution to the mechanism by which HMGB1 alters the character of the nucleosome structure and enhances ER binding affinity. The further increase in the ER binding affinity upon addition of HMGB1 reinforces the interpretation that HMGB1 most likely does weaken, to some extent, the DNA-histone interactions within the "core" nucleosome and goes beyond just disrupting the interactions between DNA and the histone tails. The Exo III digestion pattern is likewise not changed in the HMGB1-restructured nucleosomes. This indicates that the HMGB1 interactions do not obstruct Exo III from the DNA ends or impede or enhance Exo III activity. Furthermore, it would indicate that the activity of HMGB1 does not produce any nucleosome translocation. This is not unanticipated since HMGB1 exhibits no ATPase activity, in contrast to the ATP-dependent CRCs, which utilize ATPase activity to power translocation[105].

The supershift data indicate that all the core histones remain in the restructured nucleosomes, while HMGB1 is not a stable component. The latter finding is consistent with previous findings that HMGB1 exhibits a transient "hit-and-run" character (important exceptions discussed later) on DNA and isolated nucleosomes.

DIFFERENCES BETWEEN THE CANONICAL AND HMGB1-RESTRUCTURED NUCLEOSOMES

Although all three states of the nucleosome (N, N′ and N″) sediment identically in a sucrose gradient, each nucleosome state exhibits a distinctly different electrophoretic mobility in EMSA, with the order of the mobilities being N > N′ > N″.

However, the most striking effect of the nucleosome restructuring is that ER binds strongly to the (N′, N″) states. The Kd value for ER binding at the cERE when positioned at the dyad axis is about 50 nmol/L, which is about 5 times weaker that on DNA, yet about 6 times stronger than the corresponding binding on the canonical nucleosome, with an estimated Kd value about 300 nmol/L. The 6-fold increase in the ER binding affinity by HMGB1 on nucleosomal DNA is about three times the effect that HMGB1 has on ER binding to DNA[46].

It should be noted that the Kd value for ER binding at cERE appears to be independent of its location in the nucleosomal DNA. Although unpublished, the Kd values for ER binding at -20 bps and -40 bps from the nucleosome dyad axis was essentially the same as found at the dyad axis, Kd about 50 nmol/L. ER binding to a similar extent at distant and separate locations in the nucleosome raises questions about whether the binding of ER to the HMGB1-restructured nucleosomes are in accord with the "site exposure model"[106-108].

This model postulates that the ends of the DNA are in a rapid dynamic equilibrium between being histone bound (fully wrapped) and unbound (partially unwrapped) and that a binding factor can gain access to and bind stably to its binding site in the unbound state. The DNA is effectively "lifted off" the nucleosome, with the "lifting" of sequences nearer the ends being energetically less costly to dissociate from the histone octamer and therefore have a greater opportunity to bind factors than those sequences near or at the dyad. The experimental basis for the model comes from a kinetic analysis of restriction endonuclease cleavage on DNA that showed that the cleavage sites at the dyad of nucleosomal DNA were protected to a far greater extent than those near the ends[106-108].

If the energetics needed to disrupt histone-DNA interactions to "expose" the cERE at the dyad, which is up to 80 bps from the DNA ends of DNA, can be considered significantly greater than to disrupt decidedly fewer interactions up to a cERE 20 bps or 40 bps from the dyad, different levels of access for the sites could reasonably be expected and therefore different Kd values for ER binding at these distant sites would be expected. However, we find that ER binds with essentially the same affinity to these well-separated locations in the HMGB1-restructured nucleosome. This indicates that HMGB1 may efficiently facilitate global instability of the forces in the nucleosome. In this case, ER binding occurs independent of an enhancement from a "site exposure" unraveling of the DNA from the ends of the nucleosome.

The stabilities of the restructured nucleosomes (N′, N″) are distinctly different than the canonical nucleosome (N). The canonical nucleosome, N, is unaffected by temperatures up to 37 ℃ and by the NaCl and DNA levels that we investigated. On the other hand, N′ and N″ are stable at 4 ℃, but at 37 ℃, convert to N in about 1 h. This would suggest that the HMGB1 interactions, perhaps their lifetime bound to the nucleosome, is decreased with increasing temperature and therefore its effect is eliminated. Increasing levels of NaCl differentially convert N′ and N″ (the more unstable form) into the canonical form, with complete conversion of both states at about 200 mmol/L NaCl. This supports the view that the electrostatic forces in all three nucleosome states are different. This stability characteristic of the HMGB1-restructured nucleosomes, N′/N″, is similar to that observed for the altered nucleosome produced by the CRC, RSC, which also decreases with increasing NaCl levels[109]. Interestingly, NMR evidence suggests that the N-terminal domains of H3 and H4 appear to remain tightly bound to the DNA in the core particle up to about 0.3 mol/L NaCl[110]. This is about a NaCl level that is about 50% greater than that which converts the N′, N″ states to the canonical nucleosome. This is in line with the notion that the binding interactions of the histone tails of H3 and H4 with DNA in nucleosomes is important for its integrity and that these interactions may be weaker in the restructured nucleosome states that in the canonical nucleosome. The increasing levels of DNA would compete for the HMGB1 and eliminate its effect on N′, N″, consequently leading to the conversion to the canonical form. Even though the restructured nucleosomes are unstable to challenges by increasing temperature, NaCl and DNA, while the canonical nucleosomes are not, the restructured nucleosomes are stable at -20 ℃ for extended periods of time - being several months - in our hands.

ER BINDING TO TAILLESS CANONICAL NUCLEOSOMES WITHOUT THE AID OF HMGB1

Removal of the core histone tails from the canonical nucleosome eliminates about 30% of the histone proteins, many of the positive charged residues and virtually all of the tail segments that extend out from the nucleosome and can interact with the DNA backbone

and facilitate higher-order chromatin structure. The finding that the Kd value for ER binding was greatly reduced clearly indicates that the presence of the positively charged tails in the canonical nucleosomes does inhibit ER access to the cERE, presumably by either their preferred or stochastic interaction with phosphates in the DNA backbone. The finding that the Kd value was virtually the same as observed on the HMGB1-restructured nucleosomes is consistent with an HMGB1 effect that is derived, in large part, from continuously and transiently disrupting the DNA-tail interactions to increase access to the cERE.

ENERGY LANDSCAPE MODEL: EFFECT OF HMGB1 ON DYNAMICS OF NUCLEOSOME STRUCTURE

The interaction of HMGB1 protein with canonical nucleosomes (N) alters or destabilizes their structural characteristics and facilitates ER access to its ERE in the restructured conformers (N′ and N″). Challenges to these nucleosome conformational isomers by increasing NaCl or DNA levels reveal all three conformers (N, N′ and N″) are observed simultaneously in a dynamic equilibrium. A model proposed to explain the conformational changes in protein structure as a result of ligand binding - the preequilibrium/conformational selection model - can be extended to the nucleosome findings[111,112]. In this paradigm, multiple states of the nucleosomes can be considered to preexist in a statistical ensemble of conformers on the nucleosome energy landscape, with the population of the three states sensitive to the immediate microenvironment. The interaction of HMGB1 with the N conformation can be regarded as a "ligand driven" conformational selection of states that drives a population shift in the equilibrium ensemble. In the case of interest, ER binding can further influence the direction and magnitude of the population shift.

Figure 4A displays a limited representation of just two hypothetical three-dimensional energy landscapes for the equilibrium between the three conformational states - the canonical state, N, and the HMGB1-restructured states, N′ and N″. Using the conventional preparation/isolation conditions for nucleosomes, the canonical nucleosome, N, is the predominant species (energy landscape Ⅰ) with N′ and N″ kinetically trapped and at levels that are undetectable by EMSA. The presence and interaction of HMGB1 with the nucleosome drives a population shift as the interaction perturbs the distribution of states, and converts energy landscape Ⅰ into landscape Ⅱ. The new landscape now shows the population shift in which HMGB1 interaction selectively stabilizes the N′ and N″ states, leading to a significant increase in their population, with a concomitant decrease in the population of the canonical nucleosome. Once isolated, the canonical and the restructured nucleosomes are stable at low temperatures, but the N′ and N″ states can be readily converted to the canonical state by "stressful" conditions, such as increased temperature or increased levels of either NaCl or DNA. Figure 4B provides an overall picture for the reaction of ER with the various nucleosome states. Pathway 1 shows that ER does not bind to the canonical nucleosome. Pathway 2 shows that reaction of 400 nmol/L HMGB1 with the nucleosomes leads to a population shift, which effectively is a restructuring of the nucleosome that facilitates strong ER binding. Pathway 3 shows the route to isolation of the N′ and N″ states. The nucleosomes were reacted with 1600 nmol/L HMGB1 and then subjected to a sucrose gradient. The gradient fraction also contained 10 nmol/L HMGB1, which corresponds to about 2 HMGB1/nucleosome. ER binds strongly to these well-defined nucleosome states.

The interaction of HMGB1 with nucleosomes may be generally viewed in an analogous fashion to the scheme presented by Perutz *et al*[113] for small molecule interactions with hemoglobin. The interaction of HMGB1 with the nucleosome alters the forces within the nucleosome and drives the population shift to the nucleosome state in which the DNA sites are accessible to ER binding. The canonical nucleosome can be viewed as a highly constrained "tense" state of the nucleosome. As a result, its binding sites are inaccessible to TFs and this nucleosome state is effectively a functionally inactive state. On the other hand, HMGB1 interactions disrupt a level of constraints to restructure the nucleosome to a less constrained, "relaxed" state that is more accessible and permissive to TF binding.

The energy landscape presented here can be considered as only the first level in the ensemble of dynamic nucleosome states. In the most general paradigm, the impact of HMGB1 can be expected to be only one of a multitude of influences on the nucleosome state - including DNA methylation, post-translational modifications of the core histone tails, alternative forms of histone proteins and other factors that control the level of chromatin compaction. Each influence may be expected to exert a different effect, dependent on the collective (or pool) of factors that impinge on the ensemble of states. This collection of factors will expand the population of energy states within the ensemble and will further fine-tune chromatin structure and its functional capabilities. This suggests the presence of a very heterogeneous and dynamic nucleosome population within chromatin, with a varying, yet enormous capacity to respond to changes in environmental ques. These dynamic changes in nucleosome restructuring, driven by a variety of (external) forces, is reminiscent of the simple manipulation and controlled reshaping of a toy "stress ball" (by the pressure from your fingers). As a result, we refer to this dynamic nucleosome model, with its diverse forces and binding interactions affecting the changing aspects of (structural and functional) the

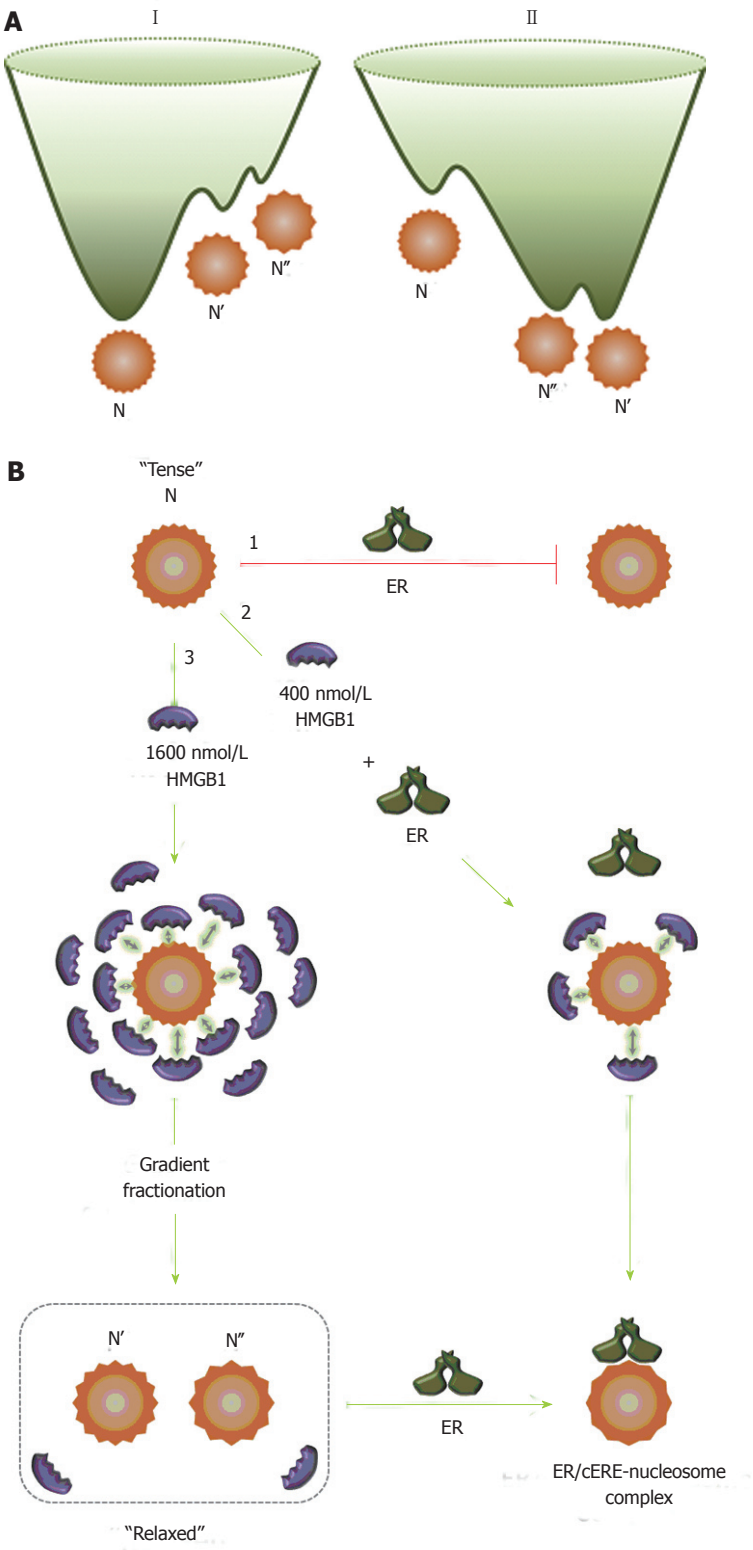


Figure 4 High mobility group protein 1 relaxes the canonical nucleosome structure and facilitates estrogen receptor binding. A: Energy landscapes for canonical (I) and HMGB1-restructured nucleosomes (II). A hypothetical representation for the energy landscape of the canonical nucleosome, N, and the HMGB1-restructured nucleosomes, N' and N''. Using conventional isolation protocols, the canonical nucleosome, N, is the predominant and thermodynamically most stable conformation. N' and N'' are in low abundance, higher energy conformational isomers that are kinetically trapped near the bottom of energy landscape I. HMGB1 interaction with N reduces intranucleosomal constraints, which resets the energy landscape (II), resulting in a population shift in which the N population significantly decreases and the population of the more "relaxed" and accessible N' and N'' states increases. The more unstable form, N'', sets in a shallower potential well than that for N'. Although interactions with HMGB1 provide the driving force to restructure N into these states, these forms remain stable and although in equilibrium with the canonical state under many solution conditions, can revert to the canonical nucleosome on challenge with increasing concentrations of NaCl and DNA; B: Interaction of the nucleosome with HMGB1 and ER. The canonical nucleosome (N) represents a "tense" and relatively inaccessible conformational isomer (pathway 1) and ER does not bind to the canonical nucleosome state. In the presence of 400 nmol/L HMGB1 (pathway 2), due to the transient and dynamic "hit and run" interaction of HMGB1 with the nucleosome, represented by arrows, the intranucleosomal constraints are relaxed, which facilitates ER binding. ER binds to the nucleosome to form ER/cERE nucleosome complex. In the presence of 1600 nmol/L (pathway 3), the intranucleosomal constraints are relaxed due to increased "hit and run" interaction of HMGB1. After gradient fractionation, the restructured nucleosomes (N' and N'') are isolated and contain only low nmol/L levels of HMGB1, which maintain the more accessible and "relaxed" conformational isomers (N' and N'') that permit ER binding. HMGB1: High mobility group protein 1; ER: Estrogen receptor.

nucleosome states, as the "stress ball" model.

THE COOPERATIVE OR CONFLICTING ROLES OF HMGB1 AND HISTONE H1 PROTEINS IN TRANSCRIPTIONAL REGULATION

It is generally thought that HMGB1 and histone H1 compete for binding in, or about, the linker region of the nucleosome and that HMGB1 provides a positive influence on transcription, while histone H1 acts counter to that. Although most *in vitro* studies agree with both the binding competition and transcriptional regulation, this may be too rigid a picture for *in vivo* findings[80,87].

Ju *et al*[88] find HMGB1 in required for transcriptional activation at the pS2 promoter. They find that as HMGB1 enters the complex, it displaces linker histone H1, which

is consistent with HMGB1 and histone H1 competing for the same or an overlapping site - presumably on the linker DNA within the nucleosome[88,89].

On the other hand, El Gazzar *et al*[90] present the unique finding both HMGB1 and H1 are found simultaneously in the TNF-α promoter and are required for endotoxin-mediated silencing of TNF-α promoter in THP-1 human promonocytes.

These studies bring out a challenge to our current thinking about the extent to which *in vitro* findings can be translated to the *in vivo* state, in at least two respects. First, both these studies and the report by Poser *et al*[54], used chromatin immunoprecipitation (ChIP) to reveal that HMGB1 was a stably bound component of the transcriptional machinery. This is unlike the transient binding characteristically found *in vitro* for HMGB1 interactions with one or two isolated components, such as DNA or nucleosomes. The *in vivo* studies suggest that HMGB1 makes multiple interactions in these larger complexes that increases their residence times so that they can be revealed by a ChIP analysis.

Second, El Gazzar *et al*[90] find that both HMGB1 and histone H1 reside together simultaneously at the TNF-α promoter. This indicates that HMGB1 and histone H1 are not competing for the same singular site (linker DNA) on the nucleosome and that, again, in such large promoter complexes, more extensive and different interactions can be expected.

COMPARISON OF ER, PROGESTERONE RECEPTOR AND GR BINDING TO THEIR RESPONSE ELEMENTS IN NUCLEOSOMAL DNA

ER, progesterone receptor (PR) and GR exhibit a similar modular structure, bind to palindromic response elements and bind to these response elements as homodimers. As a result, they are designated as Class I steroid hormone receptors. However, the ER findings, together with previous data on GR and (unpublished) data for PR from our lab, present a credible challenge to the generally held view that members of the steroid receptor family exhibit similar binding characteristics and bind strongly to their response elements within the nucleosomal DNA[50,114]. This led us to compare the effect of HMGB1 on their binding characteristics (Table 2). For both the GR and PR studies, the same DNA that was used in the ER studies was employed, except that the response element was changed to GRE, which both PR and GR bind to. Although PR binds strongly to the GRE in DNA, its binding profile was similar to that for ER in that it does not bind to its response element (GRE) when located at the dyad in the nucleosomal DNA. However, quite unlike the robust effect on ER binding, the presence of HMGB1 did not affect the PR binding affinity. Previous studies indicated that PR binds to the sequence in the mouse mammary tumor virus (MMTV) in a nucleosome, with its binding affinity on DNA being only about a factor of 3-5 stronger than in a nucleosome. This result was quite different than in our comparative findings and may be due to the different response elements used[51]. Again using this same DNA, it was previously shown that GR binds strongly to GRE in DNA and the binding on nucleosomal DNA was reduced by only a factor of about 3, suggesting that nucleosome only marginally inhibits GR binding and suggesting that HMGB1 would probably have only a small, if any, effect on GR binding affinity[50]. This comparative data indicate that HMGB1 exerts a very different effect on the binding affinity for these three steroid hormone receptors at the nucleosomal level. HMGB1 enhances only the binding affinity for ER. This is consistent with recent findings and the proposal that HMGB1 facilitates ER to bind by a variety of modes, which is quite unlike that of other steroid hormone receptors[46-48,93]. This is also consistent with the finding that although HMGB1 enhances GR binding to the GRE in MMTV DNA, the influence of HMGB1 on GR binding to MMTV chromatin was minimal[115].

Table 2 The effect of high mobility group protein 1 on the binding of steroid hormone receptors to their response elements

	Kd (nmol/L) values		
Receptor	DNA	Nucleosome	(Nucleosome/HMGB1)
ER	10	Approximately 300	Approximately 50
PR	Approximately 2	Approximately 300	Approximately 300
GR	Comparable (no specific Kd values)		No data

HMGB1: High mobility group protein 1; ER: Estrogen receptor; PR: Progesterone receptor; GR: Glucocorticoid receptor.

Interestingly, these results also in accord with the findings that HMGB1 is an active participant in the activation of the estrogen responsive *pS2* gene by ER, in addition to that for the AR, RAR and the T3R[88].

THE ABCS OF A PROPOSED MECHANISM OF ACTION FOR HMGB1 IN RESTRUCTURING THE CANONICAL NUCLEOSOME

HMGB1 has three major modular domains. The A- and the B-boxes are basic and will electrostatically bind to the highly bent nucleosomal DNA. The C-terminal domain is completely acidic and can target positively charged residues or regions in the histone proteins. We propose a model in which the A- and/or B-boxes in HMGB1 can interact in the minor groove of DNA to bend or provide a dynamic flexure in the DNA, disrupt electrostatic interactions and reduce the affinity and life-time of the core histone-DNA constraints. In contrast to the effect of proteins binding in the major groove, minor

groove binders, such as HMGB1, can produce these bends and reduce the rigidity of DNA by asymmetric phosphate neutralization, insertion of protein side-chains in between the base pairs and release bound waters and counter ions. These interactions will clearly have similarities to those also reported for the action of the FACT complex on nucleosomes[40,116].

The acidic C-terminus of HMGB1 can play a role also as it can interact with the positively charged core histone tails to reduce the residence time of the tail interactions with DNA and thereby widen the window of opportunity for greater ER access to the cERE. This is in line with the finding that the removal of the core histone tails, or alternatively, the acetylation of lysine residues in the tails did not alter the DNase I 10 bp pattern and did increase the accessibility of transcription factors for their target sequences[100,117-120]. In addition, the C-terminal of HMGB1 was shown to play an essential role in stimulating transcription and that it interacted with the N-terminus of histone H3, which may specifically alter H3 interactions with DNA[82,84,85,121]. The tails are also implicated in interacting with other nucleosomes to build higher-order chromatin structure. Whether HMGB1 disruption of the histone tail contacts with DNA will have any effect on local higher-order structure will be interesting to explore.

The binding of regulatory factors, such as ER, to their response element, act to promote the recruitment of coregulatory factors that serve as "operations managers" on post-translational modification of the tails in the core histones. This permits them to communicate with the transcriptional machinery at the promoter to regulate the transcriptome for estrogen-responsive genes. However, this simple scenario leads us to confront a causality dilemma that asks that if the restructuring or remodeling factors first bind nonspecifically to permit the transcription factor access, what is the mechanism by which the factor targets the site of interest? On the other hand, if the transcription factor targets an inaccessible site, how can it gain access and bind to it without the help of a remodeling factor? HMGB1 protein is an unusual protein in that it is estimated that there are as many as a million copies in the cell. This being the case, it may, simply by a stochastic nature, be somewhat effective in opening up some regions of chromatin. However, it was shown that ER and HMGB1 form a complex in solution, with HMGB1 binding to the carboxyl terminal end (CTE) of ER, which is immediately adjacent to the DBD domain[44]. This is a situation in which the regulatory factor (ER) chaperones a restructuring factor (HMGB1) to the response element target to provide an effective mechanism to restructure the nucleosome for ER to bind to a more accessible site. This novel avenue would increase the level of HMGB1 at the ERE target site to initiate a restructuring of the nucleosome, while ER then binds to the more accessible site in a step-wise or concerted fashion.

It has been shown that the activity of multiple CRCs is often required to make a site accessible to a regulatory factor. In addition, different CRCs exhibit different capabilities and with the expected redundancies in this spectrum of factors, one can expect that HMGB1 may collaborate with some of these coregulatory factors at this early stage of transcriptional activation. One might imagine that many of these enormous remodeling/coregulatory complexes are really "amalgams" of essential, collaborative factors, with a composition that may vary depending on the temporal state of the cell, the availability of factors and the variety of signals that are occurring at that moment in time[33,35].

The pS2 promoter has the cERE and the TATA box on adjacent nucleosomes, T and E, respectively. This type of ER chaperoning in HMGB1 to the ERE may have consequences in its essential role in transcriptional activation at the pS2 promoter in that it may provide a driving force for ER and HMGB1 binding simultaneously and perhaps an obvious avenue to compete with and expel H1 from the nucleosome early in the activation process[52,88]. In this, perhaps, unusual alignment of the regulatory response element and the TATA box on adjacent nucleosomes in the pS2 promoter, the acidic C-terminus of HMGB1 may have competitive demands on it. It has been shown to interact strongly with the TBP, with evidence that this involves a hydrogen bonding interaction between the acidic residues in HMGB1 and the glutamine (Q) tract in TBP[71].

The binding of ER to cERE was previously shown to bend the cERE toward ER[122,123]. However, when DNA is bound in the nucleosome, the cERE is physically bent in the opposite direction, with the bend toward the histone octamer. The flexure in the DNA that is introduced by the HMGB1 interaction will increase the probability that the DNA and the cERE sequence will have a greater lifetime at or near a bending angle that would be more favorable or amenable to ER binding in its preferred direction.

CONCLUSION

HMGB1 appears to play a role in a multitude of transactions that take place on DNA within chromatin. Its divergent structural domains provide it with the opportunity to interact with DNA and a host of proteins to help mold nucleoprotein complexes into optimum structures to perform their functions. An optimally oriented cERE in a nucleosome proves to be inaccessible to ER. HMGB1 can act on the canonical nucleosome to alter the internal forces between the DNA and the core histones in a nonenzymatic manner to reveal two stable, physically distinct nucleosome conformers. This restructures the nucleosome in a way that facilitates ER access to the cERE and results in a strong binding affinity. The initial stage of characterizing the HMGB1-restructured nucleosome has led to a proposal for HMGB1 acting on an ensemble of nucleosome conformers, in which HMGB1 alters the population of nucleosome states by a "ligand driven" conformational

selection process. The current level of characterization also permits us to propose mechanisms by which HMGB1 may act to restructure the nucleosome and lead to the activation of transcription.

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Chromatin boundary elements organize genomic architecture and developmental gene regulation in Drosophila *Hox* clusters

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Abstract

The three-dimensional (3D) organization of the eukaryotic genome is critical for its proper function. Evidence suggests that extensive chromatin loops form the building blocks of the genomic architecture, separating genes and gene clusters into distinct functional domains. These loops are anchored in part by a special type of DNA elements called chromatin boundary elements (CBEs). CBEs were originally found to insulate neighboring genes by blocking influences of transcriptional enhancers or the spread of silent chromatin. However, recent results show that chromatin loops can also play a positive role in gene regulation by looping out intervening DNA and "delivering" remote enhancers to gene promoters. In addition, studies from human and model organisms indicate that the configuration of chromatin loops, many of which are tethered by CBEs, is dynamically regulated during cell differentiation. In particular, a recent work by Li *et al* has shown that the SF1 boundary, located in the Drosophila Hox cluster, regulates local genes by tethering different subsets of chromatin loops: One subset enclose a neighboring gene ftz, limiting its access by the surrounding Scr enhancers and restrict the spread of repressive histones during early embryogenesis; and the other loops subdivide the Scr regulatory region into independent domains of enhancer accessibility. The enhancer-blocking activity of these CBE elements varies greatly in strength and tissue distribution. Further, tandem pairing of SF1 and SF2 facilitate the bypass of distal enhancers in transgenic flies, providing a mechanism for endogenous enhancers to circumvent genomic interruptions resulting from chromosomal rearrangement. This study demonstrates how a network of chromatin boundaries, centrally organized by SF1, can remodel the 3D genome to facilitate gene regulation during development.

Key words: Chromatin boundary element; Insulator; CTCF; Chromatin loop domains; Drosophila; *Hox* genes

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Core tip: Genomic organization in higher eukaryotes needs to fulfill at least three distinct functions: Gene compaction, gene insulation and gene regulation. Chromatin loops appear to be a common structural unit that serves all these functions. A recent study has characterized a series of chromatin boundary elements (CBEs) in the Drosophila Hox cluster. Selective and dynamic interactions between these CBEs tether chromatin loops that not only insulate neighboring genes, but also organize enhancer traffic to regulate gene expression during development.

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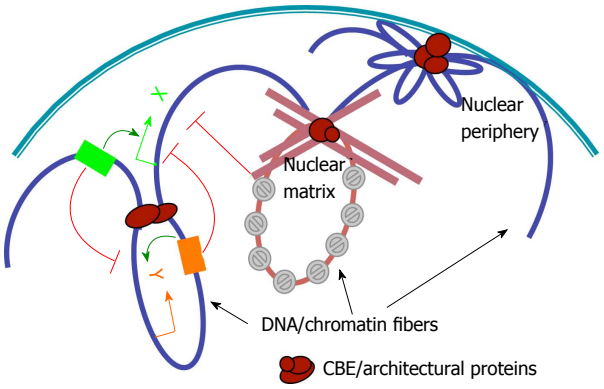


Figure 1 The roles of chromatin boundary elements in organizing genomic and nuclear architecture. Chromatin boundary element (CBE) complexes (brown ovals) may interact with each other (left), nuclear matrix (middle) or nuclear envelope (right) to tether chromatin loops that modulate enhancer-promoter interactions (curved arrows), and block the spread of silent chromatin (grey forbidden circles). Green and orange arrows indicate promoters of hypothetical genes X and Y, respectively. Green and orange boxes, enhancer elements for genes X and Y, respectively.

INTRODUCTION

Transcriptional regulation plays a pivotal role in controlling gene activity during development, physiological responses and diseases. The current paradigm of eukaryotic transcriptional regulation emphasizes the assembly of activator complexes at distal regulatory DNA elements called enhancers[1-4]. Studies have shown that communication between these enhancers and their target promoters also constitutes a critical and highly regulated step towards transcription activation. Although the mechanisms of such communication are not fully understood, studies have shown that distal regulatory elements looping to gene promoters can trigger transcriptional activation or repression[5-7]. Mounting evidence suggests that configuration of chromatin fibers in the three-dimensional (3D) space can profoundly affect the access of regulatory sequences to genes during cell differentiation (Figure 1)[6,8-12].

ROLE OF CHROMATIN BOUNDARIES IN ORGANIZING 3D CHROMATIN ARCHITECTURE

The mechanisms that regulate chromatin loop formation are poorly understood. Evidence converges on a type of specialized regulatory DNA called chromatin boundary elements (CBEs), also known as insulators. These elements were originally identified as DNA sequences that separate neighboring genomic domains[13,14]. They also interrupt enhancer-promoter communications without affecting the activities of these elements *per se*[9,15-20]. Boundary-like elements have been found from yeast to humans, often between divergently expressed genes. This is consistent with their functional role in maintaining independent domains of gene regulation. The best-characterized CBEs include the vertebrate beta-globin insulator, the Drosophila Gypsy insulator, and the boundaries from the Drosophila *Hox* loci[21-27]. The initial clue that CBEs may function by pairing with each other and tether chromatin loops came when their enhancer-blocking activity was found to depend on the position, orientation and arrangement of these elements[28-37]. In addition, the loop domains tethered by CBEs in the Drosophila *Hox* complexes, including Fab-7, Fab-8 and SF1, have also been shown to defined domains of distinct histone modifications that correlate with local gene activity[27,38,39]. Importantly, CBEs can play multi-faceted roles in gene regulation by either blocking or promoting enhancer-promoter interactions, depending on the topology and configuration of these loops[27,36,40-42]. The recent advent of genome-wide chromosomal-capture technology (3C, 4C, 5C and Hi-C, Figure 2) and protein association (ChIP) methods has provided powerful tools for assessing the spatial organization of the chromatin fibers and genomic conformation *in vivo*[8,43-51]. Recent reports indicate that CBE sequences, such as CTCF binding sites, correlate well with borders of Topological Associating Domains (TADs), which are megabase-sized units of genomic interaction domains[8,10,36,48,51-53]. These findings suggest that the interactions among CBEs/insulators may tether long-range chromatin loops that underlie much of the genomic architecture.

Diverse DNA sequences and protein factors have been associated with CBE function and genomic architecture. The most-conserved and best-characterized CBE/architectural protein is CTCF, a zinc-finger DNA-binding protein[18,19,48,50,54-56]. It mediates enhancer-blocking activity and underpins long-range chromatin loops in both Drosophila and vertebrates. Cohesin and condensin complexes, in conjunction with other CBE factors, are also well-conserved machinery that mediates long-range chromatin interactions from yeast to humans[50,57-59]. Additional classes of CBE complexes have been identified

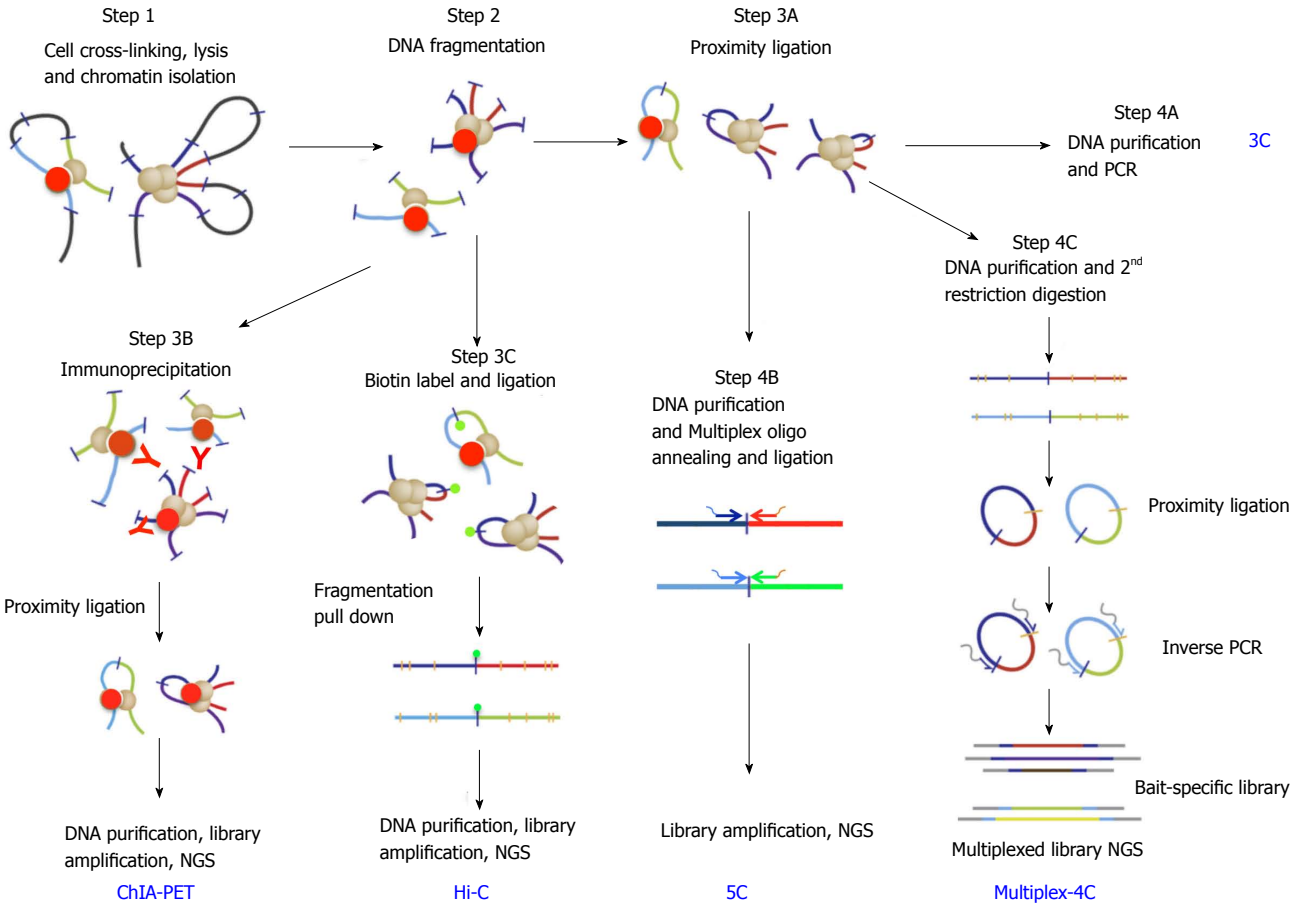


Figure 2 Key steps in chromosomal conformation capture protocols (adapted from Stadhouders *et al*[46], 2013). Bold blue upper case letters denote capture protocols and black lower case phrases denote the key steps. Steps 1 and 2 (cell crosslinking, chromatin preparation and fragmentation) are common to all capture protocols. Following these, the ends of captured DNA fragments are ligated under highly diluted condition (step 3A, proximity ligation), and junctions of captured DNAs are detected by PCR using pray- and bait-specific primers (step 4A, 3C). Alternatively, capture events can be selected by immunoprecipitation using antibody against specific architectural proteins (step 3B for ChIA-PET). Capture events can be also enriched by pull-down of Biotin-labeled ligation junction (step 3C for Hi-C). After purification, libraries of junction DNAs are amplified and sequenced by next-generation-sequencing (NGS). After step 3A, library of bait- and pray-specific primers can be used in conjunction (step 4B for 5C) to generate libraries of selected capture junctions for NGS sequencing. Bait-specific primers can also be used to generate a capture library (step 4C for 4C or multiplex 4C).

in Drosophila, anchored by the SuHw, BEAF, GAGA and ZW5 DNA-binding factors, respectively (for recent reviews see[60]). The Drosophila centrosomal protein CP190 associates with these complexes and contributes to their boundary and architectural function[59-63]. In addition, gene promoters, retrotransposons, and house keeping genes have also been shown to associate with CBE or architectural functions[48,64-66].

REGULATED *VS* STATIC CHROMATIN ARCHITECTURE

Maps of global genomic interactions (Hi-C) have been generated from numerous mammalian and Drosophila cell lines. These maps indicate that the TAD organization is a pervasive feature of the interphase nuclei[8,10,47,48,60]. Further, megabase-sized TAD domains appear to be relatively stable across different cell lineages, even conserved across species[48]. Consistent with this, constitutive and robust binding of CBE/architectural proteins collocalizes with TAD borders[51]. In contrast, dynamic reconfiguration of chromatin loops at sub-TAD (inter-TAD) scale appears to underlie much of the regulatory interactions[5,6,8,67-69]. Such dynamic reorganization may be critical for establishing distinct gene activity and nuclear organizations that are cell fate specific[60,68,70]. A recent work on the Drosophila *Hox* gene regulation revealed that transient and cell-specific chromatin loops coincide with domains of enhancer and promoter access, as well as domains of repressive histone modifications[27].

An important question that now arises is how to distinguish the static structural roles of chromatin loops in genomic architecture from the regulated chromatin loops that are tissue- and developmental stage-specific. It is unclear whether the two types of architecture utilize different cis- and trans-acting components. Significantly, most of CBE/architectural proteins identified so far are ubiquitous and constitutively expressed, raising questions about their roles in regulation.

DEVELOPMENTALLY REGULATED CHROMATIN LOOP DOMAINS IN THE DROSOPHILA *HOX* CLUSTER

A recent manuscript by Li *et al*[27] provided evidence that developmentally regulated chromatin loop domains are involved in gene regulation in the Drosophila *Hox* cluster. *Hox/HOM* genes control animal segment identity along the anterior-posterior body axis. The complexes represent the most conserved gene family, not only in gene function, gene regulation, but also in gene organization. Indeed, the striking "collinear" relationship between the order of *Hox* genes on the chromosome and the order of their expression domains along the body axis has often been cited as the defining feature of the animal kingdom. In a previous publication, the group has reported the identification of SF1, a CBE located within the Drosophila *Hox* cluster between Sex comb reduced (*Scr*) and its neighbor fushi tarazu (*ftz*, Figure 3A[26,27,71]). SF1 was shown to contain a strong enhancer-blocking activity in the early Drosophila embryos. It also exhibits a strong activity in protecting the transgenic miniwhite reporter against the influences of neighboring chromatin in late development. In the current study, the authors used the 3C technique to probe for points of contact that SF1 makes in the surrounding genomic regions. The search had lead to the identification of several novel CBEs, located downstream of *ftz*[27]. In particular, SF1 pairs transiently but strongly with an SF2 CBE to enclose *ftz* in a chromatin loop in the 4-8 h embryos (Figure 3B)[27]. This *ftz* loop coincides with a domain of chromatin depleted in repressive histone marks including H3K27Me3 and H3K9Me3 (grey circles, Figure 3B). The loop also corresponds to a domain of restricted access to the *ftz* promoter by the surrounding *Hox* enhancers (curved arrows, Figure 3B). In the 12-16h embryos, SF1 dissociates from SF2, resulting in a spread of repressive marks into the *ftz* domain and a dramatic increase in interference to the *ftz* promoter by the neighboring regulatory sequences[27,72]. These results suggest that the loop tethered by SF1 and SF2 plays a key role in insulating both *Scr* and *ftz* regulation. Importantly, pairing of SF1 and SF2 in tandem allows a distal enhancer to "bypass" the block by both CBEs, providing a mechanism for the *Scr* enhancers located downstream of *ftz* to "leap" over *ftz,* and communicate with the *Scr* promoter (green ovals, Figure 3B[45,73-75]). Besides the SF1-SF2 pairing, SF1 also anchors other chromatin loops, which may subdivide and facilitate the *Scr* early and late regulatory elements[27]. Furthermore, evidence was provided that the activities of some of these novel CBEs are tissue restricted (for details see[27]). These results suggest that attributes of chromatin conformation can be regulated in a tissue-, stage- and gene-specific fashion to direct transcriptional outcome. Interestingly, the SF1-SF2 interval represent an evolutionarily conserved genomic block (Powell Conserved Region) that houses the entire *ftz* gene and is in an inverted orientation in several Drosophila species (Grey arrows, bottom of Figure 3B[76]). These observations suggest that chromatin loops may insulate the communications between genes and their regulatory sequences from the chromosome rearrangements, resulting in intermingling and interdependence of the genomic "modules" during evolution.

The above study has provided strong evidence that CBE-tethered chromatin loops can direct enhancer traffic during development. However, several questions remain to be addressed. First, in order to demonstrate the *in vivo* function of SF1 and SF2-tethered chromatin loops in *ftz* and *Scr* regulation, it is critical to examine the chromatin configuration and *ftz* and *Scr* expression patterns in mutant animals where SF1 or SF2 is deleted. In particular, since the chromatin loop tethered by SF1 and SF2 was hypothesized to restrict enhancer access to the ftz promoter and impede the spread of silent chromatin into the active ftz domain, one would expect to see increased capture of the ftz promoter by the neighboring *Scr* enhancers, and a higher level of repressive histone marks within the ftz region in mutant animals. Consequently, reduced or ectopic expression of the *ftz* gene may be observed. Similarly, the SF1-SF2 loop was postulated to facilitate the *Scr* distal enhancers to their promoter. Deletion of SF1 or SF2 would disrupt the loop configuration and the *Scr* enhancer-promoter interactions, leading to changes in the *Scr* expression.

In the above study the authors also noted that the SF1-SF2 chromatin loop coincides with the inverted ftz region in several Drosophila species. Furthermore, several genomic rearrangements in the Antennapedia Hox complex besides the ftz domain also coincide with potential CBE-tethered chromatin loops. The authors hypothesized that chromatin loops tethered by boundary elements may promote genomic crossover and at the same time insulate any potential deleterious impacts of these rearrangements to the surrounding genes. The hypothesis can be tested from several angles. Demonstration of corresponding loop domains in these Drosophila species, validation of boundary activity that flank the loop domains, and general correlation between genomic rearrangements and CBE-tethered chromatin loops would go a long way proving this hypothesis.

MAJOR GAPS IN KNOWLEDGE AND FUTURE STUDIES

Despite recent advances in our understanding of chromatin structure and genomic organization, how key features of our genetic material re regulate development, physiological and pathological responses remain poorly understood. Below are three major gaps in our understanding of the mechanisms and function of genomic architecture.

Animal model studies

Although conformation capture technologies have

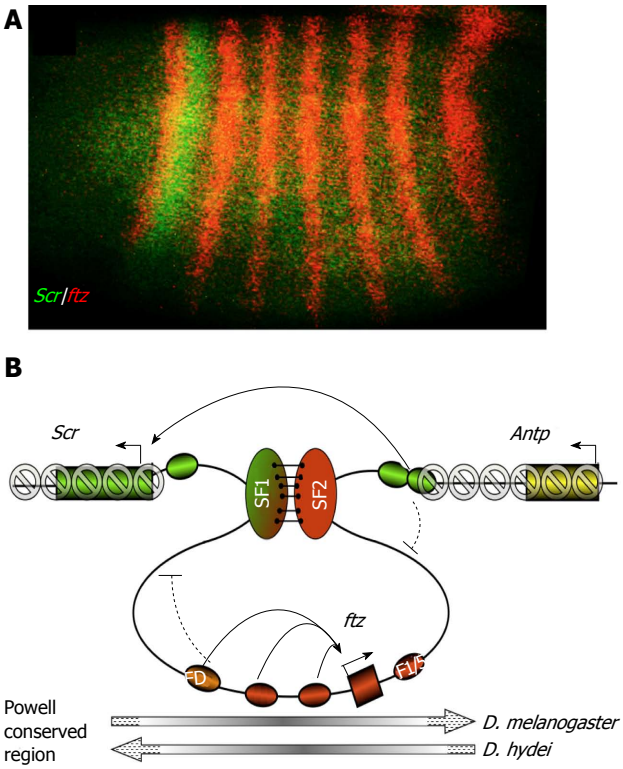


Figure 3 Hypothetical function of the SF1-SF2 chromatin loop in *Scr* and *ftz* gene regulation (adapted from Li *et al*[27], 2015). A: Segmental expression patter of a Drosophila *Hox* gene *Scr* (green). A neighbor non-*Hox* gene *ftz* is expressed in pair-rule stripes (red); B: A model for developmentally regulated SF1-SF2 chromatin loop in modulating enhancer-promoter communications and preventing spread of silent chromatin in the Drosophila *Hox* complex. The chromatin loop anchored by the interaction between SF1 and SF2 (green and red vertical ovals, respectively) during early Drosophila embryogenesis can restrict the access of *ftz* and *Scr* enhancers (red and green ovals, respectively) to neighboring gene promoters (curved block signs). The SF1-SF2 loop also prevents the spreading of the repressive H3K9Me3 and H3K27Me3 histone modification (grey forbidden circles) into the active *ftz* gene. Pairing of SF1 and SF2 may facilitate "enhancer bypass", connect the *Scr* with its distal enhancers (green ovals) in 3D in the early Drosophila embryo. The *ftz* gene domain is present in opposite orientation in various Drosophila species (grey long arrows, bottom). FD: *Ftz* distal enhancer; F1/5: *Ftz* stripe 1/5 enhancer; *Antp*: *Antennapedia*, a *Hox* gene located distal to *ftz*.

provided a critical means for assessing the genomic interactome and architecture, the methods have so far been largely applied to cultured cell lines. This is due to the need of quantities of homogeneous cell population for using the mapping technology. These studies can provide important information into aspects of nuclear and genomic organization. However, the maps do not faithfully reflect the characteristics and dynamics of animal cells and tissues during development. On the other hand, maps have been generated from mixed tissues, such as those from whole embryos. Those are not ideal either, as they reflect an average of the interactions from many cell types, and may suffer from low signal to noise ratio[77]. Future studies using cells from authentic animal models with highly defined tissue and developmental identity would be essential. Purification methods that can be applied to diverse cell and tissue types, and the use of single cell technologies in mapping genomic interactions should provide critical insights into the organization and function of genomic organization and nuclear architecture in a more physiologically relevant setting.

Causal relationship to gene regulation

Another gap in our knowledge is in the causal relationship between chromatin configuration and gene regulation. Much of the current studies correlate global TAD structures with domains of active or silent chromatin. Numerous studies have also correlated chromatin loop organization with local enhancer-promoter interactions during gene activation[27,78,79]. However, to demonstrate that these changes in chromosomal configuration play a primary role in regulating enhancer-promoter interactions or in organizing chromatin domains, rather than simply correlating with these events, it would be essential to examine these interactions and domains in mutants where the corresponding genomic configurations are disrupted. In addition, future studies should also aim to provide a more quantitative description how changes in chromatin configuration instruct or permit gene regulation events.

Mechanism of tissue-specific regulation

The protein factors that control developmental stage- and tissue-specific chromatin loops are poorly characterized. In particular, majority of the currently known CBE/architectural proteins are constitutively and broadly expressed in animal tissues. Therefore, regulated chromatin conformation may require novel factors that are more restricted in their temporal or spatial distribution. Recent studies have suggested that such factors may associate with chromatin and with each other weakly, and loop within a shorter range[27,78,79]. As a result, their association and function may only be revealed from ChIP experiments and conformation captures of higher reading depth, and possibly from purified or enriched source tissues. Combinations of molecular genetic and biochemical approaches may be required to identify these proteins.

CONCLUSION

The human genome is highly organized in the three dimensional space. CBEs represent an important mechanism that organizes such complex architecture. Functionally, these elements separate neighboring genes to ensure their independent regulation. Boundary elements also tether chromatin loops to promote communications between distant genetic elements. As such, chromatin boundary activity is essential for proper genomic function including transcriptional regulation. Despite such critical roles, how CBE activities and chromatin loop formation are regulated, especially in a developmental context, is still poorly documented and poorly understood. Current gaps in our knowledge

are in their roles in authentic animal models, in their instructing rather permitting genomic interactions during transcriptional regulation, and in their cis and trans-components that can mediate tissue- and stage-specific genomic architecture. Future studies that combine single cell technology, live imaging and other genomic, cell biological and biochemical approaches should mend these gaps and further elucidate this novel mechanism that control our genomic output.

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MINIREVIEWS

Mechanisms of the alternative activation of macrophages and non-coding RNAs in the development of radiation-induced lung fibrosis

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Abstract

Radiation-induced lung fibrosis (RILF) is a common side effect of thoracic irradiation therapy and leads to high mortality rates after cancer treatment. Radiation injury induces inflammatory M1 macrophage polarization leading to radiation pneumonitis, the first stage of RILF progression. Fibrosis occurs due to the transition of M1 macrophages to the anti-inflammatory pro-fibrotic M2 phenotype, and the resulting imbalance of macrophage regulated inflammatory signaling. Non-coding RNA signaling has been shown to play a large role in the regulation of the M2 mediated signaling pathways that are associated with the development and progression of fibrosis. While many studies show the link between M2 macrophages and fibrosis, there are only a few that explore their distinct role and the regulation of their signaling by non-coding RNA in RILF. In this review we summarize the current body of knowledge describing the roles of M2 macrophages in RILF, with an emphasis on the expression and functions of non-coding RNAs.

Key words: Macrophages; M1; M2; Non-coding RNA; MicroRNA; Long-noncoding RNAs; Radiation-induced lung fibrosis; Fibrosis

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Core tip: We discuss the mechanisms of initiation and progression of radiation-induced lung fibrosis. First we summarize the role of M2 macrophages in the initiation and development of pulmonary fibrosis with an emphasis on their function in radiation-induced lung fibrosis. We then examine the growing evidence describing non-coding RNAs in the development and progression of radiation-induced lung fibrosis.

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INTRODUCTION

Ionizing radiation (IR) is one of the most common methods of treatment for breast and lung cancer[1]. While effective at treating thoracic cancers, IR damages the cells of the lung epithelium, resulting in the chronic accumulation of inflammatory cytokines. In 10%-20% of patients who receive thoracic radiotherapy, the dysregulation of the immune microenvironment results in radiation-induced lung fibrosis (RILF)[2]. RILF is a progressive disease that occurs in two distinct phases. The first stage of RILF is pneumonitis, which occurs within the first six-months post-treatment. Pneumonitis is characterized by shortness of breath, cough, and fever. Patients with severe radiation pneumonitis experience an almost 50% mortality rate[3]. After 6-12 mo (up to two years after irradiation) pneumonitis progresses to fibrosis[4]. Fibrosis is the result of excess wound healing, characterized by the accumulation of fibroblasts and myofibroblasts, and high levels of extracellular matrix (ECM) deposition and ECM remodeling[5].

Many factors affect the development and progression of RILF. These include radiation dose, volume of the irradiated parenchyma, adjuvant use of chemotherapy, age, and genetic predisposition of the patient. Furthermore, IR induces the activation of several cell types which affect RILF in a variety of ways, including pneumocytes, endothelial cells, myofibroblasts and immune cells[4]. The most important cells for the development of RILF are immune cells, which secrete fibrosis activating cytokines, and fibroblasts, which are the predominant cell type of the stromal microenvironment and are necessary for the production and maintenance of the extracellular membrane and wound healing response[6]. While the mechanisms of RILF development and progression remain poorly understood, research into general fibrosis has laid a strong foundation for further work. Significant evidence demonstrates the critical role that macrophage polarization plays in the development of a pro-fibrotic microenvironment, and early studies have demonstrated the impact that radiation has on the balance of M1 and M2 macrophages. Moreover, noncoding RNAs have been shown to be critical regulators of most signaling pathways, including in fibrosis development and RILF.

Macrophage polarization

Macrophages are phagocytic white blood cells that have important roles in inflammation and wound healing. They are necessary in the innate immune response, and function in adaptive immunity as well through recruitment of other immune cells. When dysregulated, macrophages and macrophage activated signaling pathways play a key role in the development of RILF[7]. There are independent macrophage populations in most tissues, including alveolar macrophages in the lungs. The origin of tissue resident macrophages is not fully understood. Initial reports described tissue resident macrophage populations that were renewed through the constant recruitment and differentiation of bone-marrow derived circulating monocytes[8]. However, recent data has shown that mature macrophage populations may also originate during fetal development and are maintained through self-renewal within each tissue[9,10]. The true origin of tissue specific macrophages is likely a combination of the two. Primitive macrophages are derived from the yolk-sac, and are directly formed through a rapid differentiation mechanism without monocyte formation. However, after maturation the primary macrophage source switches to embryonic progenitor cells, which dilute the population of original yolk-sac derived macrophages[11,12].

Macrophages are known for their heterogeneity and plasticity, and classified based on their phenotypic differences in response to stimuli. They are separated into two general groups, classically activated M1 macrophages and alternatively activated M2 macrophages[13]. M1 macrophages are inflammatory, antigen presenting cells that are activated by stimulation with interferon-γ (IFN-γ), toll-like receptor (TLR) ligands and microbial products such as lipopolysaccharide (LPS)[14]. They can be identified by their expression of CD80, CD86, IL-1R, TLR2, TLR4 and iNOS, and secrete inflammatory cytokines and chemokines including TNF-α, IL-1β, IL-6, IL-12, IL-23, CCL2-CCL5, and CCL8-CCL11. M1 macrophages also produce toxic intermediates such as nitric oxide (NO) and reactive oxygen intermediates[15]. There are three sub-groups of M2 macrophages. M2a are induced by IL4 and IL13, M2b by antigen-antibody immune complexes and LPS, and M2c by IL-10 and transforming growth factor beta (TGF-β). M2a and M2c macrophages secrete low levels of inflammatory cytokines, while M2b produce inflammatory cytokines while still protecting from LPS. Th2 cytokines IL-4 and IL-13 preferentially stimulate arginase-1 activity in pro-fibrotic M2 macrophages[16]. Expression of mannose receptor-1 (CD206), the lectin-binding protein Ym1, the resistin-like protein Fizz1, and the chemokine CCL18 are also associated with M2 macrophages. All M2 macrophages promote angiogenesis and tissue remodeling, and regulate inflammation and immune responses[15].

M2 macrophages secrete pro-fibrotic growth factors including TGF-β, fibroblast growth factor, platelet derived growth factor (PDGFα), and vascular endothelial growth factor (VEGF). Stimulation by these cytokines enhances fibroblast proliferation, and can induce differentiation into myofibroblasts, a highly active fibroblast-like cell. Myofibroblasts' rapid proliferation, excessive deposition of ECM proteins such as collagen and fibronectin, and high contractile ability are critical to the development of

fibrosis[17].

Macrophage polarization is predominantly governed by several signaling pathways: The C-Jun N-terminal kinase signaling pathway, PI3K/AKT signaling pathway, Notch signaling pathway and the JAK/STAT signaling pathway[18]. While these signaling pathways are activated by their respective ligands and cytokines, non-coding RNA signaling also plays a large role in their regulation[19].

Non-coding RNAs

When the human genome project was completed, researchers found that less than 2% of the human genome is composed of protein-coding genes[20]. The large majority of non-protein coding genes are still actively transcribed and make up a new class of molecule, non-coding RNAs (ncRNA). There are many different species of ncRNA that are commonly divided based on size into short noncoding RNA and long-noncoding RNAs (lncRNA).

To date, the most commonly studied ncRNA are microRNAs, a short (approximately 22 nucleotides) noncoding RNA molecules that binds specifically to a 6-9 base pair seed sequence in the 3' untranslated region (UTR) of messenger RNAs. miRNAs are transcribed primarily by RNA polymerase Ⅱ, and after nuclear processing and cleavage are exported to the cytosol. In the cytosol miRNA precursors undergo further cleaving before being incorporated into the RNA induced silencing complex (RISC). miRNAs then guide the RISC to specific mRNAs through binding to the 3'UTR seed sequence, and silence the mRNA through degradation or inhibition of translation *via* steric hindrance[21]. Since their discovery, miRNAs have been widely studied in cancer and disease, and have been shown to regulate numerous physiological processes at the post-transcriptional level.

LncRNAs are an abundant type of ncRNA that is increasingly well studied. lncRNA are defined as any non-coding RNA that is > 200 base pairs in length. The majority of lncRNAs are transcribed by RNA polymerase Ⅱ, followed by polyadenylation, splicing, and the addition of a 5' Cap. lncRNA have great functional diversity, and interact with genomic DNA, other non-coding RNAs (including miRNA), mRNA and proteins while including functioning as epigenetic modulators, scaffold molecules, or as competitive endogenous sponges[22].

Despite the prevalence and high morbidity of RILF, very few studies have interrogated the processes of RILF development. It is increasingly evident that RILF occurs through a model of progressive macrophage polarization and temporal regulation of cytokines and ncRNAs in complex signaling pathways. Therefore, it is critical that further studies are performed using *in vivo* models of RILF to determine the intricate mechanisms behind RILF. Here, we review the function of macrophages in RILF with a focus on the roles of alternatively activated M2 macrophages and discuss the involvement of ncRNAs in RILF development and progression.

MACROPHAGES PROMOTE LUNG FIBROSIS

Lung fibrosis is caused by repetitive injury to the epithelial cells lining the alveoli, resulting in scarring of the lung tissue. A delicate balance between pro- and anti-inflammatory macrophages maintains the physiological roles of wound healing and inflammation, and is necessary to avoid fibrosis. Animal studies and clinical data suggest that M1 macrophages are associated with pneumonitis, and alternatively activated M2 and M2-like macrophages are associated with lung fibrosis.

Role of M2 macrophages in lung fibrosis in animal models

Animal models have been commonly used to demonstrate the involvement of alternatively activated alveolar macrophages in the development and progression of lung fibrosis. These studies provide insights into the macrophage polarization pathways and identify the crucial roles of M2 and M2-like macrophages in lung fibrosis.

One animal model used to study the mechanisms of lung fibrosis is Cu,Zn-SOD$^{-/-}$ mice, which do not develop fibrosis[23]. In wild-type populations, mitochondrial Cu,Zn-superoxide dismutase (Cu,Zn-SOD)-mediated production of H_2O_2 leads to DNA damage and the development of lung fibrosis. Cu,Zn-SOD is able to facilitate the nuclear translocation and enhance the transcriptional activation of STAT6 through the modulation of the redox status of Cys^{528} in the SH2 domain of STAT6, resulting in polarization of macrophages towards the M2 phenotype. Furthermore, CuZnSOD-transgenic (Cu,Zn-SOD^{Tg}) mice, which have more than a 3-fold increase of Cu,Zn-SOD protein expression and activity, had high activation of pro-fibrotic signaling pathways in their lungs. This resulted in a pro-fibrotic microenvironment and enhanced development of lung fibrosis. These data describe the role of Cu,Zn-SOD in M2 polarization, and suggest that polarization of macrophages towards the M2 phenotype in a Th2-independent manner contributes to the development of lung fibrosis[24].

In a recent study, conditional Src homology domain-containing tyrosine phosphatase (Shp2) mutant mice were generated by selective removal of Shp2 in monocytes/macrophages through lysozyme M promoter-driven Cre recombinase. These mice were used to investigate the role of Shp2 in M2 polarization and the development of lung fibrosis. When Shp2 knockout was induced in mature mouse macrophages, IL-4 mediated M2 polarization was increased. Moreover, arginase activity, a marker of M2 polarization, was enhanced in Shp2 conditional mutant mice after intraperitoneal (i.p.) injection of chitin, an inducer of

M2 macrophage polarization. Finally, when treated with bleomycin, Shp2 mutant mice were more susceptible to develop inflammation and lung fibrosis compared to control mice. Further mechanistic studies showed that loss of Shp2 leads to the association of JAK1 with IL-4Rα, causing IL-4-mediated JAK1/STAT6 activation and eventually M2 polarization. These data suggest that Shp2 is necessary to prevent the development of lung fibrosis through the inhibition of M2 polarization[25].

To investigate the function of increased IL-9 in lung fibrosis, Arras *et al*[26] compared the development of fibrosis in transgenic IL-9 overexpressing C57BL/6 mice *vs* their wild-type counterparts after 5 mg silica (DQ12) was implanted intratracheally. When the mice were sacrificed after 4 and 6 mo of DQ12 implantation, the transgenic mice had a decreased level of silica-induced lung fibrosis compared to their wild-type counterparts as evidenced by the histologic examination and measurement of lung hydroxyproline content. To further confirm the effect of increased IL-9 production in fibrosis, they compared wild-type C57BL/6 mice that were i.p. injected either with recombinant IL-9 or with PBS three times a week for 2 mo to mice that had been implanted with DQ12 and also received IL-9 during the second month of implantation. Mice were sacrificed after 2, 4 and 6 mo of DQ12 implantation and the results showed that IL-9 dosed mice had a decreased level of silica-induced lung fibrosis compared to their non-dosed counterparts. Moreover, the silica-induced fibrosis that developed in wild-type mice was associated with Th2 markers including the upregulation of IL-4 in inflammatory alveolar macrophages. On the other hand, in the IL-9 transgenic mouse model IL-4 levels were not changed significantly in macrophage populations. These results suggest that upregulation of IL-9 exerts an anti-fibrogenic effect in silica-induced lung fibrosis through the inhibition of M2 macrophage polarization[26].

Studies of a mouse model with long-term over-expression of IL-10 provided evidence for the role of IL-10 in the induction of lung fibrosis. Bronchoalveolar lavage (BAL) samples of mice overexpressing IL-10 had enhanced infiltration of T and B-lymphocytes as well as increased number of collagen-producing cells and fibrocytes. Additionally, CCR2 and its ligand, CCL2 were found to be significantly upregulated in IL-10 overexpressing mice. Interestingly, the proportion of M2 macrophages was significantly higher in both BAL and whole lung tissue of IL-10 overexpressing mice compared to wild-type ones, and administration of anti-CCL2 antiserum decreased fibrosis. These results suggest that chronic overexpression of IL-10 in mice causes the induction of lung fibrosis, increased M2 macrophages and high levels of CCR2 and CCL2[27].

Clinical data supporting the role of M2 macrophages in lung fibrosis

Clinical data also support the role of alternatively activated alveolar macrophages in lung fibrosis. Alveolar macrophages of patients with lung fibrosis had increased expression of CD206 as well as enhanced production of pro-inflammatory cytokines CCL17, CCL18 and CCL22. To confirm M2 polarization during lung inflammation, they stimulated normal human alveolar macrophages *in vitro* with Th2 cytokine IL-4 by itself or with both IL-4 and IL-10. Stimulation with IL-4 induced the polarization towards the M2-phenotype, demonstrating that healthy alveolar macrophages could be induced for M2 polarization. Furthermore, co-stimulation with IL-10 increased the IL-4 mediated expression of CCL18 and IL-1RA and decreased the production of CCL17 and CCL22. Additional data showed that IL-10 differentially regulates CCL18 and IL-1RA expression *in vitro,* suggesting that it effects IL-4 polarization of M2 macrophages[28].

Patients with lung fibrosis have increased *CCL18* gene and protein expression, which is a marker of alternative activation. Expression of CCL18 is increased in normal alveolar macrophages when they were stimulated with Th2 cytokines or co-cultured with human lung fibroblasts. In addition, co-culture of normal lung fibroblasts with cell supernatants from alternatively activated alveolar macrophages of lung fibrosis patients increased collagen production compared to co-culture of cell supernatants from alveolar macrophages of healthy individuals. These results indicate that alveolar macrophages of patients with lung fibrosis display an alternative activation phenotype[29].

A recent study investigated the role of macrophages in acute exacerbation (AE) of idiopathic pulmonary fibrosis (IPF). The levels of several cytokines including IL-1ra, CCL2, CCL17, CCL18, CCL22, TNF-α, IL-1β, CXCL1 and IL-8 were measured in BAL fluids of IPF patients with or without AE. The results showed that pro-inflammatory cytokines CXCL1 and IL-8 as well as all tested M2 cytokines were upregulated in BAL-cells from IPF patients. Furthermore, they found that increased production of CCL18 in BAL-cells were a strong indicator for the development of AE. Their data suggest that M2 macrophage activation has a large impact on the development of IPF acute exacerbation[30].

M2 macrophages in RILF

To test the hypothesis that classically activated macrophages (M1) are associated with radiation-induced pneumonitis and alternatively activated macrophages (M2) are associated with RILF, C57BL/6 mice were treated with 12 Gy of X-rays to their thoracic region. Control and irradiated mice were sacrificed at different time-points ranging from 1 h to 24 wk, and the expression of M1 and M2 markers, inducible nitric oxide synthase (iNOS) and arginase type 1 (Arg-1) respectively, were assessed at the mRNA and protein level. The analyses showed the enhanced expression of iNOS at the early time-points post irradiation (the pneumonitis stage), and enhanced expression of Arg-1 at later time points (the fibrotic phase). Further experiments confirmed that these markers were differentially expressed and were specific to macrophages[31]. The same group in a similar

study showed that the expression of factors that are associated with Th2-immune response including GATA-3, hydroxyproline, IL-3, and Arg-1 reached their maximum levels at week 16 post-irradiation. These data indicate that thoracic irradiation first induces Th1 polarization during pneumonitis, followed by transition to Th2 polarization at later time points that correspond to fibrosis. While the immediate response to irradiation involves classically activated macrophages, alternatively activated macrophages are predominant during fibrosis and likely have an important role in its development and progression[32].

Ym-1 positive M2 macrophages were shown to be elevated in response to radiation in murine lungs. Furthermore, fibrotic lungs had higher level of gal-3 expression, which is mainly expressed by M2 macrophages. The proliferation of fibroblasts and expression of α-SMA was induced by mouse recombinant gal-3. *In vitro* studies showed that when gal-3 was knocked-down the radiation-induced expression of YM-1 in Raw 264.7 cells was also decreased, indicating that gal-3 might be involved in the development of RILF through the regulation of the alternative activation of M2 macrophages[33].

The Th2-type immune system response has been suggested to be involved in the development of lung fibrosis[34,35]. IL-4, which is a Th2 cytokine, is induced in fibrosis and enhances extracellular matrix protein synthesis, including collagen and fibronectin[36], polarization of T cells toward the Th2 phenotype, and alternative activation of macrophages[37]. Furthermore, when rats were treated with a single 20 Gy dose of IR to the thoracic region, IL-4 levels increased and reached their plateau 3 wk after irradiation. The cellular origin of IL-4 was determined to be mainly the macrophages[38]. To study the role of radiation in the activation of type-2 immune response, mice with Lewis lung cancer received radiation therapy alone or radiation combined with Th1 immunomodulator unmethylated cytosine-phosphorothioate-guanine containing oligodeoxynucleotide (CpG-ODN). The mice were sacrificed 3-wk post-irradiation and the immune status of tumors as well as the histological grade of lung fibrosis was assessed. Lung fibrosis in tumor-bearing mice was more progressed compared to normal mice after radiation treatment. Th2-immune response associated markers hydroxyproline and gata-3 had higher expression in the lung tissues of tumor-bearing mice post-radiation. Furthermore, CpG-ODN weakened fibrosis by significantly decreasing gata-3 expression. The levels of IL-13 and IL-5 were increased, while the levels of INF-γ and IL-12 expression was decreased in irradiated tumor-bearing mice, which was reversed with CpG-ODN treatment. These results indicate that Type-2 immunity in tumors effects the development and progress of RILF[39] and provides further evidence for the association of M2 macrophages, Th2 immune system and development of RILF.

To investigate the role of fractionated irradiation (FIR) on macrophage recruitment and phenotype, human monocyte derived-macrophages were treated with a FIR of 2 Gy/d, mimicking the radiotherapy that cancer patients would go through. Although their DNA was damaged upon irradiation, the macrophages remained viable and metabolically active and at 10 Gy cumulative dose a significant increase in pro-inflammatory macrophage markers CD80, CD86 and HLA-DR was observed, while anti-inflammatory markers CD163, MRC1, VCAN and IL-10 decreased. These results suggest that FIR facilitates the polarization of macrophages towards pro-inflammatory phenotype[40].

Accumulating evidence clearly indicate the role of alternative activation of M2 and M2-like macrophages in development and progress of lung fibrosis; however, the mechanisms leading to M2 polarization are poorly identified and further work is necessary to detail the role of M2 macrophages in RILF.

NON-CODING RNA IN FIBROSIS

While non-coding RNAs have been well characterized in cancer, relatively few have been shown to directly influence RILF. In addition to directly affecting known fibrotic signaling pathways, it is likely that non-coding RNA regulation of macrophage polarization and induction of pro-fibrotic alternatively activated macrophages affects RILF. Therefore, we will focus on miRNAs and lncRNAs with defined roles in RILF, pulmonary fibrosis and alternative activation of alveolar macrophages (Figure 1). Please refer to the cited reviews for a broader review of the roles of non-coding RNAs in fibrosis[41-43] and macrophage polarization[19].

miRNA in RILF

To study the roles of miRNAs in RILF, Xie *et al*[44] treated adult male rats with 20 Gy of radiation. Using miRNA microarray analysis, they examined the differential miRNA expression at 3, 12, and 26 wk post-irradiation. While the authors observed minor physiological changes after 3 wk, significant fibrosis was not seen until the 26-wk time point. At the 3-wk time point, the authors found 7 upregulated miRNAs and 4 downregulated, at 12 wk 10 upregulated and 4 downregulated, and at 26 wk 7 upregulated and 4 downregulated. The authors validated the expression levels of only two miRNAs, miR-21 and let-7i and identified novel temporal regulation of miRNAs during fibrosis initiation and progression. At the 3-wk time-point let-7i was highly expressed and miR-21 had low expression, whereas after 26 wk let-7i expression decreased and miR-21 was upregulated. Furthermore, let-7i targets TGFBR1, inhibiting TGF-β signaling, while miR-21 degrades the TGF-β inhibitor SMAD7. Moreover, miR-21 has been shown to be upregulated in several models of fibrosis[45,46], and to be activated by pro-fibrotic TGF-β. The authors postulate that the temporal regulation of let-7i and miR-21 is a mechanism for regulating TGF-β signaling. Early inhibition of TGF-β allows for lung regeneration and healing, followed by an

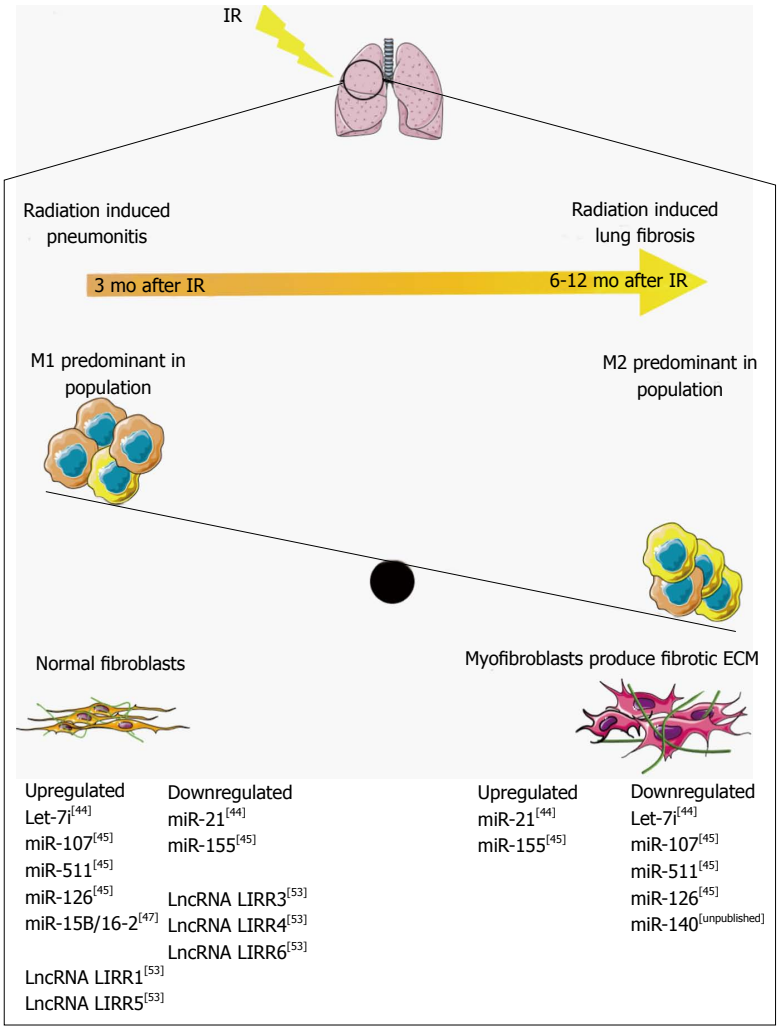


Figure 1 Non-coding RNAs regulation of macrophage polarization in radiation-induced lung fibrosis. Radiation pneumonitis develops approximately 3 mo after ionizing radiation, resulting from the accumulation of M1 macrophages. Six to twelve months after ionizing radiation (IR) the accumulation of M2 macrophages activates myofibroblast differentiation, resulting in a fibrotic microenvironment and radiation-induced lung fibrosis (RILF). Non-coding RNAs regulate the pathways involved in RILF, and are temporally regulated through RILF development and progression. ECM: Extracellular matrix. This figure was produced using Servier medical art, available from http://www.servier.com/Powerpoint-image-bank.

increase in TGF-β signaling that leads to lung fibrosis[44].

Kalash *et al*[47] performed similar animal model time-point studies. They irradiated C67BL/6NTac mice with 20 Gy to the thoracic area, and at 2, 14, 28, 75, 110, 150 and 200 d post-irradiation isolated RNA from the lungs of irradiated and control mice. Using RT-PCR, they examined miRNA expression, focusing on miRNAs known to target toll-like receptor 4 (*Tlr4*, an activator of the innate immune system), vascular endothelial growth factor α (*Vegfa*, a pro-angiogenic factor), and TGF-β, three transcripts that they had observed to be differentially expressed in irradiated tissues. They found that miR-107 and miR-511, both of which are known to target *Tlr4*, were upregulated in the first 50 day after irradiation, followed by downregulation during the later time points that correspond with fibrosis. miR-126 has been shown to stimulate expression of *Vegfa*, and was upregulated during the immediate post-irradiation inflammatory phase, but expression decreased during later stages. Interestingly, the levels of *Vegfa* remained elevated throughout the entire time course, indicating that miR-126 regulation of *Vegfa* is not important during RILF, and that miR-126 may have some other action in the development of RILF. Finally, miR-155, which targets *Tgfb*, was decreased during the early inflammatory phase, but starting at day 75 miR-155 levels were increased. While *Tgfb* expression matches that of miR-155 before day 75, it is upregulated in conjunction with miR-155 in the late fibrotic phase. This study demonstrates the significant differences in several distinct miRNAs that happens both in the inflammatory phase immediately post-irradiation, and in the late term fibrotic phases. It also demonstrates complexity of miRNA-mRNA regulatory networks in RILF. As the expression changes of mRNA and miRNA do not always match their previously defined functions, this points to the involvement of other currently unknown factors.

Ezzie *et al*[48] directly used irradiated cell models to examine miRNA function. miR-15b/16-2 was previously shown to be upregulated in fibrotic regions of human lung tissue, and to target TGF-β signaling inhibitor SMAD7. To examine the role of miR-15b/16-2 in radiation-induced fibrosis, Rahman *et al*[49] treated human lung epithelial cells with 1 Gy of radiation, and after 24 h isolated RNA for RT-PCR. In contrast to previous findings showing chronic upregulation of miR-15b/16-2 in fibrotic tissue, they showed that miR-15b/16-2 was induced directly after irradiation followed by a gradual decrease. Moreover, they identified Wip1 as a novel target of miR-15b/16-2. Wip1 inactivates the

DNA damage response through dephosphorylation of p53, γ-H2AX, and ATM. Upregulation of miR-15b/16-2 therefore activates the DNA damage response, protecting cells from radiation damage. It is likely that while miR-15b/16-2 is initially activated to protect the cell from radiation-induced fibrosis, it's activation results in increased TGF-β signaling through degradation of SMAD7.

We have recently shown that miR-140 plays a key role in protection of RILF[50]. We irradiated the thoracic region of C57BL/6 mice with 13 Gy, and after 1 year sacrificed them for histological examination. RNA *in situ* staining using a DIG labeled probe revealed that irradiated lung tissue had higher expression of miR-140, and through *in vitro* characterization we found that miR-140 expression is activated by nuclear factor-erythroid2-related factor 2 (NRF2). NRF2 is a transcription factor that activates antioxidant and anti-inflammatory genes, and is upregulated in response to the free radicals created after IR[50]. Recently we have continued these studies, and found that lung tissue with RILF isolated from C57BL/6 mice one year after 13 Gy of radiation had significantly abrogated miR-140 expression. Moreover, we treated lung fibroblasts isolated from wild-type and miR-140 knock-out mice[51] with 20 Gy of radiation, and demonstrated that miR-140 knock-out fibroblasts were more susceptible to radiation-induced fibrosis as evidenced by increased myofibroblast population, TGF-β expression and enhanced contraction ability (unpublished data). miR-140 has been demonstrated to target the TGF-β pathway through degradation of SMAD3 in chondrocytes and TGF-β receptor TGFBR1 in preadipocytes[52]. We have also identified that miR-140 also targets fibronectin, a key member of the extracellular matrix, in pulmonary fibroblasts (unpublished data). This indicates that miR-140 regulates RILF in multiple ways, both through inhibition of myofibroblast differentiation and through degradation of fibronectin, which is highly upregulated in fibrotic tissue and a key factor in development of the stiff extracellular matrix.

lncRNAs in RILF

One study has examined the expression changes of lncRNAs in response to lung irradiation. Twenty-four hours after exposing C57BL/6 mice to 12 Gy of whole body irradiation, lncRNAs LIRR1 and LIRR5 were upregulated whereas LIRR2, 3, 4, and 6 were downregulated. The authors chose lncRNA LIRR1 for further study. LIRR1 is on chromosome 1, and is co-expressed with 27 coding genes. Through *in vitro* studies using the human bronchial epithelial cell line BEAS-2B, lncRNA LIRR1 upregulation was validated, and they demonstrated that overexpression of LIRR1 affects the radiosensitivity of BEAS-2B cells. After exposing BEAS-2B to 4 Gy of IR, they found decreased cell survival, and an increased number of cells in the G1 phase. To further interrogate DNA repair, they examined γ-H2AX foci formation and found it was significantly increased in irradiated cells that overexpressed LIRR1. Finally, LIRR1 overexpression decreased the protein expression of several DNA Repair proteins, including KU70, KU80, and RAD50. While the authors suggest that LIRR1 mediates the cellular DNA damage response after irradiation, the more specific mechanisms of this regulation remain unknown[53].

CONCLUSION

It is clear that both macrophages and ncRNA are critical for the initiation and progression of radiation induced lung fibrosis. While radiation first induces inflammatory M1 macrophages leading to pneumonitis, dysregulation of M1 inflammatory signaling pathways leads to the transition to M2 macrophages, prompting the development of fibrosis. Non-coding RNAs appear to have distinct and critical roles in this transition and the development of RILF through regulation of the key inflammatory and fibrotic signaling pathways. However, while fibrosis continues to be thoroughly investigated, there are few published studies examining the specific mechanisms occurring due to exposure to radiation that lead to fibrosis. RILF is an important and deadly pathology of thoracic irradiation, however the lack of mechanistic information means there are no effective treatments. Due to the difficulty and length of *in vivo* radiation studies, most investigations of the mechanisms of fibrosis use methods other than radiation to induction fibrosis. However, long-term radiation work is necessary to truly investigate the mechanisms of RILF. These studies will likely lead to the discovery of key molecular targets in the protection and treatment of RILF.

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