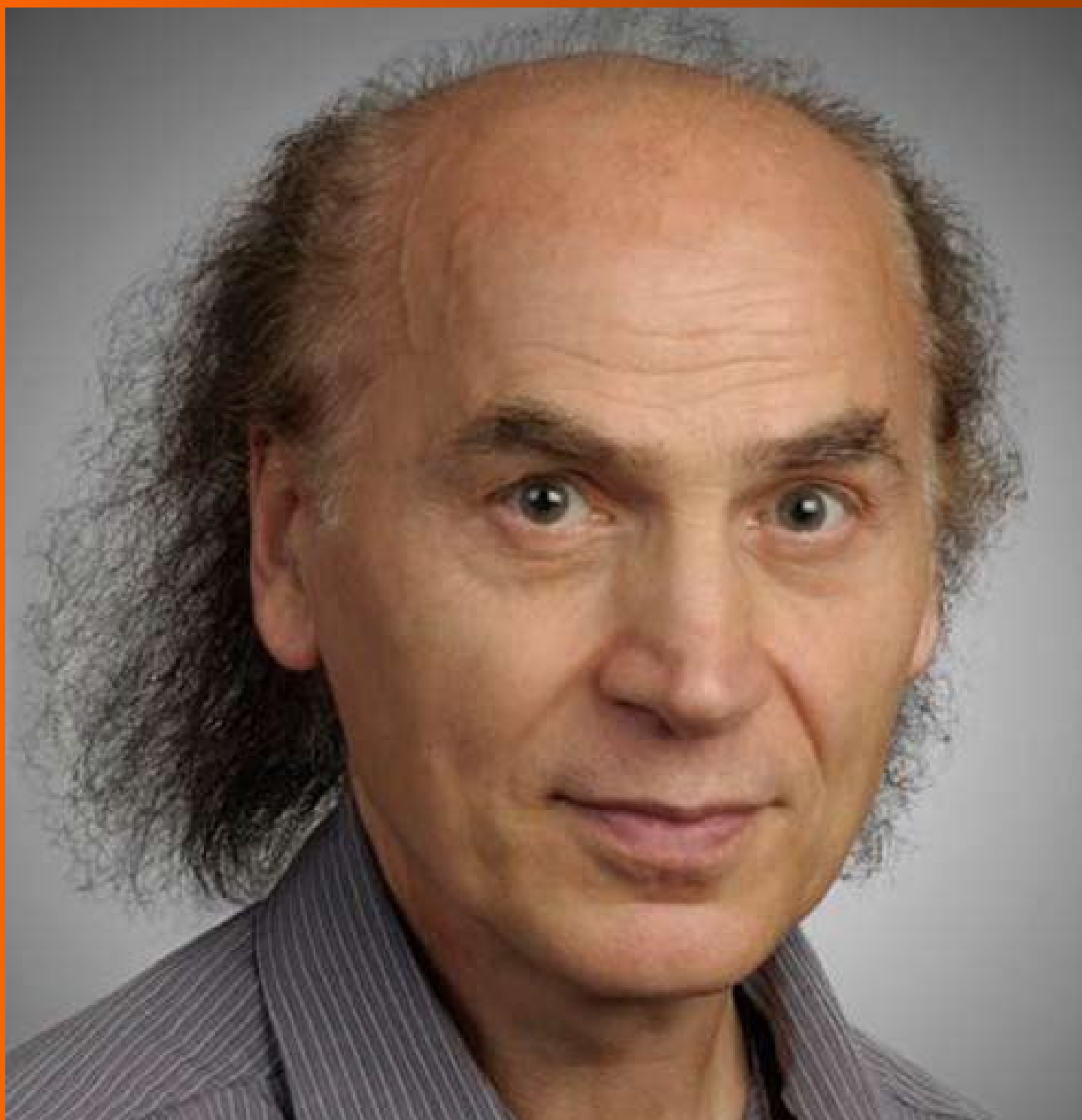
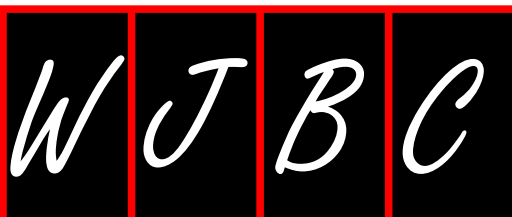


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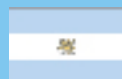
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Mechanism of DNA damage tolerance

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Abstract

DNA damage may compromise genome integrity and lead to cell death. Cells have evolved a variety of processes to respond to DNA damage including damage repair and tolerance mechanisms, as well as damage checkpoints. The DNA damage tolerance (DDT) pathway promotes the bypass of single-stranded DNA lesions encountered by DNA polymerases during DNA

replication. This prevents the stalling of DNA replication. Two mechanistically distinct DDT branches have been characterized. One is translesion synthesis (TLS) in which a replicative DNA polymerase is temporarily replaced by a specialized TLS polymerase that has the ability to replicate across DNA lesions. TLS is mechanistically simple and straightforward, but it is intrinsically error-prone. The other is the error-free template switching (TS) mechanism in which the stalled nascent strand switches from the damaged template to the undamaged newly synthesized sister strand for extension past the lesion. Error-free TS is a complex but preferable process for bypassing DNA lesions. However, our current understanding of this pathway is sketchy. An increasing number of factors are being found to participate or regulate this important mechanism, which is the focus of this editorial.

Key words: DNA damage tolerance; Template switching; DNA damage bypass; DNA replication; Replicative stress; Translesion synthesis; Ubiquitination; Sumoylation

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Core tip: DNA damage may compromise genome integrity and lead to cell death. Cells have evolved a variety of processes to respond to DNA damage including damage repair and tolerance mechanisms. The DNA damage tolerance (DDT) pathway promotes the bypass of single-stranded DNA lesions encountered by DNA polymerases during DNA replication. This prevents the stalling of DNA replication. Two mechanistically distinct DDT branches, translesion synthesis and template switching have been characterized. However, our current understanding of DDT is far from complete and that of template switching is especially sketchy. This editorial focuses on recently identified components and regulators of DDT.

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DNA DAMAGE TOLERANCE MECHANISMS

The genomic DNA of a living organism is subject to damage by both internal and external chemical and physical agents, leading to various types of lesions. Cells employ DNA damage repair and tolerance/bypass mechanisms as well as damage checkpoints to respond to these lesions^[1]. DNA double-stranded breaks (DSBs) can be repaired by homologous recombination (HR) or non-homologous end joining, while single-stranded DNA (ssDNA) lesions are subject to repair by nucleotide excision repair, base excision repair, or DNA mismatch repair^[2-6]. DNA damage such as base modification, if left unrepaired in S phase of the cell cycle, stalls the progression of DNA replication, because the replicative polymerases are not able to recognize the modified bases and use them as template for nucleotide incorporation. The DNA damage tolerance (DDT) mechanism (also known as DNA damage bypass or post-replication repair) enables DNA replication to circumvent the lesions, thereby allowing the completion of DNA replication, and leaving the damages to be repaired later^[7,8].

Recent studies indicate that DNA damage makes DNA synthesis on both the leading and lagging strands discontinuous as a result of the uncoupling of DNA polymerase and DNA helicase or the reinitiation of DNA synthesis at a distance from the lesion^[9-11]. This creates a ssDNA gap behind the replication fork with the DNA lesion at the 3' end of the gap (Figure 1A). This ssDNA gap can be filled by two distinct DDT mechanisms. One is translesion synthesis (TLS) in which the replicative DNA polymerase is temporarily replaced by a special TLS polymerase pol ζ or η that can replicate across DNA lesions^[12] (Figure 1B). As TLS polymerases lack proofreading activity and have flexible active sites that can recognize modified nucleotides, they may incorporate the wrong nucleotides (Figure 1B). As such, TLS synthesis is potentially mutagenic and error-prone. In fact, TLS is a major source of cellular mutagenesis. The other DDT process is template switching (TS) in which the stalled nascent strand switches temporarily to the newly synthesized undamaged sister strand for replication over the lesion (Figure 1C and D). Therefore, TS is an error-free process. Pairing between the two newly synthesized strands in TS is promoted by strand invasion (Figure 1C). The resulting structure is then turned into a sister chromatid junction (SCJ) after gap filling using the damage-free sister strand as a template (Figure 1D). SCJ is later resolved to yield two duplex DNA strands, completing the damage bypass process (Figure 1E). Note that both TLS and TS take place behind the

replication fork, and may happen during or after DNA replication. There is evidence suggesting that TS starts early in S phase, whereas TLS doesn't start until late S phase^[13-15].

The sliding DNA clamp PCNA plays a central role in DDT regulation as its post-translational modifications act as a molecular switch to control the choice of DDT pathways^[16]. PCNA is a ring-shaped homotrimer that is loaded to primed DNA template by the clamp loader RFC. It encircles DNA to act as a sliding platform for recruiting many factors involved in DNA replication and DNA damage response. For example, PCNA interacts with replicative DNA polymerases (ϵ and δ) to serve as their processivity factor. DNA damage such as base modification encountered by the replication fork induces the ubiquitination of PCNA^[17] (Figure 2). PCNA is first mono-ubiquitinated at lysine 164 (K164) by the E2-E3 ubiquitinase Rad6/Rad18^[17], and can be further poly-ubiquitinated *via* the formation of K63-linked ubiquitin chains by another E2-E3 ubiquitinase Rad5/Ubc13/Mms2^[17] (Figure 2). Mono-ubiquitinated PCNA specifically promotes TLS as it preferentially binds TLS polymerase ζ or η , which is believed to facilitate the replacement of replicative polymerases with TLS polymerases^[16,18] (Figure 2). On the other hand, poly-ubiquitinated PCNA activates the Rad5-dependent error-free TS pathway (Figure 2), but the underlying mechanism has yet to be elucidated^[16].

COMPONENTS OF THE DDT MACHINERY AND CROSS-TALK BETWEEN DDT AND OTHER DNA DAMAGE RESPONSE MECHANISMS

More and more factors are being implicated in DDT, especially the error-free TS branch of DDT (Table 1). The fact that many of these factors are known to also function in other DNA damage repair and checkpoint mechanisms suggests an extensive crosstalk between DDT and these pathways.

MRX complex has 3'-5' exonuclease activity and ssDNA endonuclease activity, and is known to act in DSB recognition and processing^[19]. Recent epistasis analyses place MRX in both the TLS and TS branches of DDT^[20]. Moreover, there is evidence for a physical interaction between MRX and Rad18^[20]. These findings suggest that MRX acts early in DDT, likely to process the 3' end of the ssDNA gap at stalled replication fork and help recruit Rad18^[20].

Ino80 chromatin remodeling complex is known to associate with replication forks and aid in fork progression and recovery of stalled forks^[21-23]. Ino80 was also found to facilitate Rad18 recruitment to stalled forks, PCNA poly-ubiquitination, Rad51 recruitment and formation of SCJ intermediate of error-free DDT^[23]. Therefore, Ino80 plays a role in error-free DDT during DNA replication in S phase. Whether it also

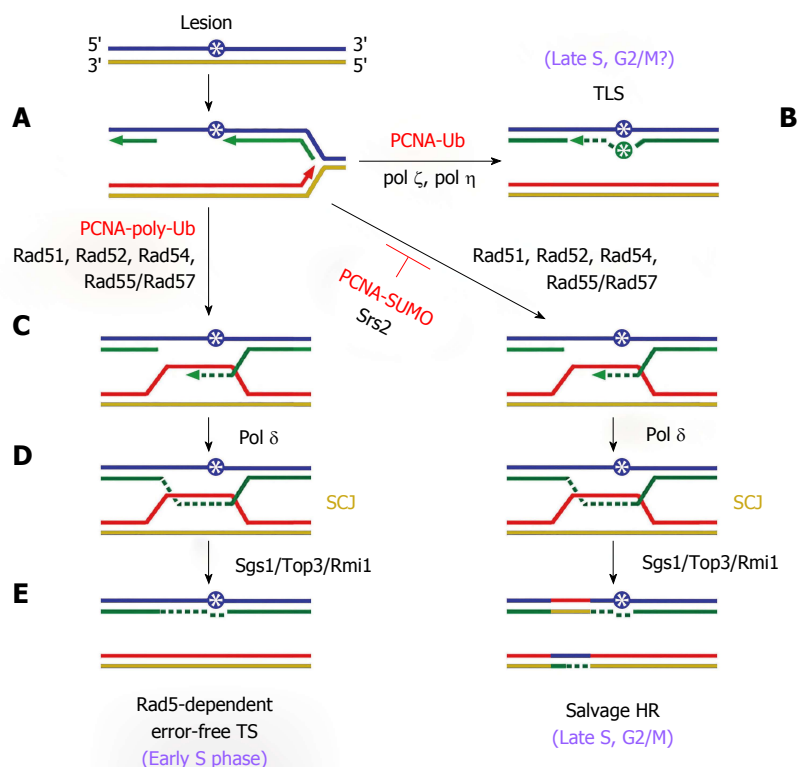


Figure 1 DNA damage tolerance mechanisms. DNA damage (asterisk in blue circle) stalls DNA replication. A: Reinitiation of DNA synthesis results in the formation of a ssDNA gap. Shown is a ssDNA gap on the lagging strand; B: PCNA mono-ubiquitination induces a switch from replicative polymerase to a TLS polymerase Pol ζ or Pol η , resulting in gap filling via TLS, which may incorporate the wrong nucleotide (asterisk in green circle); C-E: PCNA poly-ubiquitination activates the Rad5-dependent error-free TS pathway. Gap filling is achieved by strand invasion mediated by Rad51, Rad52, Rad54, and Rad55/Rad57 and repair synthesis carried out by Pol δ (C), followed by the formation of SCJ (D), and resolution of the SCJ by Sgs1/Top3/Rmi1 (E). DDT can also proceed through the salvage HR pathway that also produces the SCJ intermediate. The salvage pathway is hyper-recombinogenic and prone to crossover as indicated. It is normally inhibited by sumoylated PCNA and Srs2. G2: Gap 2; M: Mitotic; PCNA: Proliferating cell nuclear antigen; S: Synthesis; SUMO: Small ubiquitin-like modifier; TLS: Translesion synthesis; SCJ: Sister chromatid junction; Ub: Ubiquitin.

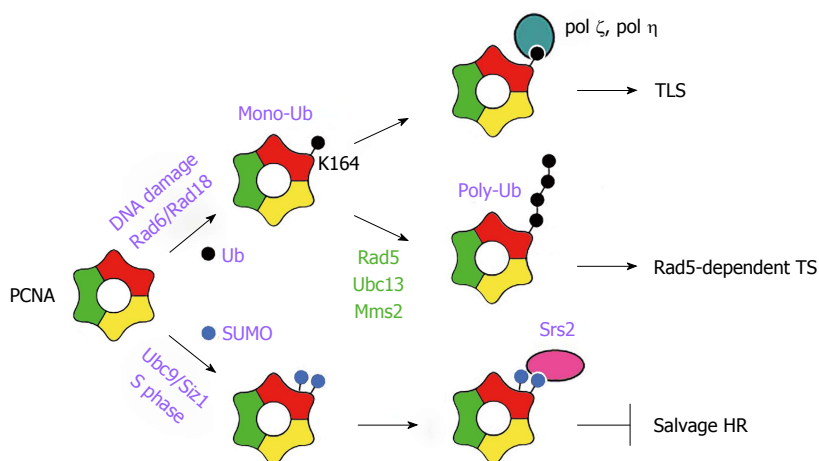


Figure 2 Regulation of DNA damage tolerance by proliferating cell nuclear antigen modification. PCNA is a homotrimer that can be modified at K164 by either ubiquitin or SUMO. It can also be sumoylated at K127. PCNA mono-ubiquitination (mono-Ub) by Rad6/Rad18 facilitates the recruitment of TLS polymerases thereby promoting TLS. Extension of mono-Ub with a K63-linked Ub chain (poly-Ub) by Rad5/Ubc13/Mms2 promotes Rad5-dependent error-free TS pathway. PCNA sumoylation recruits Srs2 that inhibits the salvage HR pathway. HR: Homologous recombination; K: Lysine; PCNA: Proliferating cell nuclear antigen; S: Synthesis; SUMO: Small ubiquitin-like modifier; TLS: Translesion synthesis; TS: Template switching; Ub: Ubiquitin.

plays a role in TLS has not been examined.

Exo1 is a major 5'-3' nuclease responsible for the resection of dsDNA break ends, and functions in DNA damage repair, recombination, replication, and

telomere integrity^[24,25]. There is increasing genetic evidence implicating Exo1 and its nuclease activity in error-free TS pathway^[20,26-28]. Moreover, Exo1 was found to be required for the formation of SCJ

Table 1 Components and regulators of DNA damage tolerance¹

Factor/complex	Functions/properties
Rad6/Rad18	E2 ubiquitin conjugase/E3 ubiquitin ligase complex; promotes mono-ubiquitination of PCNA at lysine 164 (PCNA-K164)
Rad5/Mms2/Ubc13	E3 ubiquitin ligase/E2 ubiquitin conjugase complex; promotes poly-ubiquitination of PCNA-K164; has ATPase activity
Pol30	Forms a homotrimer (PCNA); its ubiquitination and sumoylation regulates the choice of DDT mechanism
Ubc9/Siz1	E2 SUMO conjugase/E3 SUMO ligase complex; promotes PCNA sumoylation
DNA polymerase ζ	TLS polymerase; consists of subunits Rev3, Rev7, Pol31 and Pol32 with Rev3 being the catalytic subunit
Rev1	dCMP transferase; required for TLS by Pol ζ
DNA polymerase η (Rad30)	TLS polymerase
DNA polymerase δ (Pol3/Pol31/Pol32)	Lagging strand DNA polymerase; Pol3 is the catalytic subunit; promotes repair synthesis in error-free DDT; Pol32 is not essential for DNA replication but is required for repair synthesis
Rad51	Components of HR responsible for homology search and strand invasion; Rad51 molecules bind ssDNA to form a presynaptic filament important for subsequent strand invasion; both Rad55 and Rad57 are paralogs of Rad51; Rad55/Rad57 stabilizes Rad51
Rad52	nucleoprotein filament and inhibits Srs2 helicase activity
Rad54	
Rad55/Rad57	
Srs2	3'-5' helicase and translocase; disrupts Rad51 nucleoprotein filaments; inhibits salvage HR pathway thereby channeling DNA lesions to Rad5 pathway
Sgs1/Top3/Rmi1	Resolves SCJ intermediates generated in error-free DDT
RFA (replication factor A) (Rfa1/Rfa2/Rfa3)	ssDNA binding complex; is involved in DNA replication, repair and recombination; RFA-ssDNA nucleofilament helps recruit Rad18
Exo1	5'-3' exonuclease; promotes resection of dsDNA break ends; functions in DNA damage repair, recombination, replication, and telomere integrity; is required for SCJ formation in error-free DDT
9-1-1 complex (Ddc1/Mec3/Rad17)	Ring shaped DNA clamp; is involved in DNA check point signaling; facilitates Pol ζ recruitment; is required for SCJ formation in error-free DDT; interacts with Exo1
MRX complex (Mre11-Rad50-Xrs2)	3'-5' exonuclease and ssDNA endonuclease; is potentially involved in both TLS and TS branches of DDT; interacts with Rad18
Shu complex (Shu1/Shu2/Csm2/Psy3)	Is involved in error-free DDT; interacts with Rad55/Rad57
Ino80	Chromatin remodeler; associates with replication forks and promotes fork progression; required for Rad18 and Rad51 recruitment to replications forks, PCNA poly-ubiquitination and SCJ formation
Elg1	Unloads PCNA from chromatin during DNA replication; regulates choice of DDT pathway
Hmo1	HMGB homolog; binds ssDNA and DNA with an altered conformation; bends DNA; interacts with Elg1; may help channel DNA lesions to Rad5 pathway; required for SCJ formation in S phase
Fun30	Chromatin remodeler; facilitates long-range resection of dsDNA ends; potentially regulates choice of DDT pathway

¹Listed are DDT factors in *S. cerevisiae*. ATPase: Adenosine triphosphatase; dCMP: Deoxycytidine monophosphate; DDT: DNA damage tolerance; dsDNA: Double stranded DNA; HMGB: High-mobility group protein B; HR: Homologous recombination; K: Lysine; PCNA: Proliferating cell nuclear antigen; RFA: Replication factor A; S: Synthesis; SCJ: Sister chromatid junction; ssDNA: Single stranded DNA; SUMO: Small ubiquitin-like modifier; TLS: Translesion synthesis; TS: Template switching; Ub: Ubiquitin.

intermediate of error-free DDT^[27].

9-1-1 complex resembles PCNA in assuming a ring structure that can encircle DNA^[29]. 9-1-1 has been long established as a DNA damage sensor and a component of checkpoint signaling. It also plays a role in TLS by facilitating the recruitment of TLS polymerase pol ζ ^[30]. Recent genetic evidence also implicates 9-1-1 in error-free DDT^[28]. 9-1-1 is required for SCJ formation^[28]. 9-1-1's function in error-free DDT is separate from its canonical role in G1/S and S phase checkpoint signaling. 9-1-1 is loaded by Rad24 to 5' end of ssDNA region/gap and is known to associate with many checkpoint and repair proteins^[29]. Recently, 9-1-1 was found to also bind Exo1 and stimulate its end resection activity^[28,31]. This raises the possibility that 9-1-1 recruits Exo1 to the 5' end of the ssDNA gap behind stalled replication fork to extend the gap^[28].

Error-free DDT is intimately linked to HR as a subset of HR factors has been shown to play roles in it. These include Rad51, Rad52, Rad54, Rad55 and Rad57 that are believed to carry out homology search, strand

invasion and pairing of newly synthesized strands from the two sister chromatids^[27,32,33]. Rad51 molecules bind the stalled nascent DNA strand to form a presynaptic filament that is later involved in strand invasion^[34], and the Rad51 paralogs Rad55 and Rad57 form a complex to facilitate the formation or maintenance of Rad51 filament^[35,36].

DNA polymerase δ carries out DNA synthesis for gap filling after the stalled strand switches template during error-free DDT^[27]. This results in the formation of a SCJ (Figure 1D) that is resolved by Sgs1/Top3/Rmi1 complex, completing the error-free DDT process^[37-39].

SALVAGE HOMOLOGOUS RECOMBINATION PATHWAY OF DDT

Besides the Rad5-dependent error-free DDT pathway, there is an alternative HR-mediated DDT mechanism referred to as the salvage HR pathway that does not depend on PCNA ubiquitination^[27,28,40-42]. This pathway, if allowed to act during DNA replication, would generate

hyper-recombinogenic intermediates that may lead to chromosome rearrangement and genome instability. The existence of the salvage HR pathway was suggested by the finding that *RAD52* is a high copy suppressor of replicative stress-sensitivity of a mutant in which PCNA cannot be ubiquitinated^[40]. Consistently, deletion of the “antirecombinase” Srs2 suppresses the sensitivity of cells lacking Rad6 to DNA damage^[43]. This suppression is dependent on the HR repair pathway since it is blocked when *RAD51*, *RAD52*, *RAD54*, *RAD55* or *RAD57* is absent^[43]. Moreover, this suppression was found to be specific for Rad5-dependent DDT^[40,44-46]. These findings are consistent with the notion that the salvage HR pathway functions independently of the Rad5-dependent error-free TS pathway to bypass DNA lesions, and is normally hindered by Srs2.

Srs2 is a DNA helicase that has the ability to disrupt Rad51-ssDNA nucleofilament, and is thus called an “antirecombinase”^[47,48]. The role of Srs2 in inhibiting recombination during replication is closely linked to the sumoylation of PCNA at K164 or K127 by Ubc9/Siz1^[40,45]. Unlike PCNA ubiquitination that is induced by DNA damage, PCNA sumoylation is independent of DNA damage^[17]. Sumoylation of PCNA is cell cycle dependent as it only occurs in S phase. Srs2 was found to preferentially interact with sumoylated PCNA, leading to the notion that sumoylated PCNA recruits Srs2 to replicating DNA to prevent unwanted recombination during normal DNA replication, and blocks salvage HR pathway in the presence of replicative stress^[40,45].

The Rad5-dependent pathway and salvage HR pathway both employ template switching to bypass DNA lesions encountered by the replication fork. As such, it is not surprising that these two pathways share many components such as HR factors Rad51, Rad52, Rad54, Rad55 and Rad57. Both pathways generate SCJ intermediates that are resolved by Sgs1/Top3/Rmi1^[27,49]. It was found recently that the 9-1-1 complex is required for SCJ formation in both the Rad5-dependent and salvage HR pathways^[28], suggesting that the putative 9-1-1 mediated processing of the ssDNA gap is needed for each pathway.

Despite their mechanistic similarity, the Rad5 and salvage HR pathways clearly differ in that the former is dependent on Rad5/Ubc13/Mms2 mediated PCNA poly-ubiquitination and the latter is not. In addition, they are distinguishable regarding their timing of action in the cell cycle. There is evidence indicating that the Rad5 pathway operates in early S phase, whereas salvage pathway acts in late S or G2/M phase^[28,41,49]. This is presumably because the salvage HR pathway is normally kept in check in S phase by sumoylated PCNA and Srs2, and only becomes derepressed when the level of PCNA sumoylation starts to decline in late S phase^[28,49,50]. Moreover, the salvage pathway is hyper-recombinogenic and thus has a high propensity to yield chromosomal rearrangement, but the Rad5 pathway is not. This might, at least in part, stem from

the fact that salvage pathway acts in the absence of Srs2 while the Rad5 pathway operates in the presence of Srs2. Since Srs2 is known to suppress crossover in HR and promote noncrossover HR^[51-53], it is likely that the salvage pathway is prone to crossover and the Rad5 pathway is free of crossover. Lastly, Srs2 has recently been found to also limit D-loop extension, or repair synthesis, during HR and template switching^[54], which may also contribute to the prevention of DNA rearrangement in Rad5 dependent error-free DDT.

REGULATION OF THE CHOICE OF DDT PATHWAY

The choice of DDT pathway greatly impacts genome integrity as mutagenesis by TLS and hyper-recombination by salvage HR pathway can lead to the accumulation of harmful mutations and chromosomal rearrangements^[55,56]. There is increasing evidence for the existence of multiple factors/mechanisms that ensure the preferential employment of the Rad5-dependent error-free pathway for DNA damage bypass. First, TLS, Rad5 pathway and salvage HR pathway seem to operate within distinct time windows in relation to DNA replication, with Rad5 pathway acting in early S phase, and TLS and salvage HR pathway acting in late S or G2/M phase^[13,14,28,41]. Thus Rad5 pathway is the earliest DDT mechanism available to deal with DNA lesions encountered by replication forks (Table 1).

Secondly, Srs2 is believed to channel DNA lesions to Rad5 pathway by inhibiting salvage HR pathway. This is based on the finding that Srs2 deletion (or blocking PCNA sumoylation) suppresses *rad5Δ* hypersensitivity to DNA damage in a Rad51-dependent fashion^[40,44,45]. Since the salvage HR pathway is normally kept in check in S phase by PCNA sumoylation and Srs2, lack of Srs2 function would allow the salvage HR pathway to work ectopically in S phase. Alternatively, or in addition, lack of Srs2 function increases the “scheduled” function of salvage HR pathway in late S and G2/M phase. Regardless of its possible timing, the derepression of the salvage HR pathway allows *rad5Δ* cells to effectively bypass DNA lesions and stay viable, albeit at a cost of genome stability.

As Srs2 has a general antirecombinase activity of disrupting Rad51-ssDNA nucleofilament, it should in theory also inhibit the Rad5 pathway as it inhibits the salvage HR pathway. However, Rad5 pathway is apparently not subject to inhibition by Srs2. What then prevents Srs2 from blocking Rad5 pathway? Recent discoveries on potential functions of the Shu complex and Rad55/Rad57 in error-free DDT provided a possible answer to this question. The Shu complex consists of Shu1, Shu2, Psy3 and Csm2, and genetic evidence has implicated it in error-free DNA damage repair and DDT^[57-59]. The Csm2 subunit of Shu complex associates with the auxiliary HR factor Rad55/Rad57. Importantly, Rad55/Rad57 promotes the formation of Rad51 nucleofilament and may counter

the anti-recombination function of Srs2^[35,36,60-62]. It is possible that Shu complex contributes to error-free DDT by recruiting Rad55/Rad57^[63]. Shu complex itself may be recruited to stalled DNA replication fork directly or indirectly by PCNA poly-ubiquitination. In short, Shu complex may help prevent Srs2 from inhibiting Rad5-dependent pathway. The notion that Srs2 specifically inhibits the salvage HR pathway is in full accordance with *srs2Δ* mediated suppression of the hypersensitivity to replicative stress of cells lacking Rad5 pathway^[40,44,45]. An alternative explanation for why Rad5 pathway is not subject to inhibition by Srs2 is that ubiquitination of K164 of PCNA by Rad5/Ubc13/Mms2 competes with PCNA-K164 sumoylation that is necessary for Srs2 recruitment^[18].

Interestingly, similar to Srs2 deletion, deletion of Elg1, Hmo1 or Fun30 also suppresses hypersensitivity to replicative stress of cells lacking Rad5 pathway, suggesting that these three functionally diverse proteins also regulate the choice of DDT pathway^[49,64,65]. Elg1 is homologous to Rfc1, the largest subunit of PCNA loader RFC^[16]. Elg1 associates with the other subunits of RFC (Rfc2 thorough Rfc5) to form a RFC like complex (Elg1-RLC) that is known to play a role in genome stability^[66]. Elg1 deletion causes an abnormal accumulation of sumoylated and unmodified PCNA on replicating chromatin^[64,67]. Elg1 physically interacts with PCNA with a preference for sumoylated PCNA^[64]. Elg1-RLC can unload unmodified and sumoylated PCNA from chromatin during DNA replication^[64,67]. Given that sumoylated PCNA recruits Srs2, it is not surprising that Srs2 also accumulates on chromatin in the absence of Elg1^[64]. Although these findings suggest a role of Elg1 in regulating the error-free DDT pathway by controlling the level of chromatin-associated PCNA, they do not readily provide an explanation of *elg1Δ* mediated suppression of *rad5Δ* hypersensitivity to replicative stress.

Hmo1 is a chromatin architecture protein homologous to HMGB (High mobility group box) protein^[68]. It preferentially binds ssDNA or DNA with altered conformations, and has the ability to bend DNA^[69,70]. Hmo1 was found to physically interact with Elg1^[49]. However, genetic evidence suggests Hmo1 to act in parallel with Elg1 (and Srs2) in regulating DDT pathway choice^[49]. Hmo1 is suggested to facilitate the channeling of DNA lesions to the Rad5-dependent pathway and prevent the salvage HR pathway and TLS. There is evidence suggesting that the regulatory function of Hmo1 in DDT is dependent on its DNA bending activity^[49]. In addition to its potential role in regulating DDT pathway choice, Hmo1 was suggested to function specifically in the Rad5-dependent pathway. This was based on the finding that Hmo1 is required for SCJ formation in S phase, which coincides with the time window of Rad5 pathway, but is not required for SCJ formation in late S or G2/M phase of the cell cycle. It was thought that Hmo1 facilitates SCJ formation by binding and stabilizing sister chromatid bridges and hemicatenanes formed at replication forks with ssDNA

gaps^[49]. Note that unlike Hmo1, Elg1 and Srs2 are not required for SCJ formation, and are therefore unlikely to be participants of the error-free DDT process^[49].

Fun30 is a chromatin remodeler that has been shown to facilitate long-range resection of dsDNA ends that proceeds HR^[71-73]. This is believed to underlie its function in promoting DSB repair^[71-74]. We obtained evidence that implicates Fun30 in the regulation of DDT^[65]. Fun30 deletion suppresses *rad5Δ* hypersensitivity to replicative stress, which is unlikely due to a reduction in DNA end resection^[65]. This suppression is dependent on Rad51 but not TLS or DNA damage repair pathways including nucleotide excision repair, base excision repair and non-homologous end joining^[65]. It is possible that Fun30 negatively regulates the salvage HR pathway as Srs2 does.

WORKING MODEL FOR DDT MACHINERY

We incorporate recent findings on DDT described above in a working model as follows. When a DNA lesion stalls the replication fork in S phase, a ssDNA gap forms behind the fork (Figure 1A). RFA then binds ssDNA to form RFA-ssDNA nucleofilament that recruits Rad18, thereby starting the DDT process^[11,75]. RFA-ssDNA filament also activates the replication checkpoint^[76]. Ino80 chromatin remodeler that normally associates with replication forks to promote fork progression remodels chromatin at stalled replication fork, resulting in a chromatin environment that facilitates Rad18 recruitment. MRX complex may process the ssDNA gap by resecting its 3' end, and also by helping to recruit Rad18 *via* physical interaction.

Rad18 recruited to the ssDNA gap then binds Rad6 to form a E2-E3 ubiquitinase that mono-ubiquitinates K164 on PCNA that is associated with the 3' end of the gap. At this point, mono-ubiquitinated PCNA can lead to TLS or error free TS branches of DDT. On the one hand, mono-ubiquitinated PCNA can promote TLS by facilitating the replacement of replicative polymerases bound to PCNA with TLS polymerases (Figure 1B). 9-1-1 also plays a role in TLS by facilitating the recruitment of TLS polymerase pol ζ. On the other hand, Rad6/Rad18 complex may recruit Rad5/Ubc13/Mms2 E2-E3 ubiquitinase *via* physical interaction between Rad18 and Rad5^[77]. Rad5/Ubc13/Mms2 further ubiquitinates mono-ubiquitinated PCNA. Poly-ubiquitinated PCNA then signals for error-free TS. 9-1-1 loaded to the 5' end of the ssDNA gap facilitates error-free TS by recruiting Exo1 for gap expansion, which is presumably important for subsequent events in error-free DDT.

Rad51 binds to the stalled nascent DNA strand to form a Rad51-ssDNA presynaptic filament. The extent and stability of this filament is determined by a balance between the opposing actions of Srs2 and Rad55/Rad57. Shu complex may help recruit Rad55/Rad57 and Rad51. With the help of Rad52 and Rad54, the Rad51 presynaptic filament then searches for

homology and invades the DNA duplex of the sister chromatid, and pairs with the nascent complementary sister strand (Figure 1C). Hmo1 may facilitate strand invasion by binding and stabilizing sister chromatid bridges and hemicatenanes.

After annealing with its sister strand, the nascent strand from the damaged DNA duplex is extended by replicative DNA polymerase pol δ over the DNA lesion (Figure 1C). This D-loop extension or repair synthesis is limited and transient, as it is hindered by Srs2. After the completion of repair synthesis, the 3' end of the newly synthesized sequence switches back to the ssDNA gap on the damaged DNA. This effectively fills the ssDNA gap and results in the formation of an SCJ structure (Figure 1D). SCJ is then resolved by Sgs1/Top3/Rmi1 complex to form two duplex DNA strands (Figure 1E), concluding the error-free TS process.

CONCLUSION

More than four decades of studies of DNA damage tolerance/bypass have yielded significant advances in our knowledge of this important process including the characterization of the TLS polymerases and the discovery of PCNA ubiquitination as a molecular switch for TLS and TS pathways. However, the current understanding of DDT especially error-free DDT is far from complete, and many questions remain unanswered: (1) What determines the choice between TLS and Rad5-dependent TS? Specifically, what triggers poly-ubiquitination of mono-ubiquitinated PCNA? What promotes the interaction between Rad5 and Rad18? (2) How PCNA poly-ubiquitination activates downstream TS events? Does it directly recruit a component(s) involved in error-free DDT? (3) Are the three subunits of PCNA subject to independent or coordinated ubiquitination and sumoylation? (4) Does stalling DNA replication induce changes in local chromatin structure that are necessary for error-free DDT? (5) How do Elg1, Hmo1 and Fun30 regulate the choice of DDT pathway? (6) Are recently identified DDT components MRX complex, 9-1-1, Shu complex and Hmo1 directly or indirectly involved in DDT? (7) Does ssDNA gap at stalled replication fork have to be expanded by MRX complex and/or Exo1 for DDT to occur? (8) What determines the putative time windows in the cell cycle in which TLS, Rad5 pathway and salvage HR pathway operate? (9) What prevents the Rad5 pathway from being hyper-recombinogenic? and (10) Does 9-1-1 complex associate with the 5' end of the ssDNA gap at stalled replication fork and help recruit DDT components such as Exo1? Addressing these questions would help yield a comprehensive understanding of the molecular mechanism and regulation of DDT.

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DNA damage responses in cancer stem cells: Implications for cancer therapeutic strategies

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Abstract

The identification of cancer stem cells (CSCs) that are responsible for tumor initiation, growth, metastasis, and therapeutic resistance might lead to a new thinking on cancer treatments. Similar to stem cells,

CSCs also display high resistance to radiotherapy and chemotherapy with genotoxic agents. Thus, conventional therapy may shrink the tumor volume but cannot eliminate cancer. Eradication of CSCs represents a novel therapeutic strategy. CSCs possess a highly efficient DNA damage response (DDR) system, which is considered as a contributor to the resistance of these cells from exposures to DNA damaging agents. Targeting of enhanced DDR in CSCs is thus proposed to facilitate the eradication of CSCs by conventional therapeutics. To achieve this aim, a better understanding of the cellular responses to DNA damage in CSCs is needed. In addition to the protein kinases and enzymes that are involved in DDR, other processes that affect the DDR including chromatin remodeling should also be explored.

Key words: Cancer stem cell; DNA damage response; DNA repair; Cancer therapy

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Core tip: Cancer stem cells (CSCs) are thought to be at the root of cancer recurrence and therapeutic resistance. Eradication of CSCs could be a key to successful cancer therapeutics. Studies have shown that CSCs possess highly efficient ability to process DNA damage. This elevated DNA damage response (DDR) is believed to be responsible for the resistance to DNA damaging agents. Thus, targeting enhanced DDR in CSCs may be a promising strategy to facilitate elimination of these highly tumorigenic cells by conventional therapies.

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INTRODUCTION

Many tumors contain phenotypically and functionally heterogeneous cancer cells. Among them, a minority of cell subpopulation, termed cancer stem cell (CSC), which possesses the self-renewal capacity and is able to generate the heterogeneous lineage of cancer cells, has been believed to be responsible for the initiation and maintenance of the tumors. Besides their self-renewal and differentiation properties shared with stem cells, CSCs are characterized with high tumorigenic potential and are also referred to as tumor-initiating cells^[1]. Recent data suggest that CSCs are more resistant to chemotherapy and radiotherapy than non-stem cells^[2]. Relatively successful cancer treatments shrink the bulk of tumor but often fail to eliminate the CSCs, resulting in the recurrence of tumors. Combined with their high tumorigenic potential, CSCs are believed to be responsible for tumor therapy resistance, tumor relapse, and metastasis.

Many chemotherapeutic drugs, such as platinum-based agents as well as radiotherapy, kill cancer cells by inducing DNA damage. Therefore, cells that efficiently repair DNA damage can potentially survive therapeutic agents. Indeed, prompt activation of DNA damage responses (DDR) and enhanced DNA repair capacity have been implicated as contributors to increased resistance to therapy in the CSC population in gliomas and breast cancer^[3,4].

DDR

Cells incur a large number of DNA lesions from endogenous processes. Many lesions can block transcription, DNA replication, and chromosome segregation, resulting in cell death or gene mutations. Fortunately, cells have evolved an intricate machinery to deal with these lesions. This machinery, known as the DDR, can recognize these DNA lesions and repair them. Meanwhile, cells with genome injury can be arrested at specific checkpoints to allow repair of lesions before they are converted into permanent mutations. When damage is too significant, a cell may undergo apoptosis to avoid the passing of damage to their progeny. Although DDR is critical to the maintenance of genome stability, highly efficient DNA repair machinery can reduce the efficacy of DNA damaging agents in treating cancers. DNA damaging agents used for cancer therapy can induce various DNA lesions, such as covalent crosslinks between DNA bases (cisplatin, carboplatin, oxaliplatin and mitomycin C), base alkylation (methyl methanesulphonate and temozolomide), DNA single-strand breaks (SSB) and double-strand breaks (DSB) (camptothecin and etoposide). The DDR pathways in cancer cells can greatly affect the consequences of cancer treatment with DNA-damaging agents (Figure 1).

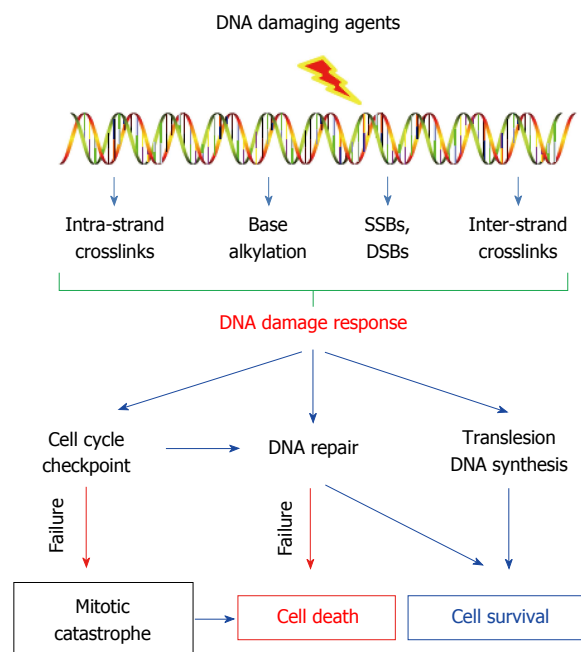


Figure 1 DNA damage responses and consequences in cancer treatment with DNA-damaging agents. DNA-damaging agent-induced DNA lesions can (1) activate cell cycle checkpoint to arrest the cell cycle progression to allow time for repair before the damage is passed on to daughter cells; (2) activate specific DNA repair pathways to remove the lesions; (3) or enables continuous functioning of replication by bypassing the DNA lesions through translesion DNA synthesis. Thus, highly efficient DDR can promote the survival of cancer cells upon DNA-damaging agent treatment.

Repair of intra-strand crosslinks

Cisplatin induces primarily 1, 2-intra-strand crosslinks between adjoining purines in DNA, *e.g.*, *cis*-Pt(NH₃)₂d(GpG) (Pt-GG), with Pt bound to two adjacent guanines, and *cis*-Pt(NH₃)₂d(ApG) (Pt-AG), with Pt bound to an adenine and an adjacent guanine. These damages contribute 90% of the total damage introduced by cisplatin^[5-8]. The intra-strand crosslinks induced by platinum-based therapeutic agents are mainly processed by nucleotide excision repair (NER), which is carried out by a minimal set of proteins including XPA, XPC-RAD23B, XPG, RPA, ERCC1-XPF, TFIIH, PCNA, DNA polymerase δ or ϵ , and DNA ligase I, in human cells^[9,10]. These proteins are sequentially assembled at the site of DNA lesions in chromatin^[11-13], where they carry out the dual incision of the damage-containing DNA. Approximately 24-32 nucleotides are then removed, and a new fragment is synthesized to restore the DNA structure^[14-16].

Repair of base alkylation and SSB

Many alkylating agents such as cyclophosphamide and temozolomide induce DNA damage by attaching an alkyl group to adenine or guanine, leading to replication fork stalling and subsequent apoptosis^[17]. These DNA lesions are mainly repaired by base excision repair (BER). In BER, the alkylated bases

are first excised from the DNA strand by the lesion specific DNA repair enzymes known as glycosylases. The resulting abasic sites or apyrimidinic/apurinic (AP) sites are further recognized and excised by an AP endonuclease, APE1. The resulted SSBs can be bound by poly(ADP-ribose) polymerase 1 (PARP1), which synthesizes PAR polymers attached to itself and other repair factors^[18,19]. Afterwards, BER proteins such as DNA polymerase β and XRCC1-DNA ligase α are recruited to sites of DNA damage for DNA synthesis and gap filling. If the AP site is not repaired, the SSB will persist at the site of damage, cause replication fork collapse during the S-phase, and subsequently is converted to DSB.

Repair of DSBs

DSBs arise from ionizing radiation, free radicals, topoisomerase inhibitors, and replication of a SSB. Homologous recombination (HR) and nonhomologous end joining (NHEJ) are the two major DSB repair pathways that have been studied extensively. In NHEJ, the DSB ends are simply reconnected without the use of a repair template. This process requires Ku70, Ku80, DNA-dependent protein kinases (DNA-PKs), XRCC4, and DNA ligase IV^[20]. The Ku70/Ku80 heterodimer first binds to DNA ends and recruits DNA-PKs, which function to keep the broken DNA ends in close proximity and to recruit end-processing factors, such as Artemis, polynucleotide kinase/phosphatase (PNKP), APE1, and tyrosyl-DNA phosphodiesterase 1 (TDP1), to prepare the DNA ends for ligation by XRCC4-DNA ligase IV^[21].

NHEJ is active throughout the cell cycle. In contrast, HR-directed DSB repair is confined to the late S and G2 phases of proliferating cells when sister chromatids are available as repair templates. HR repair is initiated by the generation of 3' single-stranded DNA (ssDNA) by resection of the 5' end, and the binding of replication protein A (RPA). This initial processing of the DNA ends involves the activity of several nucleases, *e.g.*, the MRN (MRE11-RAD50-NBS1) complex, Bloom's syndrome helicase (BLM), CtBP-interacting protein (CtIP), exonuclease 1 (EXO1), and DNA replication ATP-dependent helicase (DNA2)^[22]. A recombinase, *e.g.*, RAD51 in eukaryotes, is loaded onto these specifically processed DNA ends to displace RPA to form a RAD51-ssDNA nucleofilament with the assist of breast cancer 1 (BRCA1), BRCA2, and several RAD51 paralogues^[20]. The RAD51-ssDNA nucleofilament can invade homologous duplex DNA to search for and couple with a homologous sequence elsewhere in the genome to form a displacement D-loop in which DNA synthesis is initiated to replace the DNA surrounding the former break site.

Repair of inter-strand crosslinks

Inter-strand crosslinks (ICLs) are able to inhibit transcription and replication by blocking DNA strand

separation. Among the chemotherapeutic agents, cisplatin, cyclophosphamide, melphalan, and mitomycin C (MMC) are considered inducers of ICLs^[23]. The repair of ICLs is complex and involves a coordination of several DNA repair pathways such as NER, HR, Translesion synthesis (TLS), and Fanconi anemia (FA) pathway^[23]. ICLs can be recognized by NER machinery and incised by ERCC1-XPF endonucleases, resulting in unhooking of the lesion, which is bypassed by TLS. When ICLs encounter a replication fork during S phase, the collapse of the replication fork can lead to formation of DSB, which can be further processed by HR^[24]. However, the collapse of the replication fork can be avoided by FA complementation group M (FANCM)-mediated fork regression or stabilization.

Translesion DNA synthesis

TLS is a general DNA damage tolerance mechanism, which allows the cell to replicate over DNA lesions. DNA lesions that block replication can be bypassed by specialized low-fidelity DNA polymerases, which are capable of replicating across DNA damage. The most abundant class of these DNA polymerases belongs to the Y-family, including Pol η , Pol κ , Pol ι , and REV1^[25]. TLS is facilitated by PCNA mono-ubiquitylation, which is regulated by Rad6-Rad18 E2-E3 ubiquitylating enzymes and USP1 deubiquitylating enzyme at sites of DNA damage^[26-28]. During TLS, the first polymerase inserts a nucleotide opposite the lesion and the second polymerase, usually DNA polymerase ζ , extends beyond the lesion. TLS plays an important role in the development of cisplatin resistance and is critical in the repair of ICLs^[29].

Activation of cell cycle checkpoint machinery

Cell cycle checkpoints can be activated to arrest cell cycle progression when DNA damage or replication stalls are sensed, so that cells will have sufficient time to repair the damage before it is passed on to daughter cells. The checkpoint pathways in the mammalian cells can be regulated by the ATM (Ataxia Telangiectasia mutated) and ATR (ATM and Rad3-related) protein kinases. ATM and ATR phosphorylate a number of substrates such as Chk1 and Chk2. The activated Chk1 and Chk2 further phosphorylate p53 to stabilize the protein, enhancing the effects of p53 in response to DNA damage. Cdc25A is also phosphorylated by Chk1 and Chk2, leading to a rapid degradation of G1/S transition activators, and enforcing the G1-arrested state^[30]. Cdc25C phosphorylation by Chk1 and Chk2 triggers G2 checkpoints to make sure DNA is fully replicated and undamaged before cells undergo chromosome condensation and nuclear division. Targeting cell cycle checkpoints has been proposed as a novel anticancer strategy. ATM inhibitors (such as KU55933) have been shown to effectively increase the sensitivity of cancer cells to DNA damaging agents^[31]. The inhibitors of Chk1, Chk2, and Cdc25 have also

been suggested to enhance the cytotoxicity of various antitumor agents^[32].

DDR IN CSCS

Stem cells persist and function throughout the entire life of an organism to regulate tissue homeostasis and regeneration. The functional importunity and the long life of stem cells suggest that they must be armed to maintain genomic integrity in unique ways different from those of somatic cells^[2]. Indeed, human embryonic stem (ES) cells have been shown to display high levels of DNA repair capacity^[33]. While reduced DNA repair capacity and loss of genomic integrity have been linked to differentiation or impaired function of stem cells^[34]. However, contradictory results also showed that human ES cells are deficient in DNA repair capacity and cell cycle arrest but use high propensity to apoptosis as a strategy to ensure genomic integrity^[35].

Given that CSCs share common properties with stem cells and are resistant to the treatment with various DNA damaging agents, CSCs were also proposed to possess the elevated DDR to process DNA damage more efficiently than bulk cancer cells. It has been reported that CD133+ glioma stem cells (GSCs) contribute to glioma radio-resistance and tumor regeneration through enhanced cell cycle checkpoint response and DNA repair. This is further supported by the finding that the radio-resistance of CD133+ GSCs can be overcome with a specific inhibitor of the Chk1 and Chk2 checkpoint kinases^[3]. Further investigation demonstrated that L1CAM (CD171), which is preferentially expressed and is induced by DNA damage in GSCs, is responsible for the enhanced activation of cell cycle checkpoint and radio-resistance of GSCs by upregulating NBS1 expression^[36]. Glioblastoma-initiating cells (GICs) also exhibit enhanced basal activation of SSB repair due to enhanced activation of the key SSB repair player PARP1. The growth, self-renewal, and DNA damage repair capacity of GICs can be inhibited by PARP inhibition, leading to an enhanced sensitization of GICs to radiation^[37]. In addition, GICs exhibit hypermethylation in the promoter of O⁶-methylguanine DNA methyltransferase (*MGMT*) gene. The epigenetically silenced *MGMT* might be responsible for the resistance of GICs to temozolamide^[38] due to *MGMT*'s ability to reverse alkylation at the O⁶ position of guanine and neutralize the cytotoxicity of alkylating agents such as temozolamide^[39].

In addition to glioma stem cells, enhanced DNA repair capacity was also found in other CSCs. For example, enhanced repair of DNA DSBs and upregulated expression of DSB repair genes were found in CD133+ CSCs isolated from the A549 human lung carcinoma cell line^[40]. Similarly, DDR and the expression of various repair proteins are also found to be highly up-regulated in Lin⁻CD29^{hi}CD24^{hi} tumor-initiating cells isolated from mouse mammary gland tumors,

indicating an elevated DDR in these CSCs^[41]. Significant increase in the expression of DNA repair-related genes, such as BRCA1 and RAD51, have also been observed in pancreatic putative CSCs compared with bulk cells. These spheroid cells also repair DNA breaks more efficiently than bulk cells after treatment with gemcitabine^[42]. Furthermore, a strong activation of Chk1 and cell cycle arrest were observed in patient-derived non-small-cell lung cancer (NSCLC) stem cells compared to their corresponding differentiated cells after exposure to DNA damaging agents. Treatment with Chk1 inhibitors in combination with chemotherapy dramatically reduced the NSCLC stem cell population *in vitro* by promoting premature cell cycle progression and inducing mitotic catastrophe^[43].

Although considerable reports suggest that competent DNA repair machinery is utilized by CSCs to survive DNA damaging agent treatment, some controversial observations also exist. The DSB repair capacity was found to be significantly reduced in CD133+ glioma stem cells compared with the glioma cell lines. In addition, the intra-S-phase checkpoint was absent in these CD133+ cells, albeit G2 checkpoint activation was intact. Consistent with reduced DNA repair capacity, CD133+ glioma stem cells are more sensitive to radiation than the established glioma cell lines^[44]. In another report, although CD133+ glioma stem cells were shown to display elongated cell cycle and enhanced basal activation of Chk1 and Chk2, neither BER nor SSB repair was found to be increased in CD133+ compared to CD133- glioma cells^[45]. Interestingly, Lim *et al*^[46] reported a more efficient HR repair in GICs, but neither an extended cell cycle nor augmented activation of checkpoint proteins was observed in these cells. The reduced DDR was also reported in other CSCs. For example, esophageal CSCs exhibited compromised induction of p53 and inhibited G1 checkpoint arrest, as well as attenuated DNA repair capacity upon exposure to DNA damaging agents, as compared to non-CSCs^[47]. NSCLC TICs showed an impaired DDR due to deficient activation of various checkpoint factors, *e.g.*, ATM, DNA-PKcs, Krüppel-associated protein 1 (KAP1), and FA complementation group D2 (FANCD2), leading to compromised cell cycle checkpoints^[48]. We have recently determined the NER efficiency of ovarian CSCs, defined by either CD44+CD117+ or spheroid growing in serum-free culture, after treatment with cisplatin. Surprisingly, although ovarian CSCs exhibited resistance to cisplatin treatment compared to their corresponding bulk cancer cells, no increased capability to remove cisplatin-induced DNA lesions was found in these CSCs^[49]. In contrast, we revealed an elevated expression of DNA polymerase η and constitutively high levels of mono-ubiquitylated PCNA in various ovarian CSCs, indicating that enhanced TLS could be responsible for cisplatin resistance in these cells^[49].

The controversial observations of the DDR in CSCs indicate that enhanced DDR can be a contributor in

the resistance of CSCs to DNA damaging agents, but this may not be applied to all CSCs. Due to the genetic heterogeneity in cancer, CSCs isolated from different cell lines and patients may have different responses to DNA damage. Furthermore, the heterogeneity in CSCs and uncertainty in CSC markers may also result in different subpopulations of CSCs from the same cell line or patient^[50], which may also exhibit different DDR. Another possible explanation for the conflicting results is that the different culture conditions of CSCs may affect sensitivity and responses of CSCs to DNA damaging agents as well.

CHROMATIN REMODELING IN STEM CELLS

Chromatin creates a natural barrier against access to DNA in many nuclear functions. It is now clear that chromatin modifications have an integral role in DNA damage repair. Following DNA damage, the chromatin needs to be in an "open" state in order to allow the repair factors to access the DNA molecule. Increasing evidence indicates that DDR can be extensively impacted by the modulations to chromatin structure. Chromatin structure in stem cells is different from differentiated cells. Many stem and progenitor cells have been classically described as having a typical open chromatin conformation that is mostly devoid of heterochromatin^[51]. Electron microscopy examination indicated that heterochromatin is predominant in differentiated cells but less common in ES cells^[52]. Analysis of global chromatin compaction using nuclease also indicated that chromatin is more accessible, and thus more sensitive to nuclease digestion in ES cells than differentiated cells.

In addition to the histological evidence, the idea of "open chromatin" is also supported by other analyses. For example, immunoblotting and immunofluorescence analyses have demonstrated a reduced amount of histone H3 tri-methylation on Lys9 (H3K9me3), a marker of heterochromatin, suggesting that ES cells possess considerably less heterochromatin than differentiated cells. ChIP-seq analyses further showed that heterochromatic marks H3K9me3 and H3K27me3 cover 16% and 12% of the genome of differentiated IMR90 cells, respectively, but both of them cover only 4% in human ES cells^[53]. On the other hand, it appears that ES cells have higher global level of histone acetylation, a general mark of open chromatin, as compared to differentiated cells. In addition, some indirect evidence also support "open chromatin" in ES cells. A fraction of loosely bound architectural chromatin proteins was found in ES cells by using Fluorescence recovery after photobleaching (FRAP) assay, while this fraction is absent in differentiated cells. Collectively, these findings indicate that chromatin in ES cells is globally decondensed and hyperdynamic compared with differentiated cells.

Given that stem cells use distinct characteristic of chromatin structure for regulation of self-renewal and differentiation, it is likely that histone modifications and chromatin remodeling upon DNA damage may be different between stem cells and differentiated cells. Among the limited data regarding DNA damage-induced chromatin modifications in stem cells, γ H2AX was mostly investigated. It has been reported that γ H2AX foci can be observed under microscope in an open chromatin structure even in the absence of exogenous DNA damage in pluripotent mouse ES (mES)^[54]. Pluripotent mES cells can contain more than 100 large γ H2AX foci, which is equivalent to the number of foci that are produced in differentiated cells in response to about 4 Gy X-rays. In comparison, intact differentiated mammalian cells normally exhibit fewer than 5 large γ H2AX foci per cell. It was proposed that the decondensed chromatin structure of pluripotent mES cells, mainly maintained by enhanced global histone acetylation and reduced histone methylation, as well as abundant chromatin remodeling complexes, contributes to the development of larger γ H2AX foci^[54]. Further relaxation of ES cell chromatin by lessening the level of the linker histone H1 causes ES cells hyper-resistant to DNA damage, as the DDR is enhanced in the context of open chromatin^[55]. Although DNA damage induces γ H2AX foci in both differentiated and stem cells, the distribution of γ H2AX in the nucleus of stem cells is distinct. Momcilovic *et al.*^[56] used a 405 nm laser to induce DNA damage in a defined subnuclear area of several ES cells and found that unlike in mouse embryonic fibroblasts (MEF), γ H2AX staining was not confined to the affected nuclear region in ES cells. Instead, γ H2AX specific signal was uniformly distributed through the entire nuclear area, perhaps due to the high dynamics of chromatin in pluripotent stem cells^[56].

It is noteworthy that not all stem cells have unique pattern of γ H2AX in response to DNA damage. Like differentiated cells, unirradiated hematopoietic stem cells displayed extremely low levels of γ H2AX foci, while these cells showed an immediate induction of γ H2AX foci after exposure to 2 Gy IR^[57]. Similarly, the percentage of cells with nuclear foci of γ H2AX after IR was not significantly different in stem glioma (CD133+) and non-stem glioma (CD133-) cells at both 1 and 24 h after treatment^[45]. Thus, the distinct performance of histone H2AX phosphorylation upon DNA damage in various kinds of stem cells reflects various DNA damage responses in different stem cells.

TARGETING THE DDR TO FACILITATE ERADICATION OF CSCS

Genotoxic agents including ionizing radiation (IR) and many chemotherapeutic drugs that cause DNA damage have been used for cancer therapy for many years. As discussed above, the DDR is a determinant

factor in cell sensitivity to DNA damaging agents. Thus, the protein kinases and enzymes that are involved in the DDR, such as ATM, ATR, Chk1, Chk2, and PARP1, have been identified as promising targets for cancer therapeutics^[58]. Due to the enhanced DDR in most types of CSCs, modulation of the DDR in CSCs became a new therapy strategy in the treatment with DNA damaging agents. For example, a specific inhibitor of the checkpoint kinases debromohymenialdisine (DBH) has been demonstrated to reverse the radio-resistance of GSCs defined as CD133+^[3]. PARP inhibitor (olaparib) was also able to sensitize GICs to radiation^[37]. In addition, NSCLC-stem cell survival can be dramatically reduced by Chk1 inhibitors (SB218078 and AZD7762) in combination with chemotherapy^[43]. Recently, the work in our lab revealed depleted miR-93 expression in ovarian CSCs, which is, at least partially, responsible for the upregulated DNA polymerase η expression. Transfection of miR-93 into ovarian CSCs increased their sensitivity to cisplatin treatment, probably through downregulation of DNA polymerase η and thus inhibition of TLS of CSCs^[49]. These findings provide a new avenue to facilitate the eradication of CSCs by platinum-based chemotherapeutics. However, given that adult stem cells also possess enhanced DDR, the DDR inhibitors aimed at eliminating CSCs could also sensitize the adult stem cells to DNA damaging agent, resulting in severe side effects. Therefore, identifying the differences in the DDR between adult stem cells and CSCs, and finding the Achilles' heel in the mechanisms protecting CSCs is critical to the development of potential drugs for facilitating eradication of CSCs by DNA damaging agents.

CONCLUSION

Dysregulated DDR is observed in many cancers and is responsible for the genomic instability that related to tumorigenesis and tumor progression^[59]. The DDR defects provide an Achilles's heel that can be exploited for cancer therapy^[60-63]. The enhanced DDR found in CSCs is considered a protector from the insult of DNA damaging agents. Thus, reducing DDR in CSCs would be a promising therapy strategy for achieving elimination of CSCs with the conventional radio-/chemo-therapy.

Currently, most studies are focusing on the protein kinases and enzymes that are involved in the DDR. As discussed above, the DDR in CSCs can be affected by many factors, among which chromatin remodeling plays an important role in facilitating the DDR in cells following DNA damage. Although stem cells display an open chromatin conformation, there is lack of information on the chromatin structure and chromatin remodeling in CSCs. Understanding the chromatin structure and chromatin remodeling in CSCs after the treatment with DNA damaging agents would provide a novel avenue to interfere with the DDR in this cells. Another question we need to address is how CSCs respond to DNA damaging agents *in vivo*. Most

of the current studies were conducted using *in vitro* cultured cells. However, CSCs dwell in a specialized microenvironment denoted as "stem cell niche", which contains diverse stromal cells, vascular networks, soluble factors, and extracellular matrix component. These components may affect the DDR in CSCs. In addition, the CSC niche can protect CSCs by sheltering them from diverse genotoxic insults, leading to an enhanced therapeutic resistance^[64]. Thus, the future design of therapy to eliminate CSCs needs consideration of the tumor microenvironment. Finally, the phenotypic heterogeneity of CSCs in different tumors, and even in the same tumor, should be considered^[65]. Identifying the dysregulated DDR components in different CSC subpopulations is critical to selecting targets that can be used to efficiently shut down their specific DDR.

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Topographic patterns of vascular disease: HOX proteins as determining factors?

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Abstract

Steadily increasing evidence supports the idea that genetic diversities in the vascular bed are, in addition to hemodynamic influences, a major contributing factor in determining region-specific cardiovascular disease susceptibility. Members of the phylogenetically highly conserved *Hox* gene family of developmental regulators have to be viewed as prime candidates for determining these regional genetic differences in the vasculature. During embryonic patterning, the regionally distinct and precisely choreographed expression patterns of HOX transcription factors are essential for the correct specification of positional identities. Apparently, these topographic patterns are to some degree retained in certain adult tissues, including the circulatory system. While an understanding of the functional significance of these localized *Hox* activities in adult blood vessels is only beginning to emerge, an argument can be made for a role of *Hox* genes in the maintenance of vessel wall homeostasis and functional integrity on the one hand, and in regulating the development and progression of regionally restricted vascular pathologies, on the other. Initial functional studies in animal models, as well as data from clinical studies provide some level of support for this view. The data suggest that putative genetic regulatory networks of *Hox*-dependent cardiovascular disease processes include genes of diverse functional categories (extracellular matrix remodeling, transmembrane signaling, cell cycle control, inflammatory response, transcriptional control, *etc.*), as potential targets in both vascular smooth muscle and endothelial cells, as well as cell populations residing in the adventitia.

Key words: *Hox*; Blood vessel; Cardiovascular disease; Positional identity; Endothelial cell; Vascular smooth muscle cell

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Core tip: Accumulating evidence indicates regionally restricted HOX expression patterns in the adult arterial tree that may reflect a topographic vascular HOX code for specifying positional identities in various cell types of the circulatory system. We propose that this positional information is critical for maintaining local vessel wall homeostasis and that its disruption plays an important role in the development of vascular diseases with distinct topographic preferences. This editorial discusses emerging molecular data in support of this novel concept.

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INTRODUCTION

According to epidemiological data from the World Health Organization (WHO), diseases of the circulatory system, collectively known as cardiovascular diseases (CVDs) are the leading cause of death world-wide (<http://www.who.int/mediacentre/factsheets/fs317/en>), and a great number of resources have been committed to study the pathophysiology of CVDs with the goal of improving therapeutic interventions. The most common forms of CVD include atherosclerosis of various segments of the arterial tree (coronary, carotid, and cerebral arteries, aortic arch, abdominal artery), aneurysms of the thoracic and abdominal aorta, as well as cerebral arterial aneurysms, peripheral arterial disease (PAD), and venous insufficiency.

Although certain types of CVDs share some of the same pathogenic characteristics (e.g., neointima and atherosclerotic plaque formation as a result of chronic inflammation in atherosclerosis, medial degeneration in aortic aneurysms), they usually develop at distinct anatomic locations. As the most prevalent CVD risk factors including hypertension, diabetes, smoking, alcohol, lack of physical activity, obesity, high blood cholesterol and triglycerides, and stress are all systemic, regional genetic differences in the vascular bed are likely to be critical for these topographic disease patterns, as they cannot be attributed to hemodynamic differences alone. This view is underscored by aortic homograft transplant experiments in canines, in which atherosclerosis-susceptible segments of the abdominal aorta were transplanted into the atherosclerosis-resistant thoracic aorta and *vice versa*^[1]. Examination of transplanted vessel segments subsequent to keeping the animals on an atherogenic diet revealed that the abdominal segments readily developed atherosclerotic lesions in their thoracic host environment, whereas the thoracic aortic segments continued to be free from

lesion formation in the abdominal aorta. These results are consistent with data from a large-scale clinical study involving approximately 12000 patients that were treated surgically for atherosclerotic occlusive disease with a follow-up of over 25 years^[2]. In this study the arterial bed was divided into four anatomical categories for analysis including the coronary arterial bed, the major branches of the aortic arch, the visceral branches of the abdominal aorta and the terminal abdominal aorta with its major branches. The data showed that the response to systemic risk factors is distinctly different in each of the four categories^[2].

A search for potential determinants underlying these inherent differences in the response to systemic pathogenic triggers will have to consider the circumstance that vascular smooth muscle cells (VSMCs) residing in distinct segments of the arterial tree originate from different sources depending on their anatomic location. Lineage-mapping involving chick-quail chimera identified at least seven different sources including proepicardium, secondary heart field, neural crest, mesangioblasts, somites, splanchnic mesoderm and mesothelium that contribute to defined arterial segments, in addition to various types of progenitor cells residing primarily in either the media or adventitia with a more universal distribution^[3]. Overall, this fate map reveals a segmented pattern of the arterial tree that seems to reflect the metameric patterning of the vertebrate embryo. As segment identities, and therefore diversities, are specified by members of the phylogenetically highly conserved family of *Hox* transcriptional regulators, these genes have to be viewed as prime candidates for determining different positional identities in the vascular bed that reflect regional differences in CVD susceptibility.

HOX-SPECIFIED POSITIONAL IDENTITIES IN THE CIRCULATORY SYSTEM

The conserved *Hox* gene family constitutes a genetic system of unique properties that is utilized initially during embryonic patterning for specifying positional identities along the anterior-posterior (A-P) axis^[4,5]. The mouse and human genome harbor 39 *Hox* genes that are organized into four separate clusters designated *Hoxa*, *-b*, *-c*, and *-d*. Alignment of these transcriptionally unipolar clusters based on sequence similarities reveals 13 paralogous groups of *Hox* genes that are activated sequentially in distinct A-P embryonic domains such that genes of groups 1 and 2 are expressed first in the most anterior regions, whereas group 13 *Hox* genes are activated last by following the A-P morphogenetic progression. This modus of activation generates unique domains of combinatorial *Hox* activities at any given location of the embryo that has been referred to as the *Hox* code in analogy to the postal zip code for specifying positional identities^[6]. Data obtained by large-scale gene expression profiling

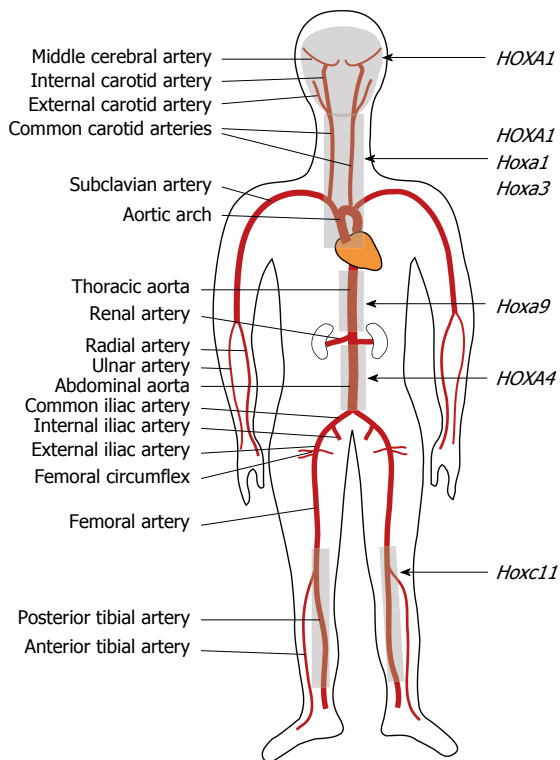


Figure 1 Map of Hox functional domains in the arterial tree. The schematic shows a rough outline of the main human arterial segments. Localization of vascular defects associated either with mutated human *HOX* or mouse *Hox* alleles as indicated at the right were used to generate a cursory map of *HOX/Hox* activity domains (grey shading) assuming that the defects observed in the mouse map to roughly equivalent anatomic positions in humans. The demarcation of the *HOXA4* activity domain is based on down-regulation of *HOXA4* expression associated with AAA. The *Hoxa9* domain in the thoracic aorta signifies an atherosclerosis-resistant segment.

of adult fibroblasts derived from different anatomic regions in humans suggest that this embryonically established topographic *Hox* code is, at least to some degree, retained in the adult^[7], where it is believed to be critical for maintaining positional identities by regulating local differentiation and signaling events.

Initial evidence for the existence of a topographic *Hox* code in the circulatory system came from *LacZ* reporter gene studies in transgenic mice that revealed remarkable regionally restricted expression patterns for *Hoxc10*, *Hoxc11*, and *Hoxa3* in subpopulations of VSMCs of the media, as well as in endothelial cells (ECs) within distinct segments of the vascular bed of young adult (6 wk), as well as 1 year old mice^[8]. Apparently, this presumptive vascular *Hox* code is instrumental in maintaining vessel wall integrity and homeostasis as indicated by the region-specific vascular remodeling events upon its interruption. Specifically, this was demonstrated by inducing changes in the vascular *Hoxc11* expression pattern that is normally restricted to the distal limb vasculature of adult mice (Figure 1). By utilizing an integrated tetracycline regulatory system and *Transgelin* (*Tagln*) promoter elements that drive expression universally in VSMCs of TetOn(*Tagln-Hoxc11*) transgenic mice upon doxycycline (dox)

induction, these mice developed severe vessel wall defects (medial thinning, elastic laminae fragmentation, intimal lesion formation) in arterial segments where *Hoxc11* is normally not expressed (carotid artery, aortic arch, thoracic aorta), whereas overexpression of *Hoxc11* in its natural vascular domain of activity, including the lower femoral artery, resulted in a drastic increase in vessel diameter but without the structural defects observed upon ectopic expression^[9]. Furthermore, human *HOX* transcriptome analysis of vascular ECs derived from different anatomic locations revealed specific *HOX* expression signatures that are believed to determine positional identities and regulate endothelial differentiation^[10].

Compared to gene expression profiling, an alternative approach to consider for mapping *Hox* activities in the circulatory system is to determine *Hox* functional domains by mutational analysis in mice and by linking human congenital vascular defects to mutant *HOX* alleles. The *Hoxa3*^{tm1Mrc} mutant is perhaps the first case in which disruption of a *Hox* gene has been linked to severe cardiovascular defects in mice that include absence of the right carotid artery and stenosis of the aortic valves, in addition to abnormalities of the cardiac chambers, as well as other developmental defects^[11]. In humans and mice, a homozygous *HOXA1/Hoxa1* mutation was linked to a complex syndrome that includes malformations of the cerebral vasculature, the internal carotid arteries, and the cardiac outflow tract in addition to neuronal defects^[12-14]. Mice homozygous for the *Hoxc11*^{tm1Mrc} targeted allele developed greatly enlarged femoral arteries that were phenotypically very similar to the enlarged vessels seen upon induced overexpression of *Hoxc11* in the distal hindlimb vasculature^[9]. Furthermore, and of potential clinical relevance is the downregulation of *HOXA4* expression associated with abdominal aortic aneurysms^[15]. Interestingly, *HOXA4* exhibits an A-P gradient in expression levels along the aorta (ibid) that might overlap with a gradient for *Hoxa9* expression, for which highest levels were found in the thoracic aorta of mice^[16] as discussed below.

HOX-REGULATED GENETIC PATHWAYS IN THE VASCULATURE - POTENTIAL CVD RELEVANCE

The vascular functions of *Hox* genes have perhaps been studied most extensively in cells of endothelial lineage within the context of angiogenesis as observed in normal physiological scenarios (e.g., wound healing) and under pathological conditions (e.g., tumorigenesis). This work has yielded useful insight into *Hox*-dependent pathways regulating EC phenotypic properties. Accordingly, all *HOX* genes of paralogous group 3 were found to stimulate angiogenesis albeit through different pathways^[17]. *HOXD3* promotes expression of *integrin* $\alpha 5$ and $\beta 3$ subunit genes in response to FGF2^[18-20];

heterodimers of these subunits with other subunits generate fibronectin receptors $\alpha 5\beta 1$ and $\alpha v\beta 3$ that facilitate adhesion and migration through fibronectin-rich granulation tissue during wound healing^[17]. Consistent with this is the *HOXD3*-regulated increase in expression of the urokinase plasminogen activator (*uPA*) gene^[18]. The human *HOXD3* paralogs *HOXA3* and *HOXB3* also promote angiogenesis although through different pathways that involve increased EC migration and expression of *Mmp14* and *uPAR* (*HOXA3*) and enhanced expression of the angiogenic ligand Ephrin A1 (*HOXB3*)^[17]. Increased EC migration in tissue culture was also observed for *HOXA9* through direct transcriptional activation of the *Ephrin receptor B4* (*EPHB4*) gene^[21]. *HOXD10*, on the other hand, was found to be inactive during angiogenesis but expressed in quiescent vascular endothelium, and pro-angiogenic factors ($\beta 4$ and $\alpha 3$ integrins, *MMP14*, *uPAR* and *cyclin D1*) were downregulated in *HOXD10*-transfected ECs^[22]. Likewise, *HOXA5* is reportedly inactive in angiogenic endothelium but expressed in quiescent ECs where *VEGFR2*, *ephrin A1*, *HIF1 α* , and *COX2* were downregulated upon transfection with *HOXA5*^[23].

As most of these *Hox* expression data and functional correlations have been obtained in cell culture systems, some of these results may not reflect the vascular patterns and functions observed *in vivo*. For example, *Hoxa3* expression is induced together with *Hoxd3* during cutaneous healing of excisional wounds in mouse^[24]. However, the same gene was found to be abundantly expressed in VSMCs of major blood vessels in addition to ECs but in a topographically restricted manner^[8].

As pointed out above, induction of ectopic *Hoxc11* expression in VSMCs of TetOn (*Tagln-Hoxc11*) transgenic mice on an FVB/NTac background resulted in severe vascular defects. Consequently, these mice offer an opportunity to start defining *Hoxc11*-regulated pathways of pathological vessel wall remodeling. Preliminary results showed induction of the apoptotic marker *CASP3*, as well as matrix protein metalloproteinase *MMP2* and *MMP9* expression in medial VSMCs of the thoracic aorta following doxycycline (dox)-induced *HOXC11* expression^[9]. Both *MMP2* and *MMP9* are involved in extracellular matrix (ECM) breakdown and restructuring of the vessel wall, as well as breakdown of the basement membrane and cell migration^[25,26], *i.e.*, events that are consistent with the formation of the type of lesion formation observed in the thoracic aorta of these mice. Interestingly, in the lower femoral artery, where *Hoxc11* overexpression resulted in an enlargement of an otherwise structurally normal vessel, only *MMP2* was induced upon dox-treatment. Accordingly, the differential histological changes observed in response to *Hoxc11* ectopic vs overexpression were mirrored by distinct differences in molecular responses, in this case *MMP9* expression, that is usually associated only with injury and inflammatory responses^[25]. Furthermore, data from

chromatin immune-precipitation (ChIP) and transient co-transfection assays suggested that *Mmp2* and *Mmp9* are transcriptionally regulated by *HOXC11*, thus linking *Hoxc11* directly to the regulation of vascular remodeling pathways involving these MMPs.

In a separate study, a search for factors that contribute to the commonly greater susceptibility to atherosclerosis in the aortic arch vs the thoracic aorta found that members of *Hox* paralogous groups 6-10 exhibit higher expression levels in the former across various mammalian species including mouse, rat, and porcine^[16]. Remarkably, essentially the same differential *Hox* expression profiles were observed in human embryonic stem cells that were differentiated *in vitro* either along the neuroectoderm pathway into aortic arch-like VSMCs or along the paraxial mesoderm pathway into thoracic aorta-like VSMCs. These data suggest that *Hox*-specified positional identities of VSMCs established during development are retained in adulthood. Apparently this positional diversity is reflected by differential responses to inflammatory stimuli that are critical for the progression of vascular diseases such as atherosclerosis. This was illustrated by the greater activity and binding to DNA of the pro-inflammatory transcription factor NF- κ B in aortic arch vs thoracic aorta VSMCs. Moreover, this NF- κ B pattern apparently is, at least in part, due to a reciprocal regulatory relationship with *Hoxa9* such that the higher levels of *HOXA9* expression in the thoracic aorta repress NF- κ B transcriptional activity, whereas the high levels of NF- κ B activity in the arch repress *Hoxa9* expression^[16,27].

As mentioned before, downregulation of already low *HOXA4* expression levels in the abdominal aorta was associated with abdominal aortic aneurysms (AAAs)^[15] that are much more prevalent than thoracic aortic aneurysms (TAAs), which themselves occur primarily in the ascending aorta, *i.e.*, the proximal region of the aortic arch^[28]. While there exist many differences in the etiology, histology, and genetics of the two diseases, it is perhaps noteworthy that both develop predominantly in vessel segments that are situated at the low ends of *Hox* expression gradients with a greater pro-inflammatory predisposition in both cases, even though the inflammatory component in TAAs is lower than in AAAs^[28]. This raises the intriguing question whether high levels of *Hoxa9* and *Hoxa4* expression may protect against the development of both aneurysms and atherosclerosis in the descending aorta, aka thoracic aorta. In that case, the further reduced *HOXA4* levels clinically associated with AAA are likely the consequence of inflammatory stimuli, a view supported by *in vitro* data showing reduced *HOXA4* expression in cultured human VSMCs and ECs treated with inflammatory cytokine IFN- γ ^[15].

Recent *in vitro* silencing experiments of *HOX* activities (*HOXB7*, *HOXC6*, *HOXC8*) in multipotent stem cells isolated from the adventitia of the human

thoracic aorta provided evidence for *Hox*-dependent differentiation into VSMCs involving epigenetic mechanisms^[29]. This raises the interesting possibility that *Hox* genes may be critical regulators of vascular cell populations that drive phenotype switching between homeostatic/regenerative vs pathological remodeling in vascular tissues in a region-specific manner.

CONCLUSION

Their persistent topographic expression patterns in post-natal vascular tissues suggest that *Hox* genes play a critical role in maintaining vessel wall homeostasis in a region-specific manner. Consequently, the idea that *Hox*-linked genetic polymorphisms affecting either their functional quality or expression levels may underlie CVD susceptibility in discrete portions of the vascular tree is a valid proposition. Albeit limited, the currently available *in vivo* data derived from analyses of *Hox* knockout and conditional *Hox* gain-of-function mutant mice, as well as from clinical studies support that view. Initial efforts of defining the genetic regulatory networks of *Hox*-dependent CVD processes implicate genes of diverse functional categories (ECM remodeling, transmembrane signaling, cell cycle control, inflammatory response, transcriptional control, etc.), as potential targets. The finding of region-specific reciprocal interactions of a *Hox* gene with a pro-inflammatory factor (NF- κ B) is intriguing and might be of universal relevance in the search for therapeutic approaches. Within this context, defining the epigenetic mechanisms controlling *Hox* activities in blood vessels will be critical as indicated by data that associate hypomethylation of CpG islands within the *HOXC11/HOXC9* genomic interval with the ectopic activation of *HOXC10* and -9 in atherosclerotic coronary arteries^[30].

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Lack of new anti-infective agents: Passing into the pre-antibiotic age?

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Abstract

The lack of newly developed antibiotics, together with

the increase in multi-resistance of relevant pathogenic bacteria in the last decades, represents an alarming signal for human health care worldwide. The number of severely infected persons increases not only in developing but also in highly industrialized countries. This relates in first line to the most severe form of a bacterial infection, sepsis and the septic shock syndrome, with high mortality on critical care units. No particular anti-sepsis drug is available, and the therapy with conventional antibiotics more and more fails to provide a survival benefit. Due to the fact that the pharmaceutical industry has withdrawn to a high degree from the development of anti-infectious agents, a huge challenge for health care is approaching in the 21st century. In this article, these problems are outlined and possible alternatives are presented which may be helpful to solve the problem.

Key words: Antimicrobial resistance; Antibiotics; Sepsis; Antimicrobial peptides; Lipoproteins; Inflammation; Cytokines; Endotoxins

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Core tip: The dramatic increase in multiresistance of relevant pathogenic bacteria can be assumed to be one of the main global health care problems of the 21st century. At the same time, the development of new antibiotics decreases considerably from which a significant lack in the health supply for combating infections can be scheduled. In this paper, alternative strategies are discussed which are focused mainly on the development and application of antimicrobial peptides. These can easily be synthesized in high amounts under moderate costs, have usually a high therapeutic index, and do not have a tendency for resistance emergence. Recent developments are presented from which it may be deduced that the most serious form of a bacterial infection, sepsis and septic shock, may be treatable in near future.

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DESCRIPTION OF THE PROBLEM

The assessment of infections has changed completely in the last 30 years. Up to the 1980's it was believed that all bacterial infections were controllable with antibiotics: "It's time to close the book of infectious diseases"^[1]. But already at the end of the 1990's, the general view changed: "Bacterial infections are a worldwide threatening of human health, in developed states they are the third frequent, in countries of the Third World "even the most frequent cause of death"^[2].

These new assessments can be summarized in the statement: "Bacterial infections are the largest health care challenges of the 21st century".

The reasons for this paradigm change are complex. One of the main reasons is certainly the drastic increase in bacterial resistance: The occurrence of bacterial drug resistance, which is - among others - caused by wrong antibiotic medication and the excessive use of antibiotics in animal husbandry, represents an increasing threatening for human health. This situation is communicated also more and more in the media, for example in a contribution by the well-renowned British BBC^[3]. The uncontrolled emission of antibiotics in mass animal husbandry, leading to a contamination of the soils and the water, represents a selection pressure on bacteria from the environment such as *Staphylococcus aureus* (*S. aureus*) and *Pseudomonas aeruginosa* (*P. aeruginosa*) with subsequent increase in resistance. As examples for the increase in resistance, it should be stated that more than 90% of the *S. aureus* strains are resistant to all penicillins and other related antibiotics, and many, *i.e.*, around 30% nowadays with increasing tendency, are resistant even to the newer methicillin-related drugs (MRSA). Further relevant resistant germs with high infection potential are beside MRSA, vancomycin-resistant *Enterococci*, fluorquinon-resistant *P. aeruginosa*, and ESBL (extended spectrum beta-lactamase)-resistant *Escherichia coli* and *Klebsiella pneumoniae*. Infections induced by multiresistant strains frequently lead to heavy infections, for example to wound, urinary, and respiratory infections which finally may lead to the life-threatening sepsis syndrome with multi-organ failure.

More than 100000 patients suffer annually from MRSA infections in Germany, with a mortality rate of up to 10% by sepsis or pneumonia ("Deutsche-Sepsisgesellschaft")^[4]. Very important germs with high infection and mortality potential are also the MDR (multi-drug resistant). *Mycobacterium tuberculosis* strains (MDRMt). These are in particular in Eastern Europa a

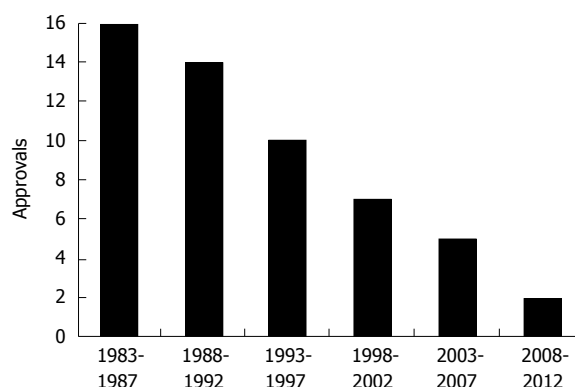


Figure 1 New systemic antibacterial agents approved by the United States Food and Drug Administration per 5-year period, through 2012. From Boucher *et al*^[6], with permission by Oxford Press.

huge threatening of human health, which does not lead to short-time infections as described above but to a long-term infirmity of infected people with a high death-rate. For these bacteria, in contrast to those described above, even the bioactive compounds from their cell wall have not been characterized so far. Another reason for the paradigm change mentioned above is that in the last decades pharmaceutical companies have brought to market only two new classes of antibiotics, which moreover have only effects against particular bacteria. This situation is expressed by the statement: "We are facing a huge crisis worldwide not having an antibiotics pipeline". (Dr. Janet Woodcock, Director of CDER at FDA)^[5]. The lack in the development of new antibiotics is illustrated in Figure 1 showing a dramatic decrease of newly approved compounds.

There is no evidence that this situation will change. In the pipeline of the pharmaceutical industry no broad-band developments are reported. This fact leads to ironic or even sarcastic formulations from experts such as the following: Who Will Mourn the Passing of the Pharmaceutical Industry^[7]?

Details of "Medicines in Development" against a variety of bacterial and viral infections, mostly by small Biotech companies, is found in the 2013 report^[8].

The failure of the pharmaceutical industry to develop new antibiotics has of course a rational background: The development of anti-infective drugs is a very laborious, long-lasting, and expensive procedure, and is frequently "crowned" by failure^[9]: Thus, for example, the development of an effective anti-sepsis medicament (see below) has been described as "Money graveyard" for pharmaceutical companies, because in more than 20 clinical phases III there was no survival benefit for patients, but the companies had invested overall some billion \$. Thus, drug development is targeted to fulfill primarily the promise of the return of investment rather than creating new anti-infective drugs.

MECHANISMS OF SEPSIS INDUCTION

Bacterial infections may give rise to a variety of different

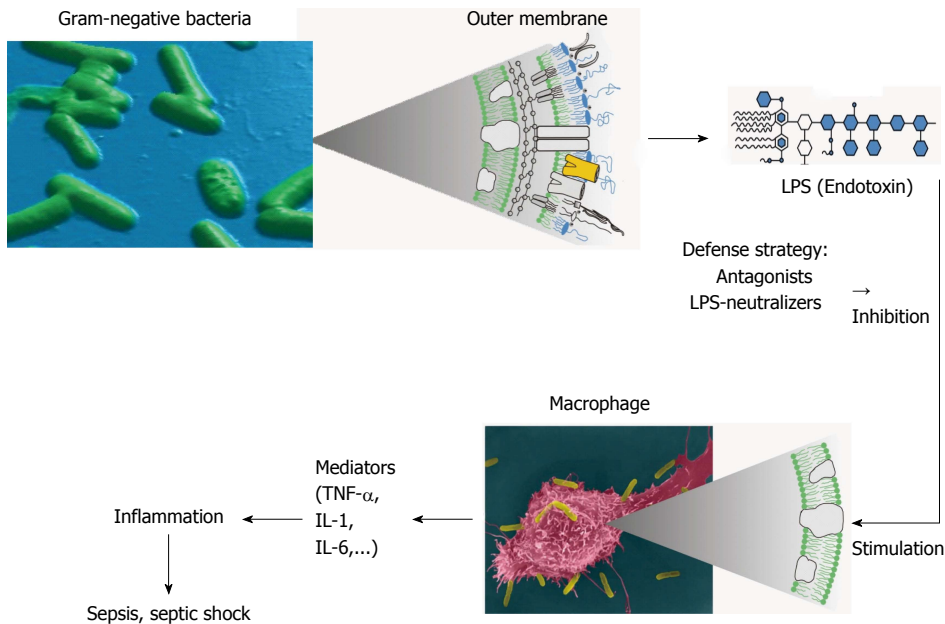


Figure 2 Mechanisms of lipopolysaccharide-induced cytokine (mediator) secretion with subsequent induction of inflammation and sepsis. Without therapeutical intervention, the interaction of the toxins leads to cell activation with subsequent release of inflammation-inducing compounds eventually resulting in sepsis. Intervention strategies may be performed by e.g., AMP and LPS/LP-neutralizers. LPS: Lipopolysaccharide; IL: Interleukin; TNF: Tumor necrosis factor.

diseases, from harmless local infections affording no treatment over more severe local infections such as skin and skin-related infections up to the most severe form, the sepsis. In contrast to other infections such as respiratory or urogenital tract diseases, sepsis is a systemic disease, *i.e.*, it is spreading over the blood stream. For this, no particular drug is available since the withdrawal of Drotrecogin- α (recombinant protein C, Xigris®) in October 2012, which anyway was only suitable for a small number of patients. The numbers known from the USA may give a hint regarding the high relevance of the septic syndrome in health care, when compared with the data from other diseases: annual death cases United States: Sepsis 215000; Myocardial Infarction 193000, Lung cancer 153000.

In selected video presentations, the dramatic kind of occurrence and frequency of sepsis are presented most impressive^[10,11]. It is well known that septic shock may occur for patients, whose bodies are weakened due to other traumata (heavy burns, accidents, surgery, *etc.*), who have a priori a weak immune system, or who are infected due to unfortunate reasons (even an inaccurate treatment of a tooth may lead to sepsis because bacteria from the mouth may infiltrate the bloodstream). It is important to note that not the bacteria and their toxins, but rather the overshooting reaction of the body to these stimuli is responsible for the induction of the sepsis syndrome. Whereas the presence of the inflammation-inducing bacterial "pathogenicity factors" (PFs) such as endotoxin [lipopolysaccharide (LPS)] for Gram-negative and lipoprotein (LP) from Gram-positive source at low concentrations is beneficial for the immune system in particular in the early childhood, the negative consequences at high concentrations lead to

the well-known cytokine storm (Figure 2)^[12].

The lack in progress in the fight against sepsis has not changed during the last decades, however, the occurrence of drug-resistant strains has severely worsened the situation. Whereas for the drug-susceptible bacteria an elaborate therapy by applying suitable antibiotics and "adjunctive" and "volume replacement" procedures in critical care units may lead to some therapeutic success, bacteria such as MRSA and ESBL - resistant strains from *Escherichia coli* and *Klebsiella pneumonia* cannot be treated adequately. Even if the immune system is potent enough to combat the bacterial invasion, the release of the bacterial toxins LPS and LP from the cell envelopes of bacteria induces the immunity to go haywire. These toxins react with cells of the immune system in a heavy way by producing so-called cytokines (mediators), which leads to a self-poisoning of the body. As shown in Figure 2, the liberation of LPS - which may take place by the action of the immune system or only by cell division of the bacteria - from the bacterial outer membrane is responsible for the heavy inflammation reaction by interacting with cell receptors, as shown here on macrophages (for LPS: Toll-like receptor 4, for LP: Toll-like receptor 2), with subsequent cell signaling.

In Figure 2 also possible intervention strategies are depicted, like antibacterial drugs, LPS neutralizers, and protein antagonists such as antibodies (see below).

Even if suitable antibiotics would be available, this frequently does not necessarily improve the health of the patients, but in contrary may worsen their state, because due to bacterial killing the bacterial toxins are now released from the bacterial cell envelopes with a

Table 1 Nomenclature of terms used in severe systemic infections

Term	Definition
Endotoxemia	Occurrence of Lipopolysaccharide (Endotoxin) in Blood
Bacteremia	Occurrence of living bacteria in blood
SIRS	Generalized hyperinflammatory reaction with different causes, Infections, Burns, Traumata
Sepsis	SIRS induced by an infection
Severe Sepsis	Sepsis with organ dysfunction
Septic Shock	Sepsis with shock

SIRS: Systemic inflammatory response syndrome.

severe increase in the inflammation reaction. "There are more and more results from animal models and from clinical studies that the antibiotic-induced release of biologically active LPS leads to a worsening of the critical state of septic patients"^[13].

A detailed characterization of the terms and symptoms, which may occur in humans due to a systemic inflammation is given in the Table 1.

REASONS FOR FAILURE OF PREVIOUS ANTI-SEPSIS THERAPIES

Regarding all previous efforts for the development of anti-septic compounds, the corresponding experimental therapies have failed to improve the clinical outcome of severe sepsis and septic shock, and sometimes even increased mortality. The targets of current and past anti-septic strategies can be divided into five categories^[9]: (1) Cell-signaling; (2) Coagulation pathway; (3) Endotoxin; (4) Immunomodulation; and (5) Specific mediators.

From the analysis of published data it can be deduced that seven agents yielded at least some improvement in clinical trials: a high-dose corticosteroid, two anti-endo-toxin therapies, a human recombinant IL-1 receptor antagonist, an intensive insulin therapy, an intravenous immunoglobulin, and a recombinant activated protein C10.

High dose corticosteroids, have been used until 1989. At the beginning, the compounds actually showed some survival benefit, but further analyses revealed that the survival rate in patients with septic shock was even lowered^[14]. IL-1 receptor antagonists were used until 1997 and again showed a survival benefit at first, which could not be confirmed in follow-up trials. The same applies to monoclonal anti-TNF α antibodies (afamilomab, Abbott), which showed some positive effects, but no overall increase in survival. The use of anti-IFN γ antibodies (INNO-202, Innogenetics) showed some benefit in monkeys (reported in 2003), but for unknown reasons was not further pursued through clinical studies. Intensive insulin therapy (IIT) is not recommended at this time because the

first encouraging results could not be transferred to other ICU-populations. Furthermore, the application of IIT resulted in significant harm to patients by severe hypoglycemia.

Details of the numerous attempts for combating sepsis and the reasons of failure are described by Fink and Shaw Warren^[15].

The common problem of the strategies mentioned above is that all concepts (with the exception of compounds that are targeting the bacteria's endotoxins) do only tackle some aspects of severe sepsis rather than addressing the body's whole inflammatory state, triggered by bacteria or rather bacterial PFs. Accordingly, these strategies had been - at best - only be partially successful.

An alternative approach relates to immunomodulation mediated by short peptides to bolster host immunity, rather than reacting directly with bacteria or their PFs. Representative compounds are defensins, cathelicidins, dermicines and derivatives thereof, which are able to stimulate the expression of cytokines and chemokines. Some data indicate that these peptides may modulate the inflammatory cascade by targeting multiple inflammatory mediators, which may lead, among others, to an alteration of cell membrane function connected with the inhibition of TLR4 signaling induced by LPS. However, no data are available that would indicate their clinical usefulness in sepsis.

The latest failed clinical trials concern two in preclinical investigations very promising compounds, talactoferrin, a recombinant lactoferrin produced by Agennix, and the LPS-antagonist Eritoran by EISAI corporation. Both compounds did not succeed in phase III studies, an explanation may be with the former compound that talactoferrin is focused mainly on Gram-negative bacteria, but may not have enough potential to neutralize endotoxins (see, *e.g.*^[16]), and with the latter that LPS-antagonists of course cannot act against Gram-positive bacteria and their toxins.

Presently, only two anti-septic compounds are under development. The approach by Medinox (United States) is in phase I / II and exploits the compound NOX100 (Irish Pharm. Healthcare Assoc., 2010), which is believed to neutralize excessive NO produced during inflammation. Again, only one side-aspect of bacterial sepsis is addressed, and accordingly, it is unlikely that the compound will be broadly efficacious and of benefit for sepsis patients. Similar applies to compound cyto-FAB (anti-TNF, AstraZeneca), which has entered clinical phase II trials (Irish Pharm. Healthcare Assoc., 2010). The current therapeutic situation is best described by the following statement: "Mostly old therapeutic concepts in a 'new design' are currently pursued, although it has been reported that 395 medicines and vaccines are under clinical evaluation, with 88 of them denoted as antibiotics/antibacterials"^[8]. As can be concluded from the above, an efficacious and safe anti-septic drug is yet to be developed.

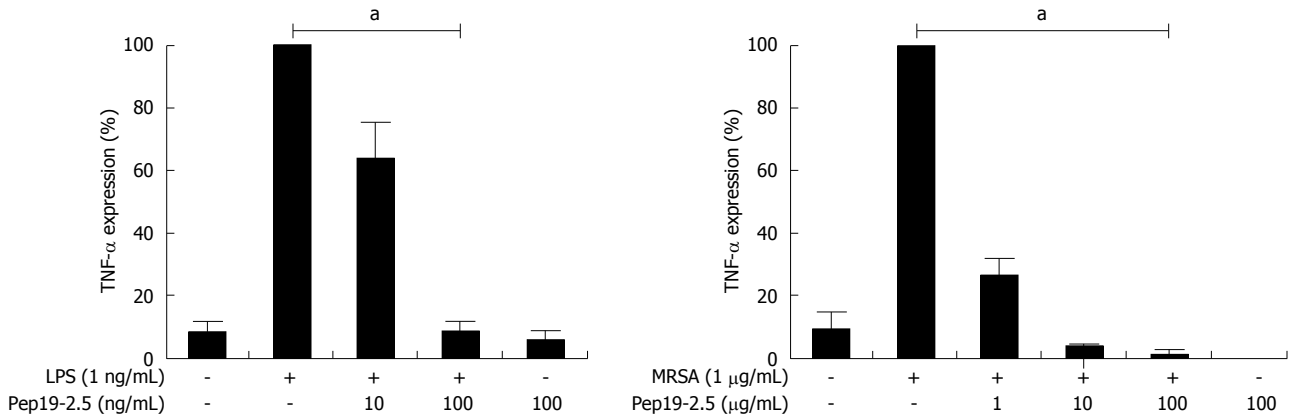


Figure 3 Determination of tumor necrosis factor- α expression levels from human lung tissue with lipopolysaccharide from *Salmonella minnesota* R60 and heat-killed MRSA combined with different concentrations of Pep19-2.5 (Aspidasept®). Depicted is the mean $\pm$ SEM of 5 independent experiments. Assuming parametric distribution data were log-transformed and groups were compared for statistical significant differences by use of One-Way Repeated Measure ANOVA ($^aP < 0.05$). From^[19] with permission of American Society for Microbiology. LPS: Lipopolysaccharide; TNF: Tumor necrosis factor.

NEW APPROACH WITH ANTIMICROBIALS. HOPE FOR THE 21st CENTURY?

At the beginning of the 1990's, more and more investigators focused on the design and synthesis of antimicrobial peptides (AMP) in the fight against bacteria and their toxins, mainly based on the use of endogenous (body-own) AMPs such as cathelicidins, defensins, and dermicins. Up to now, these activities have not led to a newly approved drug, except for daptomycin, a lipopeptide which acts only against Gram-positive bacteria. The reasons for the inefficacy are manifold: AMPs are efficacious mucosal factors, but naturally never exposed to the systemic environment, rendering them inherently toxic at concentrations required to bind the bacteria and their toxins when used systemically. A second point is the fact that the bacterial surface structures, which are the first line of attack for the AMPs, show so many variations that not a single compound is able to act as broad-band drug. Nearly all of these approaches were directed against bacteria.

We have chosen another approach: Our rational was to engineer simpler, smaller synthetic polypeptides, which are essentially non-toxic, but still capable of binding PFs. Originally based on comprehensive biophysical studies of natural occurring antimicrobial proteins (e.g., cyclic peptides from anti-*Limulus* factor, shortened peptide variants from human lactoferrin, porcine NK-lysine, human granulysin), we have developed a new class of synthetic polypeptides (lead compound: Aspidasept®), which possess potent antibacterial activities. These were originally scheduled to bind to and neutralize the lipid A part of Gram-negative LPS, but in the meantime could be shown also to neutralize Gram-positive lipopeptides/proteins^[17-19] (a neutralization of peptidoglycan and lipoteichoic acid could also be observed, but it can be highly doubted

that these are relevant PFs, as still proposed^[20]).

The neutralization of the PFs from nearly all bacteria prevents the over-shooting inflammation reactions induced by these during sepsis. A variety of *in vitro*, *in vivo* and *ex vivo* data is indicative of high inflammation-decreasing activity and efficient protection (e.g., strong weakening of infections, increased survival). As example for the inhibition of the inflammation reaction, in Figure 3 data from an *ex vivo* model of the human lung are presented. A schematic view on the processes taking place in human blood in the absence (up) and presence (down) of Aspidasept® are shown in Figure 4.

Aspidasept's scheduled therapeutic concentrations in sepsis patients are far below the concentrations at which side effects ("no observed adverse effect level", NOAEL) do occur. In fact, experiments in rats by a CRO (contract research organization) which have been conducted in accordance to ICH guideline M3, revealed that at a concentration of 0.5 mg/kg per day with infusions for 14 d no toxic effects were found. Therefore, this concentration is within the NOAEL. At 3.2 mg/kg per day, slight toxic effect occurred, indicating that this belongs to the MTD (maximum tolerated dose). Both concentrations are more than 200-fold higher than the scheduled concentration in septic patients. Therefore, these data provide for the first time the opportunity to develop an equally safe and efficacious medication against sepsis.

CONCLUSION

It cannot be expected that in the next decades new effective broad-band antibiotics will enter the market. Since the increase in multi-resistance and the demographic development - with a growing proportion of elder people - will continue, a huge burden to the health care system can be supposed. Therefore, to solve the urgent problems concomitant with lack of new medicaments and increasing resistance, new

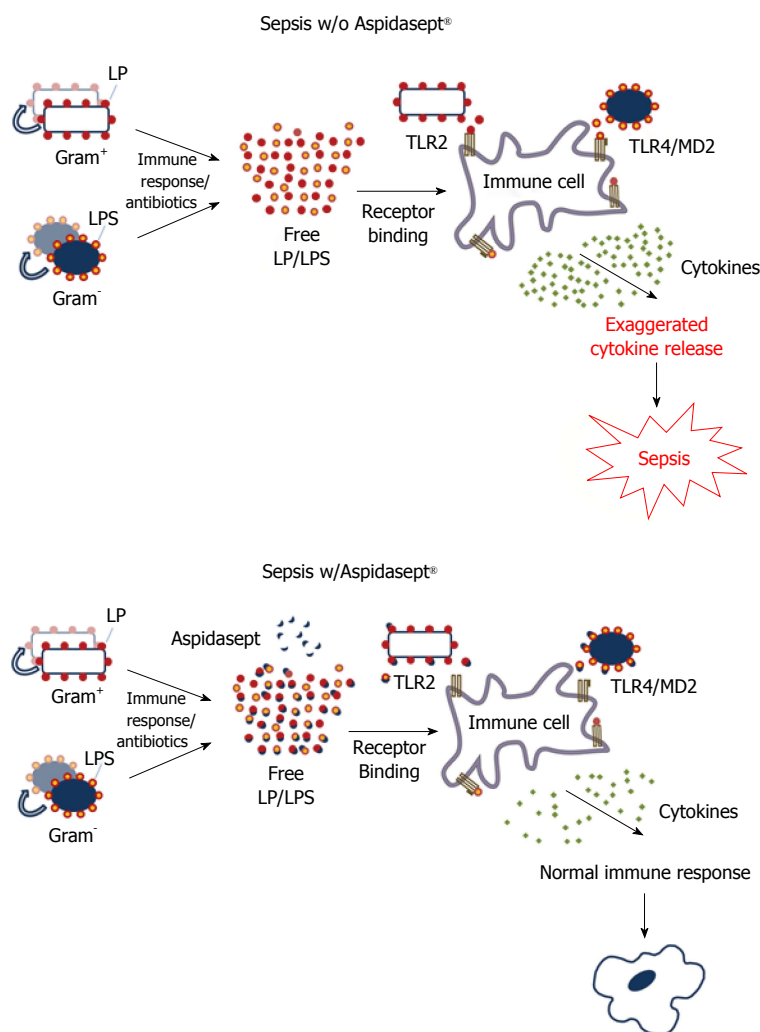


Figure 4 Compound Aspidasept® (Amino acid sequence GCKKYRRFRWKFGKFWFWG; EU, United States, and Japan patent filed) is able to inhibit very efficiently the cytokine production induced by the pathogenicity factors endotoxin/lipoproteins or by the bacteria *in vitro*, *in vivo* (various mouse models), and *ex vivo* (human lung, see Figure 3). These inhibition properties of Aspidasept® comprise various relevant bacterial strains from Gram-negative and Gram-positive origin, as well as Mycobacteria, in particular *M. tuberculosis*. Aspidasept® has low toxicity (the NOAEL, no observed adverse effect level' is 200 to 500 times higher than the planned therapeutical doses). Beside its inflammation-inhibiting activity, it is able to bind the multi-resistant bacteria thus limiting their spreading. LPS: Lipopolysaccharide.

approaches must be developed. The presented data of antimicrobial peptides with high efficiency to neutralize the bacterial toxins may be one chance for the future. Whether this development will be the only promising approach, is unclear. However, one of the main arguments for the development of AMP is the rather simple and cheap synthesis up to very large scales (kg). In upcoming clinical trials it should turn out whether the highly successful preclinical data *in vitro*, *in vivo*, and *ex vivo* can be confirmed successfully also for septic patients as significant decrease in lethality. This would be the first therapeutical approach with antimicrobial peptides in clinical practice. A success would be also insofar important, since development of resistance against these AMPs is less probable.

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Is erythroferrone finally the long sought-after systemic erythroid regulator of iron?

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Abstract

Iron metabolism is regulated on the cellular and the systemic level. Over the last decade, the liver peptide "hepcidin" has emerged as the body's key iron store regulator. The long postulated "erythroid regulator of iron", however, remained elusive. Last year, evidence was provided, that a previously described myokine "myonectin" may also function as the long sought

erythroid regulator of iron. Myonectin was therefore re-named "erythroferrone". This editorial provides a brief discussion on the two functions of erythroferrone and also briefly considers the emerging potential role of transferrin receptor 2 in erythropoiesis.

Key words: Erythroid regulator of iron; Heparin; Iron metabolism; Myonectin; Systemic iron regulation

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Core tip: Iron metabolism is regulated on the cellular and the systemic level. Over the last decade, the small, 25-amino-acid liver peptide "hepcidin" has emerged as the body's key iron store regulator. The long postulated "erythroid regulator of iron", however, remained elusive. Several candidates have been suggested in the literature, however all had to be dismissed. Last year, evidence was provided, that erythroferrone, a previously described myokine, may also function as the long sought erythroid regulator of iron. While erythroferrone appears to be an important iron regulator for erythropoiesis, transferrin receptor 2 also emerges as a player in this regulation.

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IRON AND LIFE

Iron is a very important metal for all cells; iron deficiency results in cell death, but iron overload can be similarly detrimental. Adult humans have about 3 to 5 g of iron in the body, the vast majority of which is bound to haemoglobin^[1]. The major iron storage in the body occurs in the liver, or more specifically, the

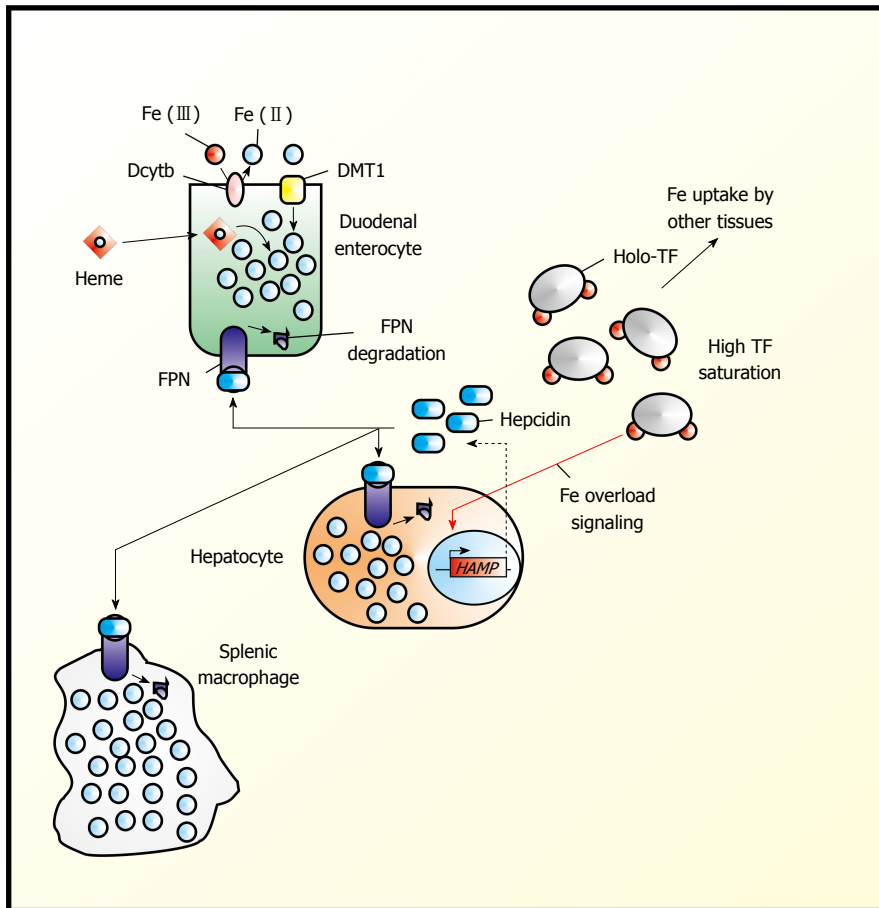


Figure 1 Hepcidin in the regulation of systemic iron homeostasis. Systemic iron (Fe) homeostasis is primarily regulated by the hepcidin-ferroportin (FPN) axis. Duodenal enterocytes import dietary Fe from several sources including heme Fe and non-heme Fe, which typically must be reduced at the level of the apical membrane by chemical reductants such as ascorbate^[26,27] or by plasma membrane oxidoreductases (e.g., Dcytb). The reduced Fe then enters a common intracellular pool of Fe. "Sensing" the level of transferrin (TF) saturation and levels of Fe stores under conditions of Fe overload, hepatocytes up-regulate the expression of hepcidin, which is then released into the plasma where it can then bind to ferroportin (FPN), thereby triggering FPN internalization and proteosomal degradation^[1].

hepatocytes, where most iron is bound to ferritin^[2]. As iron is such a double-edged sword, its metabolism has to be tightly regulated and for a long time, both, a store regulator and a erythroid regulator have been hypothesised to regulate systemic iron needs^[3]. It was also shown early on that both, iron deficiency and increased erythropoiesis were able to up-regulate iron absorption^[4,5].

HEPCIDIN AS THE IRON STORE REGULATOR

In 2000/2001, a small (25-amino-acid) hepatic antimicrobial peptide, LEAP-1 or hepcidin, was first described^[6,7]. It was soon to be connected to iron metabolism and now is widely accepted to be the "store regulator"^[5]. Murine hepcidin was found to be over-expressed during iron overload^[8]. It is synthesized in response to high iron load and secreted by the liver into the blood stream, where the majority may bind unspecifically to albumin, while a small fraction (the active fraction?) will bind specifically to β_2 -macroglobulin, from which it can be delivered to ferroportin^[9]. Once

bound to ferroportin, the only known mammalian cellular iron exporter, it will trigger proteosomal ferroportin degradation and thereby lower iron release into the circulation (Figure 1)^[1].

PROPOSED ERYTHROID REGULATORS OF IRON

The nature of the erythroid regulator on the other hand remained elusive. In order to be considered the erythroid regulator, the molecule should be independent of the total body iron stores, but up-regulated in anaemia; it should be up-regulated by erythropoietin and it should trigger the down-regulation of hepcidin. It also should be down-regulated after blood transfusion. Two candidates have been earlier identified, Growth Differentiation Factor 15 (GDF15) and Twisted Gastrulation (TWSG1)^[10,11]. The former was identified as being up-regulated in thalassaemic serum by a transcriptional profiling approach during erythropoiesis and as being able to *in vitro* suppress hepcidin expression^[10]. By transcriptome analyses of erythropoiesis, TWSG1 was identified as

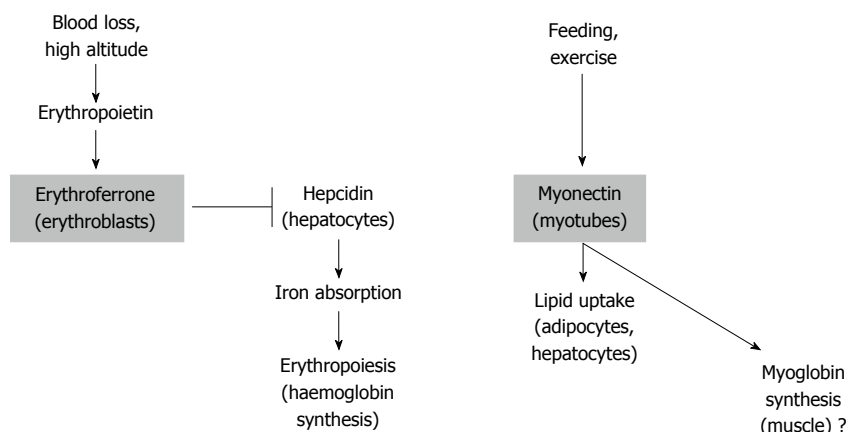


Figure 2 Two potential actions of erythroferrone. After blood loss, stress erythropoiesis is triggered by erythropoietin. This results in erythroferrone release from erythroblasts and in turn down-regulation of hepcidin. As a result, iron absorption and hemoglobin synthesis and erythropoiesis are increased (left). In response to feeding and exercise, myonectin (erythroferrone) produced by myotubes triggers fatty acid uptake by adipocytes and hepatocytes (right). Considering the function of erythroferrone in erythropoiesis, regulation of myoglobin is suggested to be a likely consequence of myonectin release (far right).

being up-regulated in early stages and again being able to (indirectly) suppress hepcidin expression^[11]. For both these factors, however, evidence that they would constitute the erythroid regulator remained weak. Such, GDF15 levels are not correlated to hepcidin levels^[12]. Even soluble transferrin receptor 1 was proposed to play the role of the erythroid regulator as it increases in iron deficiency and during increased erythropoiesis^[13,14], however, this proposal also had to be dismissed, as it has no effect on iron absorption or hepcidin expression^[14].

ERYTHROFERRONE - THE ERYTHROID REGULATOR?

Earlier this year erythroferrone, 340-amino acid soluble protein, was suggested for this role^[15]. In response to erythropoietin, erythroblasts produce erythroferrone, which in turn suppresses hepcidin expression, resulting in increased release of iron from cellular iron stores^[15]. Knockout mice for erythroferrone fail to rapidly suppress hepcidin expression in response to haemorrhage^[15]. They also show a haemoglobin deficit, indicative of an impediment of erythropoiesis^[15]. Erythroferrone expression in spleen and bone marrow increases during anaemia of inflammation and this contributes to iron mobilisation and recovery from anaemia^[16].

MYONECTIN - A MYOKINE

These data provide strong evidence for erythroferrone finally being the (or at least an) erythroid regulator of iron metabolism. However, erythroferrone had earlier been described as "myonectin" or "C1q tumor necrosis factor α -related protein isoform 15" (CTRP15)^[17]. [Unfortunately the same name "myonectin" was used in the same year for the related, but not identical "C1q tumor necrosis factor α -related protein isoform

5 (C1QTNF5)"^[18] by Lim *et al*^[19] for a myokine, the level of which is increased in insulin-resistant rats and mice and in myocytes depleted of mitochondrial DNA. This 243-amino acid soluble protein induces the phosphorylation on AMP-dependent protein kinase and acetyl-CoA carboxylase^[20]. CTRP15/myokine's circulating level was described to be tightly regulated by the metabolic state (suppressed during fasting and up-regulated after re-feeding) and predominantly expressed in skeletal muscle^[17].

A MYOKINE AS ERYTHROID REGULATOR OF IRON?

These authors did not include bone marrow or spleen in their expression analysis (the two tissues where erythropoietin significantly stimulated erythroferrone expression^[15]) and neither analysed a knockout mouse of myonectin for a muscle phenotype (which was not reported for the erythroferrone knockout mouse^[15]). Thus, it appears at first glance difficult to reconcile the two proposed functions of erythroferrone/myokine as those of a factor released into the circulation. Exercise, unless performed at high altitude, is not usually considered to stimulate erythropoiesis^[21], neither is feeding. Thus, how can the apparent conundrum be solved that a myokine, that produced during exercise and feeding, enhancing fatty acid uptake by myocytes and hepatocytes^[17] should be a master regulator of iron metabolism in erythropoiesis^[15]? There appears to be discussion on whether continuous exercise (on sea level) can raise the erythropoietin levels and therefore erythropoiesis^[22], thus there may be a way of reconciling the two functions of erythroferrone now described in the literature. Furthermore, a peptide stimulating haemoglobin formation in erythropoiesis may also have a role in myosin formation in the muscle (Figure 2).

REGULATION OF ERYTHROFERRONE BY TRANSFERRIN RECEPTOR 2

While preparing this short editorial, several papers were published that provided evidence for a role of transferrin receptor 2 in the regulation of erythropoiesis. Wallace *et al*^[23] reported that mice lacking matriptase-2 and transferrin receptor 2 are characterised by severe anaemia. These mice have a significantly increased erythroferrone expression when compared to their wild type litter mates during stress erythropoiesis. Similar results were presented by Nai *et al*^[24] showing that mice lacking both matriptase-2 and transferrin receptor 2 have increased erythrocyte counts when compared to littermates only lacking matriptase-2. When analysing mice, lacking transferrin receptor 2 in the bone marrow only, Nai *et al*^[25] reported that these mice exhibit increased numbers of nucleated erythroid cells in the bone marrow and increased erythroferrone levels in the spleen and in erythroid precursors.

CONCLUSION

C1q tumour necrosis factor α -related protein isoform 15, originally called myonectin and now referred to as erythroferrone, appears to play two different regulatory roles; that of a myokine and that of an erythroid regulator of iron. On the whole, evidence for its function as erythroid regulator of iron appears to be stronger than that of its function as myonectin. Transferrin receptor 2 also plays a regulatory role in erythropoiesis, down-regulating erythroferrone and thereby erythropoiesis. Transferrin receptor 2 may be involved in the iron sensing that results in the regulation of red blood cell formation.

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Multifunctional facets of retrovirus integrase

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Abstract

The retrovirus integrase (IN) is responsible for integration of the reverse transcribed linear cDNA into

the host DNA genome. First, IN cleaves a dinucleotide from the 3' OH blunt ends of the viral DNA exposing the highly conserved CA sequence in the recessed ends. IN utilizes the 3' OH ends to catalyze the concerted integration of the two ends into opposite strands of the cellular DNA producing 4 to 6 bp staggered insertions, depending on the retrovirus species. The staggered ends are repaired by host cell machinery that results in a permanent copy of the viral DNA in the cellular genome. Besides integration, IN performs other functions in the replication cycle of several studied retroviruses. The proper organization of IN within the viral internal core is essential for the correct maturation of the virus. IN plays a major role in reverse transcription by interacting directly with the reverse transcriptase and by binding to the viral capsid protein and a cellular protein. Recruitment of several other host proteins into the viral particle are also promoted by IN. IN assists with the nuclear transport of the preintegration complex across the nuclear membrane. With several retroviruses, IN specifically interacts with different host protein factors that guide the preintegration complex to preferentially integrate the viral genome into specific regions of the host chromosomal target. Human gene therapy using retrovirus vectors is directly affected by the interactions of IN with these host factors. Inhibitors directed against the human immunodeficiency virus (HIV) IN bind within the active site of IN containing viral DNA ends thus preventing integration and subsequent HIV/AIDS.

Key words: Retrovirus integrase; Integration; Host factors; Atomic structure; Human immunodeficiency virus integrase inhibitors

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Core tip: This review examines the multifunctional properties of retrovirus integrase (IN) besides its key function of integrating the viral DNA into host chromosomes. IN has a major role in the maturation of the virus, reverse transcription and nuclear transport of the preintegration complex. IN binds to cellular cofactors

for uncoating of the core and to other cellular proteins that guide the preintegration complex to preferred regions on the host genome for integration. Understanding these IN functions has resulted in the production of clinical IN strand transfer inhibitors to prevent human immunodeficiency virus (HIV/AIDS) and development of retrovirus vectors for human gene therapy.

Grandgenett DP, Pandey KK, Bera S, Aihara H. Multifunctional facets of retrovirus integrase. *World J Biol Chem* 2015; 6(3): 83-94 Available from: URL: <http://www.wjgnet.com/1949-8454/full/v6/i3/83.htm> DOI: <http://dx.doi.org/10.4331/wjbc.v6.i3.83>

INTRODUCTION

Retroviruses are ubiquitous in nature and are found in yeast to insects and to the animal and plant kingdoms. In retroviruses, the single stranded viral RNA genome is reverse transcribed by the viral reverse transcriptase into a double strand DNA copy that is subsequently integrated into the host genome. The integration process that inserts the viral DNA into the host DNA is mediated by the viral encoded integrase (IN). Retroviruses are mobile elements that distinguish themselves from other viruses because their DNA genomes become an integral part of the host genome, an essential requirement in their replication cycle. There are slight variations in this integration process in the above mentioned biological systems, some of which are called Ty1/copia and Ty3/gypsy elements or LTR retrotransposons. This review will focus on IN found in alpharetroviruses, gammaretroviruses, lentiviruses which includes human immunodeficiency virus (HIV), and spumaviruses. The purpose of the review will be to briefly highlight the most current salient features of the many functional facets of the retrovirus IN and identify critical papers and reviews that provide in-depth information for a specific subject.

HIV is the most studied retrovirus with regards to its biology, biochemistry and pathogenesis. A general retrovirus replication cycle is depicted in Figure 1 along with location of steps for FDA approved HIV drug therapies. Each step from the binding of the virus particle to a cellular receptor to the release of a new virus particle is described in the legend. In Figure 1, IN plays a role in step 3: uncoating of the internalized viral core, step 4: reverse transcription of the viral RNA, step 5: nuclear transport of the preintegration complex (PIC), step 6: integration of viral DNA into the host genome, and step 13: maturation.

IN DOMAIN ORGANIZATION

The retrovirus IN is located at the C-terminal end of the Pol protein that is part of a larger precursor polyprotein (Gag-Pol) encapsulated within the virus particle. The retrovirus IN was first identified and

purified from an alpharetrovirus^[1] and genetically shown to be necessary for integration^[2-5]. The avian retrovirus or Rous sarcoma virus (RSV) and HIV IN proteins are 286 and 288 residues in length, respectively, while the prototype foamy virus (PFV) IN is 392 residues^[6] (Figure 2). RSV IN possesses three structural domains comprising an N-terminal domain (NTD) (residues 1-44) that contains a zinc-binding HHCC motif, a catalytic core domain (CCD) (residues 58-213) and a C-terminal domain (CTD) (residues 222-268). HIV IN has similar size domains and linkers as RSV IN. Both PFV and murine leukemia virus (MLV) IN (408 residues) possess an additional N-terminal extension domain (NED) (approximately 50 residues) attached adjacent to the NTD of IN.

BIOLOGICAL FUNCTIONS OF IN

Role of IN in viral maturation

The retrovirus structural Gag proteins are approximately 80% of the total mass of the viral proteins. The reverse transcriptase (RT) and IN are derived from a separate Gag-Pol precursor polyprotein produced by translational frameshifts. This precursor polyprotein accounts for approximately 2% to 3% of the total viral protein mass (Figure 3). Despite this lower level of IN in virus particles, studies using site-directed mutagenesis of HIV IN highly suggest that deletion and missense mutations affect HIV assembly and release^[7]. The mechanisms associated with these defects are not well understood but may include activation of the viral protease located within Gag-Pol and dimerization of this precursor polyprotein.

The recent discovery of inhibitors that do not interact within the catalytic active site of HIV IN has provided unforeseen information on a structural role for IN in virus particle maturation. These inhibitors called LEDGINS^[8] or ALLINIS (allosteric IN inhibitors)^[9] prevent interactions of IN with a cellular chromatin binding protein lens epithelium-derived growth factor (LEDGF/p75), a transcriptional cofactor that guides the HIV PIC (Figure 1) to active transcription sites for integration (see host factors). In addition to inhibiting integration, significant structural defects are caused by these inhibitors resulting in abnormal formation of electron dense HIV virus cores thus producing non-infectious particles upon egress^[10-12] (Figure 1). Other studies have shown that these inhibitors significantly induce HIV IN monomers to produce inactive oligomers *in vitro*^[10,13,14]. These results highly suggest that IN plays a critical role in virus maturation (Figure 1).

Reverse transcription requires IN functions

RT and IN are linked together covalently at the C-terminal end of Gag-Pol precursor polyprotein (Figure 3). The processing of the precursor protein to RT and IN by the viral protease in the virus particle is different between RSV and HIV. For RSV, the Pol product from the polyprotein is a 95 kDa polypeptide termed β that forms

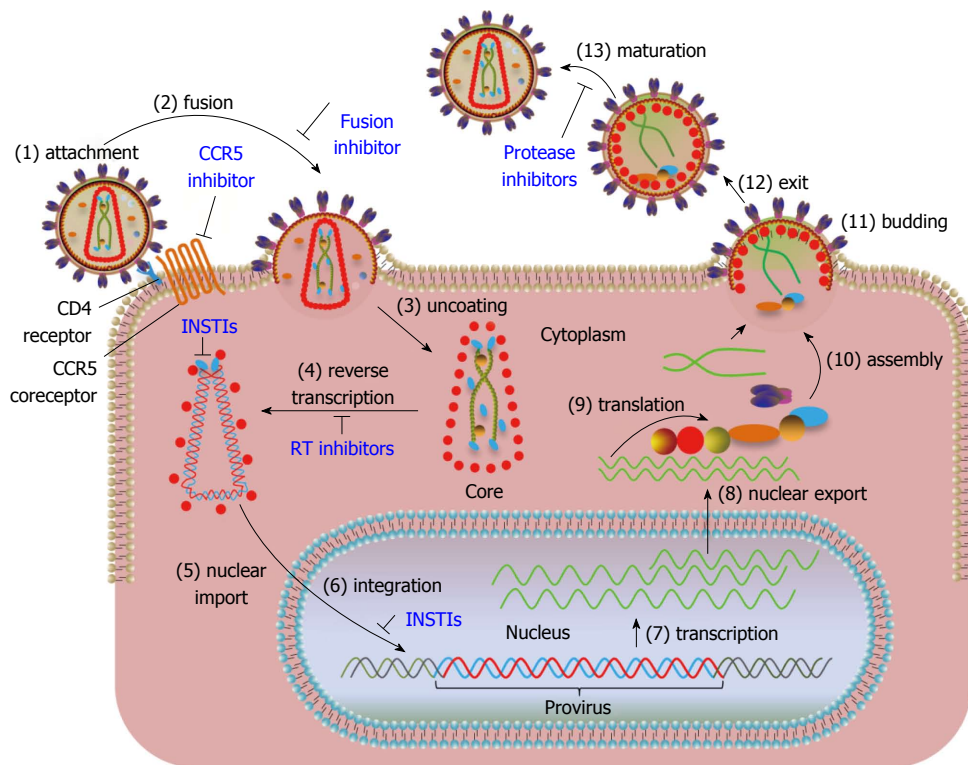


Figure 1 Overview of human immunodeficiency virus type 1 replication cycle. Steps in the viral replication cycle are numbered. The steps where FDA approved drugs exist are marked in blue. (1) The trimeric envelope glycoprotein (gp120/gp41) of human immunodeficiency virus (HIV) binds to the CD4 receptor and CCR5 co-receptors present on host cell surface. Maraviroc, a CCR5 receptor antagonist blocks the binding of viral gp120 to CCR5 and prevents the entry of virus into cells; (2) Viral membrane fuses with cellular membrane allowing the entry of the virus into the cytoplasm. Enfuvirtide, a fusion inhibitor binds to gp41 and prevents the formation of an entry pore for the capsid of the virus, thus blocking the entry of virus into the cytoplasm; (3) The virus undergoes uncoating of the core; (4) HIV RNA is reverse-transcribed by reverse transcriptase into double stranded DNA within the core. Reverse-transcriptase inhibitors block the synthesis of viral DNA; (5) Integrase (IN) cleaves two nucleotides adjacent to conserved CA nucleotides from both 3'-ends of viral DNA (referred as 3'-processing in text) and forms the preintegration complex (PIC). The PIC is transported into the nucleus through a cellular nuclear import pathway. IN strand transfer inhibitors (INSTIs) bind to the IN-DNA complex after 3'-processing rendering the cytoplasmic PIC defective for integration but not nuclear transport; (6) IN facilitates the joining of viral DNA to the host DNA (termed strand transfer in text). Integrated viral DNA is referred to as "provirus". A cellular protein, lens-epithelium derived growth factor (LEDGF/p75), preferentially targets the integration of viral DNA within transcriptionally active regions. INSTIs block the joining of processed viral DNA ends into cellular DNA; (7) The viral DNA is transcribed by cellular RNA pol II into viral RNAs; (8) Full-length and alternatively spliced RNAs are transported out of nucleus. Reverse-transcriptase inhibitors block the synthesis of viral DNA; (9) Viral proteins are synthesized from the RNA template; (10) Full-length viral RNA and newly synthesized proteins begin the assembly of new virus particles at the inner surface of cell membrane; (11) Immature viral particles bud out of the membrane and released (12); and (13) Proteolytic processing of viral proteins results in the maturation of viral particle. Protease inhibitors block the formation of mature proteins by inhibiting the proteolytic processing of the viral polyproteins.

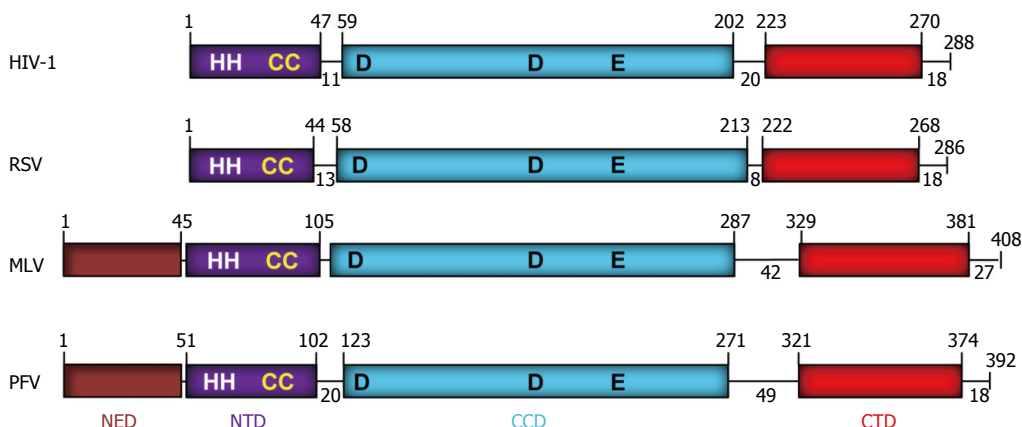


Figure 2 Domain organization of retroviral integrases. The domains (NED: N-terminal extension domain; NTD: N-terminal domain; CCD: Catalytic core domain; and CTD: C-terminal domain) are depicted with different colors. The number of residues in each domain are indicated. The domains are separated by protein linkers of various sizes. NTD contains the zinc binding HH-CC motif and the D-D-35-E motif in CCD binds Mg^{2+} which constitutes the active site. The CTD shows a topology of SH3-like domain, a characteristic feature associated with protein-protein and protein-DNA interactions. The exact MLV domain sizes have not been determined experimentally except for the CTD^[9]. A short tail of disordered amino acids is located on the C-terminal end of each IN. IN: Integrase; HIV: Human immunodeficiency virus; RSV: Rous sarcoma virus; MLV: Murine leukemia; PFV: Prototype foamy virus.

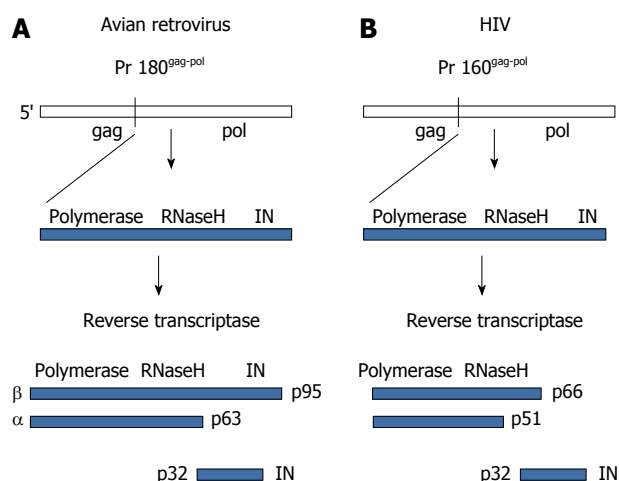


Figure 3 Structural organization and proteolytic processing of reverse transcriptase and integrase. A: The precursor Gag-Pol protein is processed by the viral protease into an intermediate polypeptide. The avian polypeptide exists as a dimer of the β subunit that is processed into the active $\alpha\beta$ subunits (RT) and integrase (IN). The α subunit contains the active polymerase and RNaseH sites while β retains IN residues. RT does not possess integration capabilities; B: Cleavage of the human immunodeficiency virus (HIV) polypeptide produces the active dimeric RT and IN. The HIV p66 subunit possesses the polymerase and RNaseH active sites. RT: Reverse transcriptase.

dimers (Figure 3). The viral protease cleaves the dimers into active RT, termed $\alpha\beta$, which causes the release and activation of the 32 kDa IN. The $\alpha\beta$ polypeptide still contains a single copy of IN residues that facilitates a processive mechanism for reverse transcription and its associated RNaseH activity. In contrast, HIV IN is released entirely from the polypeptide producing RT (p66/p51) (Figure 3), similarly to MLV IN (not shown).

Site-specific mutations introduced into the cleaved HIV IN result in reverse transcription defects but not integration^[15]. The exact mechanisms associated with these RT defects are mostly unknown. HIV IN (Figure 3) appears to have a physical interaction with RT (p66/p51) that is necessary for normal viral DNA synthesis^[16-19]. In normal HIV infected cells, IN interacts with the major structural viral capsid protein (CA) whose interactions affect the normal uncoating process of the core and thus reverse transcription^[20]. Following the above theme that viral core uncoating affects reverse transcription, recent results suggest that the cellular protein DYNLL1 is required for early viral DNA synthesis and uncoating *via* a physical interaction of DYNLL1 with HIV IN^[21]. As these authors suggested, IN-DYNLL1 interactions may be a potential target for future inhibitors.

Role of IN in the preintegration complex, its nuclear transport and in integration

Although IN has many roles in the retrovirus replication cycle, IN is the catalytic component necessary for concerted integration of the viral DNA ends into the host genome (Figures 4 and 5). A pivotal study clearly demonstrated that linear MLV DNA acts as the substrate for integration *in vivo*^[22]. The high molecular weight PIC containing a copy of the linear viral DNA was isolated

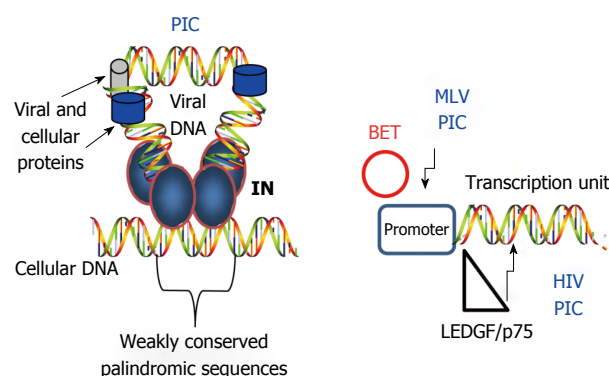


Figure 4 Selection of cellular DNA target sites for integration. Left: IN within the PIC is guided to host sites by several mechanisms including recognition of weak palindromic sequences by IN, nuclear topography of the cellular host DNA site, chromatin structure, and several kinds of associated histone tails. Only IN (labeled), RT (blue circle) and LEDGF/p75 (gray cylinder) are identified in the figure; Right: Cell proteins (BET and LEDGF/p75) influence the PIC to preferentially insert the viral DNA into specific regions of the host genome. IN: Integrase; PIC: Preintegration complex; HIV: Human immunodeficiency virus; MLV: Murine leukemia virus.

from MLV infected cells (Figures 1 and 4). This complex is capable of integrating the viral ends in a concerted fashion into a target substrate *in vitro*. DNA sequence analysis of the integrated viral DNA demonstrated that IN inserted the 3' OH ends into opposite strands of the target DNA producing the 4 bp host site duplication found associated with the integrated MLV provirus *in vivo* or a 6 bp duplication with RSV (Figure 5). The host site duplication for HIV is 5 bp.

There are numerous publications (many not indicated here) describing the integration capabilities of the PIC for MLV^[22,23] and later for HIV^[24-27] at the biochemical, genetic and inhibition of strand transfer by IN inhibitors (Figure 1). The PIC is composed of linear DNA, viral proteins IN, RT, CA, matrix (MA), viral protein R (Vpr) and several cellular associated proteins (Figure 4). In the cytoplasm, IN cleaves a dinucleotide from the 3' OH ends of blunt DNA before transport of the PIC into the nucleus (Figure 5). HIV IN also appears to play a role in the nuclear transport of the PIC along with other viral and cellular proteins^[28,29]. After transport, IN inserts the 3' OH recessed DNA ends into the cellular DNA genome. As described later, the HIV IN strand transfer inhibitors (STIs) interact within the active site of IN and the 3' processed LTR ends in the cytoplasmic PIC, thus inactivating the complex preventing integration into cellular DNA^[27]. The retrovirus PIC has provided major insights into retrovirus integration but has a severe limitation for providing sufficient quantities necessary for biochemical studies.

IN interactions with host factors and cellular target site selection

IN is the dominant factor for selection of weakly conserved palindromic sequences at the target site *in vivo*^[30-32] (Figure 4). Whole genomic sequencing of DNA target sites demonstrates that HIV IN prefers

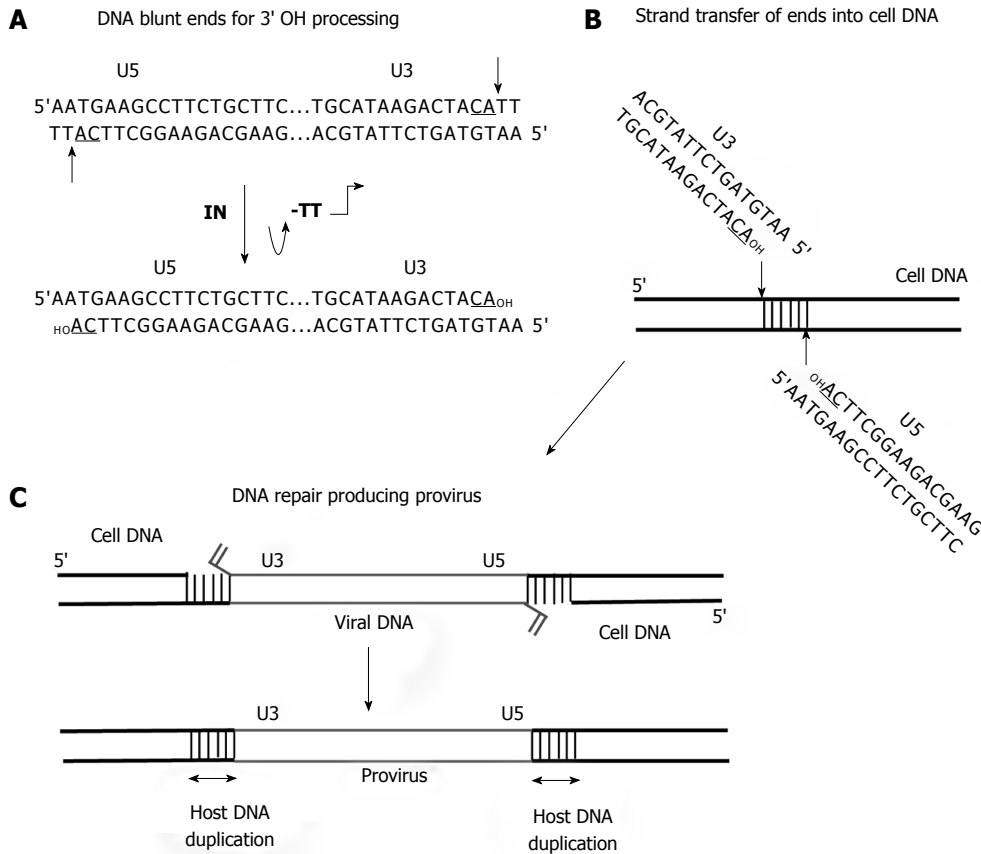


Figure 5 Requirement for 3' OH processing and strand transfer activities to facilitate concerted integration of viral DNA by integrase into target DNA. A: The linear approximately 10 kb DNA with only the ends is shown. The U3 and U5 RSV DNA blunt ends with the underlined conserved CA dinucleotide on the catalytic strand are indicated. The thin arrows indicate 3' OH processing of two nucleotides by IN producing the recessed substrate for strand transfer activity; B: Concerted integration of the viral linear DNA ends by integrase (IN) into the target DNA is by a transesterification mechanism; C: The insertion of viral DNA into the target produces a 6 bp stagger cut by RSV IN that is repaired by cellular proteins producing the integrated provirus. RSV: Rous sarcoma virus.

to integrate its viral DNA into active transcriptional units^[30] while MLV IN favors integration sites near the promoter region of genes and not into transcriptional units^[33]. Further studies demonstrated that RSV IN essentially integrates its DNA into the host genome at random sites^[34,35]. These results suggested that HIV and MLV IN interact with specific host factors that direct IN to preferred sites in the host genome.

Major studies have centered on the ability of LEDGF/p75, a transcriptional co-activator, to guide the HIV PIC to integrate its DNA into active transcriptional sites^[36-41] (Figure 4). Besides LEDGF/p75, cellular Nup153 is indispensable for the integration of HIV DNA into the peripheral edge of the nuclear DNA suggesting that nuclear topography is also an important for HIV integration site selection^[42]. For MLV IN, recent studies demonstrated that IN interacts with the chromatin BET (bromo- and extra-terminal domain) proteins (BRD2, BRD3 and BRD4) directing it to the promoter region of genes^[39,43-45] (Figure 4). To date, there are no reports that have identified cellular proteins involved in host site selection by avian retroviruses. In summary, IN is capable of integrating its viral DNA into numerous segments of the host genome but is preferentially directed to specific regions by host factors.

BIOCHEMICAL AND STRUCTURAL PROPERTIES OF IN

Solution properties of IN

Biochemical and biophysical studies of retrovirus IN has been extensive over the last 37 years. Most studies have used recombinant RSV, HIV, MLV and PFV IN. Only the avian retrovirus IN has been purified from virus particles^[1,46]. As stated above, IN cleaves a dinucleotide from both 3' OH blunt ends of the viral DNA and subsequently inserts the recessed ends into a target DNA by a transesterification reaction (Figure 5). The biochemistry of these reactions and other structural properties of IN were recently reviewed^[47].

The subunit structure of IN is varied and highly dependent on the purification process and different salt requirements to maintain solubility for each expressed recombinant IN. PFV IN is soluble (10-15 mg/mL) in 0.2 mol/L NaCl^[48,49] while RSV IN is highly soluble (30 mg/mL) in 0.2 mol/L (NH₄)₂SO₄^[50]. PFV IN is monomeric^[49] while RSV IN is a dimer in solution^[50-52]. Recombinant HIV IN has been purified as a monomer, dimer and tetramer^[47,53,54]. Recent studies suggest that the active form of HIV IN is a monomer that is responsible for assembly of the synaptic complex (SC) or intasome

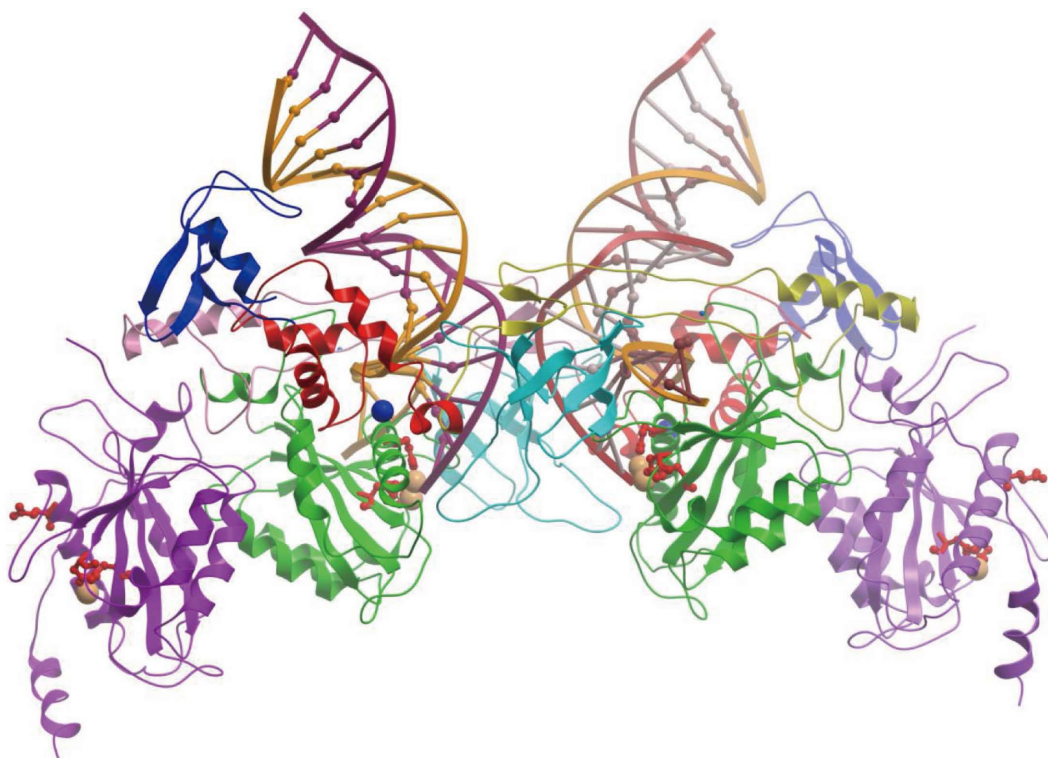


Figure 6 Prototype foamy virus intasome structure. The intasome consist of an integrase (IN) tetramer with two viral DNA ends. The 17/19 bp DNA interacts extensively with the two inner monomers of IN. Among the inner monomers, the N-terminal extension domain, N-terminal domain, catalytic core domain (CCD) and C-terminal domains are shown in blue, red, green and cyan, respectively. The DDE residues are identified as red amino acids. Among the outer protomers, only the CCD (shown in purple) was resolved. The cartoon was redrawn from the PDB ID 3OY9 in Molsoft ICM-Browser. The Zn^{2+} and Mg^{2+} metal ions are shown with blue and orange spheres, respectively.

capable of efficient concerted integration of two oligonucleotides into a target substrate *in vitro*^[53]. The HIV IN monomer purified in the presence of EDTA is converted into a tetramer in the presence of Zn^{2+} without the formation of a dimer intermediate^[53,54]. Studies have demonstrated that a tetramer of IN is associated with HIV, PFV and RSV SC capable of concerted integration^[50,55-57].

The oligomeric state of retrovirus IN play key roles in virus maturation upon release of the virus particle, enzymatic activities for 3' OH processing and concerted integration of viral DNA and possibly for reverse transcription in the cytoplasmic PIC.

Retroviral IN superfamily and PFV intasome structure

The retrovirus IN superfamily comprises proteins that are involved in a variety of activities like replication and repair of DNA, V(D)J recombination, and DNA transposition. The DDE domain of retrovirus IN (Figure 2) is highly conserved in proteins capable of similar catalytic reactions including MuA transposase, human RAG1 recombinase, the RNaseH domain on the retrovirus RT (Figure 3) and other bacterial and human endonucleases^[58,59]. Structures of many of the above protein have been resolved at the atomic level in complex with DNA. However, among the retrovirus INs only the four-domain PFV intasome (PFV IN-DNA

complex) has been resolved (Figure 6).

The crystallographic study of the full-length PFV IN with bound viral DNA revealed, for the first time, the architecture of retrovirus intasome assembly and atomic details of the IN-DNA interactions^[57,60] (Figure 6). The PFV intasome contains a tetramer of IN, which consists of a dimer of the "inner" subunits that make extensive protein-protein and protein-DNA interactions, and a pair of the "outer" subunits that appear to play structural roles. All four domains of the IN subunits and the inter-domain linkers interact extensively with DNA to bridge between the two viral DNA ends and capture the target DNA. The engagement of the viral DNA sequence and catalysis *in trans* is a feature shared with related transposase systems^[59,61].

The determination of the atomic structure of the PFV intasome greatly enhanced our understanding of IN interactions with 3' OH recessed ends in the active site and showing how a tetramer of IN catalyzes the concerted integration reaction^[57]. Other structural studies have shown how PFV IN is bound to the viral DNA in its ground state prior to 3' OH processing or strand transfer^[62]. Lastly, the PFV intasome in complex with target DNA after insertion of the 3' OH ends (strand transfer complex) demonstrated how severely the target DNA is bent upon insertion of the viral DNA ends^[60,63].

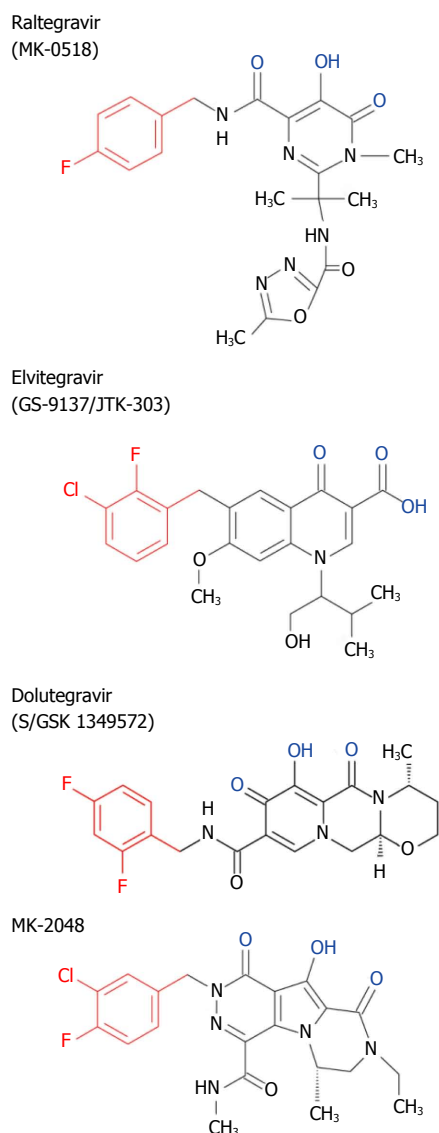


Figure 7 Chemical structure of strand transfer inhibitors. Conserved metal chelating moiety similar to diketo acid is shown in blue and a conserved halogenated benzyl group that interacts with the penultimate nucleotide of DNA is shown in red.

HIV IN INHIBITORS

Strand transfer inhibitors and their interactions within the active site of HIV IN

The requirement for integration of the viral DNA into the host genome for replication renders IN as a natural target for development of inhibitors. Numerous studies in the 1990s to identify relevant HIV IN inhibitors culminated with a study demonstrating that IN STI prevent the integration of the viral DNA into the cell genome resulting in inhibition of HIV replication in cells^[27]. Further studies established that the STI competed with target DNA binding to IN suggesting a unique conformation of IN for the strand transfer reaction^[64]. The diketo acid pharmacophore of the STI also bound to the divalent metal ion in the active site of IN^[65].

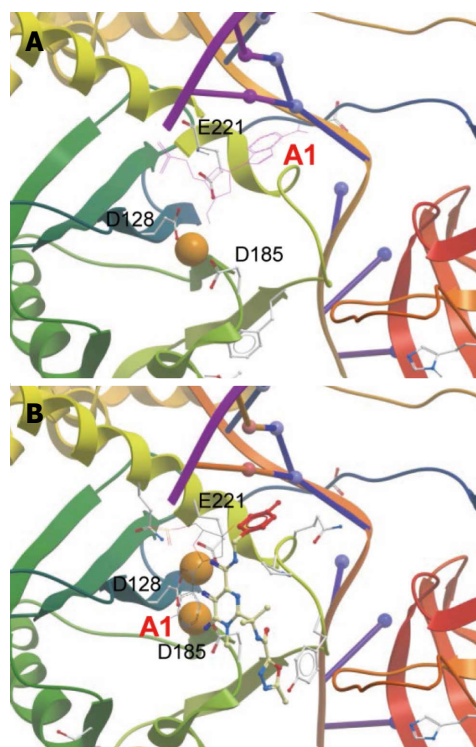


Figure 8 Active site within prototype foamy virus intasome in apo (A) and Raltegravir-bound state (B). Orange spheres indicate Mg^{2+} . Active sites residues are labeled. Only the terminal 3' adenosine (marked A1) is displayed in its chemical form to show the displacement upon RAL binding. The diketo acid group of Raltegravir (RAL) interacts with the metal ions. Adenine is π -stacked against the RAL metal-chelating scaffold. The halobenzoyl group of RAL is marked red. The figures were constructed from PDB entries 3L2R and 3OYA, respectively, in Molsoft ICM-Browser.

Crystallization studies of the four-domain PFV IN intasome without (Figure 6) and with STIs demonstrated the interactions of these inhibitors within the active site of IN^[57,62,66,67] (see Figures 7 and 8 for brief description of STIs and their mechanism). There are currently three clinical STIs approved by the FDA for treatment of HIV/AIDS (Figure 7). They are Raltegravir (RAL), Elvitegravir (EVG) and Dolutegravir (DTG). The HIV IN STIs are interfacial inhibitors whose mechanisms are similar to other compounds that target bacterial DNA gyrase and topoisomerase IV as well as anticancer drugs targeting human topoisomerases^[68,69]. Interfacial inhibitors, like the HIV STIs, bind to a site produced at the interface of two (or more) molecules bound together resulting in a functional complex. In the case of STIs, the interfaces are formed by the viral terminal CA nucleotides and specific IN residues in the PFV intasome^[57,67] (Figure 8). A recent book^[70] and several reviews of HIV IN inhibitors provide an extensive overview of the discovery of STIs and their mechanisms^[47,71-73].

HIV and RSV synaptic complexes are kinetically stabilized by STIs

The atomic structures of the three-domain HIV and RSV IN (Figure 2) in complex with viral DNA are unknown. Crystallization of these INs in complex with

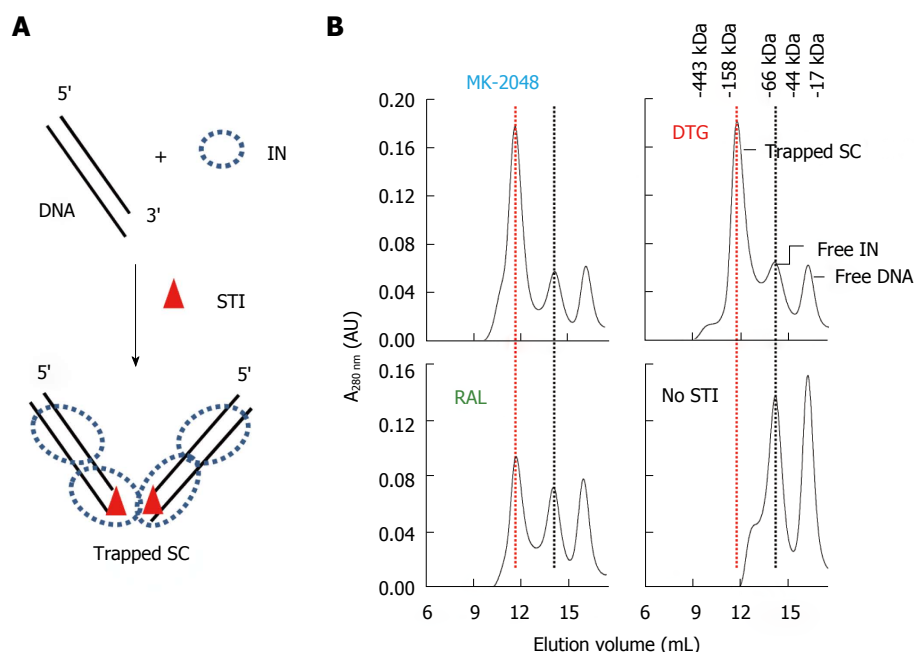


Figure 9 Identification of strand transfer inhibitor-trapped Rous sarcoma virus synaptic complex by size-exclusion chromatography. A: Schematic of Rous sarcoma virus (RSV) IN, 3' OH recessed ODN, and strand transfer inhibitor (STI) used to assemble STI-trapped synaptic complex (SC); B: MK-2048 and Dolutegravir (DTG) efficiently produce trapped SC while Raltegravir had moderate efficiency. No trapped SC is produced in the absence of STI. Elution positions of STI-trapped SC (red line), free IN and DNA are marked. Molecular weight markers are indicated.

their viral DNA substrates at high IN concentrations has not been successful for numerous biochemical reasons including aggregation of HIV IN and limited stability of the complexes.

The HIV SC at low nM concentrations can be physically trapped in the presence of HIV IN STIs as analyzed by native agarose gel electrophoresis^[74,75]. There are strong structural similarities in the CCD between HIV, PFV and RSV IN (Figure 2)^[47,51,52,57,67,76,77] suggesting that under appropriate conditions the highly soluble RSV IN could also be trapped in the presence of the appropriate oligonucleotide (ODN) substrate and STIs. Assembly of a trapped RSV SC at high IN concentrations (1.5 mg/mL; 45 μ mol/L) in solution required the presence of 3' OH recessed viral ODN ranging in sizes (16/18R to 18/20R) and DTG, RAL or MK-2048^[50] (Figure 9). DTG and MK-2048 (an investigational inhibitor) possess a significantly longer dissociative half-life than RAL within an HIV IN-DNA complex allowing these STIs to capture and stabilize the IN-DNA complex^[78,79]. Without the STI presence, the transient RSV SC produced in the concerted integration pathway could not be isolated highly suggesting the STI-trapped SC is kinetically stabilized. Efforts are ongoing to resolve the atomic structure of this kinetically stabilized STI-trapped SC and the RSV strand transfer complex.

Clinical results using HIV IN STIs and drug resistance

The previously described three clinical STIs (Figure 7) are the newest class of inhibitors to treat HIV/AIDS that must be used in combinational drug therapy with

reverse transcriptase, protease and entry inhibitors. RAL and EVG were FDA approved for clinical use in 2007 and 2012, respectively, while DTG was approved in 2013. IN STIs are superior to other regimens in first-line therapies^[80,81]. All three IN STIs have a high safety record and display strong efficacy^[81].

HIV has a high mutational rate, approximately 3×10^{-5} mutations/base pair per cycle^[82]. Thus, like with RT and protease inhibitors, the use of IN STIs has resulted in the emergence of drug-resistant viral strains that causes a loss of drug effectiveness. Both RAL and EVG possess a low genetic barrier giving rise to drug-resistant IN mutants (G140S/Q148H, N155S, Q148K, F121Y, T66I/S153Y) in tissue culture as well as in treated patients^[72,83]. In contrast, DTG has a high genetic barrier and to date significant resistant IN mutants have been nearly absent in treatment-naïve patients^[81,84,85]. The higher genetic barrier of DTG in patients may be due to its longer dissociative half-life from the HIV IN-DNA complex suggesting that DTG extended binding may be a significant factor for prevention of drug-resistance^[86]; also shown to be a factor in producing kinetically stabilized RSV SC (Figure 9). HIV carrying the rare signature IN R263K mutation (others are H51Y and E138K) observed with DTG apparently cannot co-exist in combination with many of the above classical IN resistance mutations^[87]. HIV is apparently unable to compensate for these mutations which induce a fitness cost that prevents the virus from evading inhibitor pressure.

Taken these demonstrated properties of DTG, investigators have slightly modified its structure to

formulate a long-acting injectable nanoparticle (200 nm) suitable for clinical administration on a quarterly basis^[88]. In these preclinical studies, GSK744 provided high-level protection against repeated simian/human immunodeficiency virus challenge in rhesus macaques. This study suggests that GSK744 in combination with a similar injectable RT inhibitor could decrease adherence problems associated with pre-exposure prophylaxis.

RETROVIRAL VECTORS

Human gene therapy

Retroviruses are natural vectors for transfer of genetic information between cells. For example, the insertion of an oncogene derived from one cell by a retrovirus into another cell occurs naturally^[82]. Numerous commercial and investigative retroviral vectors have been developed to accomplish a wide variety of experimental protocols as well as the study of human diseases^[89]. This permanent transfer of genetic information by retroviruses has led to an explosion of ideas for human gene therapies using gammaretroviral (MLV) and lentiviral (HIV) vectors.

The selection of DNA target sites by IN in cells is highly influenced by the interactions of IN with host cofactors and nuclear import properties of the PIC (Figure 4, see above). The selective property of MLV IN to preferentially integrate its viral DNA in the promoter regions of genes had placed a hold on human gene therapy using MLV vectors to treat SCID-X1 (x-linked severe combined immunodeficiency) in boys, which was successful^[90-92]. Insertional mutagenesis lead to T-cell acute lymphoblastic leukemia in 5 of the 20 patients caused by transactivation of LMO2 or CCND2 proto-oncogenes^[93,94]. Recently, a highly modified self-inactivating (SIN) MLV vector with deleted viral U3 enhancer sequences provided the same efficacy as the original MLV vectors and with no leukemogenesis produced after 33 mo^[95]. Future observations of these patients using this modified MLV vector will determine further use of this kind of SIN vector. SIN HIV vectors have been used in human trials and have the advantage that they can effectively transfect resting cells due to their nuclear import properties^[89,96]. Finally, SIN avian retrovirus vectors have been developed but have not been used in human gene therapy^[97]. Consistent with the observation that avian retroviruses integrate randomly into cellular DNA^[34,35], genome-wide analysis of integration sites by a SIN avian retrovirus vector in human hematopoietic stem/progenitor cells demonstrated that integration is random and not near hotspots containing proto-oncogenes like LMO2^[98].

CONCLUSION

This review has outlined the multiple functions that the retrovirus IN has in the life cycle of retroviruses

besides the key function of DNA integration. The development of the three clinical STIs (Figure 7) directed against HIV IN has provided a strong premise for supporting basic scientific research^[73]. The future development of advanced STIs for pre-exposure prophylaxis, the search for new IN inhibitors and the discovery other IN functions are currently underway. The potential utilization of retrovirus vectors for human gene therapy only adds to this future spectrum for scientific investigation of the retrovirus IN.

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Role of ZAC1 in transient neonatal diabetes mellitus and glucose metabolism

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Abstract

Transient neonatal diabetes mellitus 1 (TNDM1) is a rare genetic disorder representing with severe neonatal hyperglycaemia followed by remission within one and a

half year and adolescent relapse with type 2 diabetes in half of the patients. Genetic defects in TNDM1 comprise uniparental isodisomy of chromosome 6, duplication of the minimal TNDM1 locus at 6q24, or relaxation of genomically imprinted *ZAC1/HYMAI*. Whereas the function of HYMAI, a non-coding mRNA, is still unidentified, biochemical and molecular studies show that zinc finger protein 1 regulating apoptosis and cell cycle arrest (ZAC1) behaves as a factor with versatile transcriptional functions dependent on binding to specific GC-rich DNA motives and interconnected regulation of recruited coactivator activities. Genome-wide expression profiling enabled the isolation of a number of *Zac1* target genes known to regulate different aspects of β -cell function and peripheral insulin sensitivity. Among these, upregulation of *Ppar γ* and *Tcf4* impairs insulin-secretion and β -cell proliferation. Similarly, *Zac1*-mediated upregulation of *Socs3* may attenuate β -cell proliferation and survival by inhibition of growth factor signalling. Additionally, *Zac1* directly represses *Pac1* and *Rasgrf1* with roles in insulin secretion and β -cell proliferation. Collectively, concerted dysregulation of these target genes could contribute to the onset and course of TNDM1. Interestingly, *Zac1* overexpression in β -cells spares the effects of stimulatory G-protein signaling on insulin secretion and raises the prospect for tailored treatments in relapsed TNDM1 patients. Overall, these results suggest that progress on the molecular and cellular foundations of monogenetic forms of diabetes can advance personalized therapy in addition to deepening the understanding of insulin and glucose metabolism in general.

Key words: Diabetes mellitus; *ZAC1*; Transient neonatal diabetes mellitus 1; Genomic imprinting; Insulin; Glucose; Target genes; Tailored therapy

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Core tip: Accidents of nature leading to rare genetic diseases can provide important insights into the molecular and cellular foundations of related common diseases. Various genetic anomalies at chromosome 6q24 manifest with life-threatening transient neonatal diabetes mellitus (TNDM1). All of these genetic defects share overexpression of the maternally imprinted transcriptional regulator *ZAC1*. Genome-wide expression profiling identified a number of downstream target genes sharing a critical role in insulin secretion, β -cell proliferation, and survival. Importantly, *Zac1* overexpression in β -cells spares the effects of G-protein signaling on insulin secretion opening the prospect for tailored therapy in TNDM1 patients.

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INTRODUCTION

The individual, societal, and economic burden of the diabetes epidemic has raised steadily over the last decades despite continuous refinements in dietary and pharmacological therapies^[1]. On current trends, at least one in 10 people alive today are at risk to suffer from diabetes at some stage during their lifespan. At the same time, the total number of individuals being at risk to develop diabetes is predicted to double in a generation from 150 million in 2000 to 300 million by 2025. Existing medical treatments frequently ameliorate diabetic conditions without reinstating normal metabolism and thus leave many patients exposed to debilitating, or even life-threatening, impairments^[2]. In conjunction with genetic predisposition (particularly in some ethnicities) the epidemic derives mainly from type 2 diabetes (T2D) and associated conditions termed "diabesity" and "metabolic syndrome". These states encompass various risk factors such as obesity, overly rich nutrition, and a sedentary lifestyle^[3]. Although lifestyle modifications (balanced diet, more exercise, and weight reduction) are well-recognized to delay, or even prevent, T2D in persons at risk, such cautionary measures are notoriously hard to sustain^[4].

A large number of epidemiological and experimental studies have aimed to detect the genetic and environmental factors underlying the onset, progression, and treatment response of diabetes over the last decades. Contrary to the well-established damage of insulin-producing β -cells by autoimmune processes in type 1 diabetes (T1D), the pathophysiological basis of T2D is still incompletely understood and involves multiple cellular and physiological systems influencing each other's set points, thresholds, and metabolic

functions^[5]. Insulin resistance typically evolves during the course of T2D as result of a diminished sensitivity to insulin's metabolic regulatory functions in several peripheral tissues (fat, muscle, and liver) aggravated by reduced exercise, aging, and obesity. T2D represents a relative, rather than an absolute insulin deficiency, as a result of a progressive malfunction of β -cells to secrete sufficient amounts of insulin for sustaining efficient carbohydrate and lipid homeostasis^[6]. Conclusively, refined insight into the pathways that underlie β -cell dysfunction at a physiological and molecular scale remains desirable to advance personalized treatment of T2D.

How can we examine human β -cells when these are barely accessible? Single gene defects encoding severe β -cell dysfunction with manifest diabetes provide unique inroads to disease mechanisms if the causative gene can be isolated. In this regard, positional cloning enabled the identification of heterozygous mutations in the hepatic transcription factors HNF1A and HNF4A as the cause of early-onset diabetes^[7,8]. This important discovery drove forth the subsequent discovery of a transcription factor network underpinning normal β -cell development^[9] as well as susceptibility to T2D^[10,11]. Hence, monogenetic diseases can guide biological studies aimed to unlock the cellular and molecular basis of the specific condition under study, but may have also important implications for related multifactorial diseases.

Based on this perspective, we will discuss in this review recent progress in the molecular genetics of transient neonatal diabetes mellitus (TNDM) and how these new findings advance insight into β -cell development and physiology. Specifically, we will focus on the transcriptional regulator ZAC1 (zinc-finger protein 1 regulating apoptosis and cell cycle arrest), which is encoded by a genomically imprinted gene at 6q24 in human. TNDM is characterized by a unique clinical course manifesting prenatally with T1D and an increased risk for T2D in adolescence. Importantly, the identification of various target genes over the last years eventually enabled insight into ZAC1's role in pancreatic β -cell development and maturation and potential pharmacological treatments of newborns suffering from TNDM.

NEONATAL DIABETES MELLITUS

Neonatal diabetes mellitus (NDM), also recognized as "early-onset" diabetes mellitus, manifests unregulated hyperglycemia during the first 6 mo of life and is a rare disorder developing uniformly in all races and ethnic groups. Typically, NDM presents with intrauterine growth retardation (IUGR), reduced subcutaneous fat, failure to thrive, and low or undetectable C-peptide levels (a peptide of 31 amino acids connecting the A-chain and the B-chain of proinsulin)^[12]. NDM commonly arises from mutations in genes that play a

crucial role in pancreas development, β -cell apoptosis, insulin processing, and the regulation of insulin release^[13]. NDM has an incidence of 1 in 300000 to 500000 live births, whereby current data indicate that approximately over half (57%) of the cases are transient (TNDM), depend on initial insulin application, and resolve spontaneously in less than one and a half year, only to relapse in adolescence.

Different genetic defects have been identified as the source of TNDM; these include mutations in the potassium channel genes *ABCC8* (TNDM2; OMIM 310374) and *KCNJ11* (TNDM3; OMIM 610582)^[14], *insulin*^[15], and occasionally *HNF1B*^[16]. About two-third of all cases of TNDM derive from genetic mutations or loss of genomic imprinting at a locus on 6q24 (TNDM1; OMIM 603044)^[13] encoding *ZAC1* (alias *PLAGL1*), which is in the focus of the remainder of this review, and *HYMAI* (hydatiform mole associated and imprinted transcript) whose biological function remains unknown so far.

TNDM1

Clinical picture and course

TNDM1 was firstly described as a clinical entity in 1926^[17]. Similar to NDM, newborns suffering from TNDM1 represent with perinatal hyperglycemia, undetectable or very low insulin levels, and severe dehydration. Altogether, this life threatening condition requires immediate insulin therapy. IUGR develops in > 95% of TNDM1 cases during the third trimester and concurs with severely reduced subcutaneous fat formation in neonates^[18]. Consistent with insulin's hypothesized role in growth stimulation, postnatal substitution initiates a fast catch-up growth and normalizes TNDM1 patients' height and weight by the age of 2 years.

Additionally, TNDM1 patients can display a number of symptoms varying in penetrance and unrelated to diabetes such as macroglossia, umbilical hernia, cardiac anomalies, brain malformation, and mental impairments in lately diagnosed newborns. These features are shared among TNDM1 infants irrespective of the underlying genetic anomalies^[18].

It is important to note, that insulin dependency declines within a few months postnatally (median treatment duration 3 mo) and rarely extends for up to one and a half year. Following on, TNDM1 remits and patients represent as normoglycemic, free of glycemic compromise, and sustain normal growth^[19]. This reversible course seems compatible with a temporary rather than a persistent defect in the control of insulin synthesis and/or secretion. Yet, approximately one half of all TNDM1 patients develop in adolescence or early adulthood signs of T2D^[20] and require dietetic as well as pharmacological treatments (sulfonylurea and insulin) to ameliorate hyperglycemia. Puberty-related hormonal changes and resumption of somatic growth are known to impose an increased demand on insulin's

availability, may compromise pancreatic functions, and ultimately, lead to relapse with T2D in persons at risk.

Alternatively, the same genetic defect underlying impaired insulin secretion, and consequently IUGR, may predispose to relapse in later life^[21]. Epigenetic mechanisms, particularly DNA-methylation, are thought to mediate epigenetic programming in response to early life conditions^[22,23] and are increasingly recognized to interact with genetic risk factors.

Collectively, TNDM1 represents with neonatal hyperglycemia, infant remission, and adolescent relapse. IUGR and hyperglycemia suggest a decline in available insulin due to a deficit in β -cell number and/or compromised insulin secretion.

Molecular and clinical genetics of TNDM1

Paternal uniparental disomy of chromosome 6 (UPD6) was firstly discovered in two TNDM1 patients in 1995^[24]. The same report presciently suggested that TNDM1 is a genomic imprinting disorder^[25] - a hypothesis that has been confirmed as early as five years later^[26,27]. Expression at the TNDM1 locus on chromosome 6q24 is confined to the paternally derived allele with the maternal allele being silenced. This process, termed genomic imprinting, is initiated in the respective parental germ cells and involves DNA methylation as a central mechanism^[28]. As a result, the expressed paternal *ZAC1* allele is unmethylated at a confined region of 1 kb in size, the so called differentially methylated region (DMR), while the silenced maternal allele is methylated. The DMR straddles the shared promoter of *ZAC1* and *HYMAI* establishing monoallelic expression in healthy individuals. Contrarily, a defect in DMR methylation results in relaxation of *ZAC1/HYMAI* imprinting with biallelic expression in TNDM1^[29]. Although it has not been formally proven whether one or both of these genes underlie TNDM1, it appears decent to assign a critical role to *ZAC1* based on its molecular and cellular properties (see below). In contrast, the non-coding RNA *HYMAI* is transcribed from the same imprinted promoter and in the same orientation as *ZAC1* and is ubiquitously expressed throughout development and maturation although its function remains still unresolved^[27,30]. Notwithstanding the limited information on *HYMAI*, further studies are looked for with respect to the critical role of non-coding RNAs in the control of imprinted gene expression^[28].

In addition to the proximal promoter, *ZAC1* contains a second promoter some 50 kb upstream, which is devoid of genomic imprinting, drives biallelic *ZAC1* expression, and predominates in blood cells^[31]. Although biallelic expression may prevail in some tissues or across defined periods, monoallelic expression from the imprinted promoter is preserved during mouse development and seems to fulfil a particular role in tissues regulating glucose metabolism and/or showing dynamic changes in perinatal *Zac1* expression (see below). Still, the possibility of postnatal switches in

promoter usage^[32] during postnatal remission and/or relapse with T2D should not be dismissed and requires further analysis.

Although half of the TNDM1 patients relapse with T2D in early adulthood, *ZAC1* seems not to encode a T2D susceptibility locus. Whereas the chromosomal region 6q22-25 has been initially captured in some genome-wide association studies, particularly in the Pima Indian population^[33], this finding was refuted in a recent large case-control study in which not a single *ZAC1* nucleotide polymorphism (SNP) associated with T2D^[34]. This study lacked however sufficient power to address a parental impact on SNP effects - an important issue since investigations in the Iceland population evidenced several imprinted loci among regions with a disease association dependent on parental origin^[35].

In addition to the original discovery of UPD6, duplication of the TNDM1 region, and maternal hypomethylation at the DMR have been identified subsequently as a genetic cause of TNDM1^[36].

Children suffering from UPD6 (40%) can display additional symptoms unmasked by uniparental disomy (e.g., recessive mutations). Since UPD6 is the result of a stochastic reproductive error, it is non-hereditary. In contrast, duplication of 6q in TNDM1 patients (32%) comprises a critical region of approximately 160 kb and can be passed to offspring with manifestation in males^[37]. The majority of these duplications are submicroscopic, differ in breakpoints, but share a similar phenotype^[36].

Hypomethylation at the DMR in TNDM1 patients (28%) leads to erasure of all maternal methylated CpG residues and suggests that normal *ZAC1* gene dosage has to be severely disrupted to manifest TNDM1. It is important to note, that approximately 50% of TNDM1 patients with *ZAC1* DMR hypomethylation suffer from hypomethylation at other imprinted loci as well^[38]. This raises the possibility that defects in the imprinting machinery (e.g., loss of ZFP57, a zinc-finger transcription factor containing a KRAB domain^[39]) or imprinted gene networks (IGN) can give rise to TNDM1^[32].

In sum, TNDM1 patients show genetic defects leading to overexpression of *ZAC1* from the imprinted region of chromosome 6q24. The underlying chromosomal anomalies comprise paternal UPD6, paternally derived duplication of 6q24, and maternal methylation defects at the DMR^[20,29].

TRANSGENIC MOUSE MODELS OF TNDM1

ZAC1 overexpression in TNDM1 stimulated the creation of suitable mouse models to analyze the underlying pathology. For this purpose, a P1-derived artificial chromosome (PAC) of 175 kb comprising the entire *ZAC1* and *HYMAI* loci was integrated with multiple copies into the mouse genome^[40]. In accord with

the inheritance in human, hyperglycemia in neonate mice was confined to offspring derived from paternal transmission of the overexpressed PAC.

Interestingly, the effects from *ZAC1* overexpression in mice largely recapitulated human TNDM1 with neonate hyperglycemia, juvenile remission, and adult glucose intolerance^[40]. Yet, neonate growth was not retarded at birth nor were aged mice overt diabetic. Histological examination of pancreata from TNDM1 mice suggested a reduction in the development and existence of all endocrine cell types including β -cells concomitantly with a downregulation of the important developmental transcription factors Ngn3, Pax6, and Pdx1. Puzzlingly, in late pregnancy and postnatally transgenic β -cells assumed a rapid catch-up growth as a result of increased proliferation and/or decreased apoptosis and surpassed in numbers their normal counterparts. Irrespective of this increase in neonatal β -cell numbers, insulin content and secretion were diminished and resulted in postnatal hyperglycemia. Still, an ongoing increase in β -cell numbers enabled their duplication and reinstatement of normal glucose tolerance in juvenile mice. This compensatory increase was, however, not sustained in aged mice, which suffered from an accelerated decline in β -cell numbers and compromised insulin secretory capacity when compared to controls.

Together, these findings in mice corroborate *ZAC1/HYMAI*'s maternal imprinting status and evidence the impact of these genes on pancreatic β -cell proliferation, differentiation, and insulin secretion from late gestation through adulthood. Consistent with these data, *ZAC1* overexpression in rodent β -cell lines^[41,42] reduces glucose-stimulated insulin secretion (GSIS) as will be discussed in more detail in the section on target genes.

In contrast to *ZAC1* overexpression in pancreatic β -cells, results from *Zac1* deficient mice (*Zac1*^{+/-pat}) evidenced no obvious effects on β -cell proliferation or insulin secretion^[43]. *Zac1*^{+/-pat} mice represented with IUGR, altered bone formation, impaired pulmonary respiration, hydrocephaly, disturbed neural cell numbers, and neonatal lethality^[43,44]. Notably the same authors^[43] assigned to *Zac1* a critical role in the regulation of an IGN, which on its own may affect β -cell function and confound *Zac1* defects.

All in all, these studies support a role of *ZAC1* overexpression in TNDM1 and prompt the question through which molecular and cellular mechanisms *ZAC1* contributes to the clinical picture and course of TNDM1.

TRANSCRIPTIONAL REGULATOR ZAC1

The zinc-finger protein *Zac1* was originally isolated from clonal corticotroph tumor cells derived from mice^[45] and shortly afterwards from malignantly transformed ovary surface epithelial cells^[46]. *Zac1* overexpression studies together with appropriate knock-down experiments

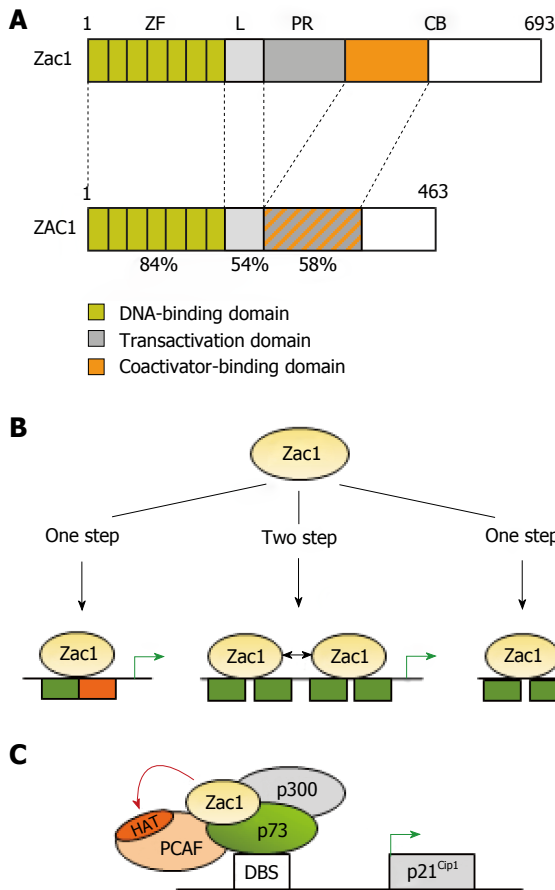


Figure 1 Transcriptional activities of Zac1. A: Schematic drawing of Zac1 proteins. Domains are boxed and numbers refer to amino acids and percentage of homology between mouse and human. The N-terminal DNA binding domain is highly conserved between human and mice and comprises seven canonical C2H2-type zinc fingers (ZF). The linker domain (L) in conjunction with the proline-rich region (PR) confers transactivation in mice, which is further enhanced through the C-terminus's coactivator binding (CB) domain. Contrarily, transactivation and coactivator recruitment jointly localize to ZAC1's C-terminus; B: Zac1 recognizes different DNA-elements to confer transcriptional regulation. Monomeric Zac1 binding to GC-rich palindromes (left) or dimerization at G/C rich direct repeat elements (middle) results in transactivation. On the other hand, monomeric Zac1 binding to G/C rich half sites causes repression (right); C: Zac1 coactivation of p73. Following recognition of its DNA-binding site (DBS), the transcription factor p73 recruits Zac1 together with the general coactivator p300 and PCAF to the *p21^{Cip1}* promoter during early neural differentiation. Due to its scaffolding function, Zac1's zinc fingers stabilize this interaction and enhance additionally PCAF's histone acetyltransferase (HAT) activity. This event enhances histone acetylation at the *p21^{Cip1}* promoter and subsequent transcription.

evidenced Zac1's role in the regulation of apoptosis and cell cycle arrest and stimulated its name-giving^[45,47,48]. In agreement with these observations, human ZAC1^[48], undergoes methylation-sensitive silencing in various tumors including ovary and mammary cancer^[46,49].

Human and mouse ZAC1 proteins consist of 463 and 693 amino acids, respectively, and share a highly conserved amino-terminal DNA-binding domain enclosing seven zinc fingers conforming with the classical C2H2-type (Figure 1A). Apart from that, a central and C-terminal domain enriched in proline and glutamic acid residues, respectively, exists solely

in mice^[48]. The region adjacent to the DNA-binding domain, termed linker-region, together with the central proline-rich region confers transactivation in mice, which is enhanced by the C-terminus' interaction with the general coactivators p300/CBP^[50]. On the other side, transactivation and coactivator recruitment are merged in ZAC1's C-terminus and illustrate how different protein motives are used species-specifically in transactivation (Figure 1A).

Monomeric Zac1 can bind to palindromic GC-rich DNA elements to confer transactivation (Figure 1B). Similarly, Zac1 dimerization at direct and reverse G/C-rich repeat DNA elements confers transactivation whereas monomeric binding to single half sites leads to repression^[51] (Figure 1B). In addition to DNA binding, zinc finger 6 and 7 interact with p300 to increase (palindromes and repeats) or decrease (half sites) its catalytic activities^[50]. This interrelationship between Zac1's DNA address code and enzymatic regulation of transcription is thought to support efficient regulation of target genes.

Moreover, Zac1 can coregulate the activity of various members of the nuclear receptor and p53 families without binding on its own to DNA due to its scaffolding function and concomitant coactivator regulation^[52-54] (Figure 1C). The p53 family comprises the eponym p53 and the relatives p63 and p73, all of which share critical roles in cell renewal and proliferation, cell fate commitment and differentiation, and ultimately apoptosis^[55]. As an example, the transcription factor p73 tethers the coactivators PCAF/p300 and Zac1 jointly to the *p21^{Cip1}* (*Cdkn1a*) promoter in a model of early neuronal differentiation. Consistent with its scaffolding function Zac1 strengthens *via* its zinc fingers the interaction between PCAF and p300 and enhances at the same time PCAF's catalytic activity (Figure 1C)^[52]. This finding evidences an authentic Zac1 coactivator function and suggests that similar mechanisms apply to Zac1-dependent nuclear receptor coregulation^[53].

In summary, Zac1 transcription factor activities are determined by sequence-specific DNA binding and interconnected regulation of coactivator activities. Additionally, Zac1 can act as coregulator for unrelated transcription factors by stabilizing complex formation with recruited coactivators and enhancement of their catalytic functions.

ZAC1 TARGET GENES IN THE PANCREAS

Zac1's role as transcription factor and coregulator offers the opportunity to gain insight into its function in TNDM1 *via* the identification of downstream target genes. In the context of this review we will pay particular attention to those targets, which are expressed in pancreatic β -cell islets and are likely to share a role in insulin and glucose metabolism. Readers interested in further Zac1 functions are referred to recent reviews^[32,56].

In support of ZAC1's potential role in TNDM1, strong immunoreactive signals were detected in the nuclei of insulin positive cells, whereas the exocrine pancreas was only sparsely stained in adult human^[42]. Refined immunohistochemistry in mice evidenced strong Zac1 signals at prenatal and neonatal stages [(embryonic day 18 (E18) and postnatal day 1 (P1)] in the endocrine pancreas of both insulin-expressing and non-expressing cells. Moreover, sparse immunoreactivity was spread throughout the exocrine pancreas. Postnatally, Zac1 immunoreactivity rapidly declined in either pancreas compartment (P4 to P12) in accord with previous reports^[40,57]. Subsequently, Zac1 expression was maintained at lower levels in insulin-positive cells in early adult mice (P42) but was largely absent in the parenchyma consistent with the findings in human^[42].

Together, these findings corroborate ZAC1 expression in insulin-positive cells in human and mice and reveal dynamic changes in perinatal expression levels.

PAC1

PAC1 (*alias ADCYAP1R1*) encodes a G-protein coupled receptor (GPCR) for the neuropeptide pituitary adenylate cyclase activating polypeptide (PACAP) and was the first Zac1 target gene to be identified^[45,58,59]. Zac1 regulates *Pac1* gene expression and related promoter reporter constructs in a cell type-specific fashion as evidenced by activation in cerebellar granule cells, epithelial kidney cells, and osteosarcoma cells vs inhibition in pituitary gonadotroph cells and pancreatic β -cells^[58,60,61] (Hoffmann A, Spengler D. Unpublished findings. 2012). The neuropeptide PACAP was firstly purified from sheep hypothalamus^[62] and has multiple functions on the neuroendocrine, endocrine, and nervous system^[63]. Pancreatic islets are richly innervated *via* pancreatic ganglia that derive from the vagus dorsal motor nucleus. Both ganglia and islets contain PACAP as well as the related vasointestinal peptide (VIP) among other neurotransmitters. This distribution suggests that PACAP can act at the same time as neurotransmitter and islet endocrine peptide^[64].

The VIP/PACAP receptor family comprises PAC1 (specific for PACAP), VPAC1, and VPAC2 (both with equal affinity for VIP and PACAP)^[65]. Pancreatic islets express all of these receptors, which couple to stimulation of adenylate cyclase^[66,67]. Increases in intracellular levels of cAMP activate protein kinase A (PKA) and guanine nucleotide exchange factors of the Epac family that enhance insulin secretion through distinct pathways involving K_{ATP} channels together with the exocytotic machinery^[68].

Intracellular glucose metabolism presents the major stimulus for GSIS. This process commences with the uptake of glucose into β -cells *via* the glucose transporter protein 2 (Glut-2). Breakdown of glucose leads to ATP production and an enhanced ATP/ADP ratio followed by closing of K_{ATP} channels. Subsequently, plasma membrane depolarization and

Ca^{2+} influx stimulate the exocytosis of insulin.

PACAP and VIP are thought to potentiate glucose-stimulated, but barely resting, insulin secretion by increasing intracellular Ca^{2+} concentrations^[64]. In accord with this hypothesis, *Pac1* null mice develop apparently normal and represent with inconspicuous resting plasma glucose and insulin levels^[69]. On the other side, the response to PACAP on GSIS was reduced by half in isolated islets from *Pac1* null mice. Moreover, these mice showed reduced GSIS following intravenous or oral glucose administration. Hence, *Pac1* seems to control the insulin-secretory response to insulin itself in addition to its potentiation. Consistent with this possibility, *Pac1* null islets showed a diminished insulin response to glucose indicating *Pac1*'s critical role for optimal GSIS during food intake^[69]. A possible explanation for this finding is that intracellular PACAP, independently of neural release, triggers PKA activation necessary for optimal calcium influx in the context of GSIS.

In contrast to PACAP's role for insulin secretion, its effects on long-term β -cell mass have been less studied. Islets commonly decline during the course of T2D and aggravate the increased demand for insulin. Interestingly, PACAP overexpression in β -cells ameliorated the effects of streptozotocin-induced cell death as evidenced by lower plasma glucose levels in transgenic mice when compared to controls^[70]. Morphometric analysis showed a tendency towards increased β -cell mass suggestive of a role for PACAP in the regulation of islet cell proliferation or differentiation. Compatible with this view, PACAP enhances the growth of pancreatic cancer cells through cAMP/PKA-dependent stimulation of the MAP kinase pathway together with the upregulation of nuclear oncogenes^[71]. Relatedly, other studies suggest that intracellular PACAP protects β -cell mass by preventing harmful exogenous insults on the β -cell^[72,73].

All in all, these studies raise the possibility that altered ZAC1 dosage in TNDM1 reduces *PAC1* expression in β -cells, GSIS, the insulin-secretory response itself, and β -cell proliferation or survival on the long term.

PPAR γ

Genome-wide expression analysis^[74] led to the identification of *Ppar γ* (peroxisome proliferator-activated receptor) as a Zac1 target gene. PPAR γ belongs to the nuclear hormone receptor family with an important function in adipogenesis, lipid and glucose metabolism, differentiation, antiproliferation, apoptosis, and inflammation^[75]. The 5'-end of *PPAR γ* is subject to alternative splicing that produces two protein isoforms differing in 30 amino acids present in the PPAR γ 2, but absent in the PPAR γ 1, amino terminus^[76]. PPAR γ 2 expression prevails in adipose tissue, whereas PPAR γ 1 is more widely expressed comprising heart and skeletal muscle, kidney, liver, spleen, colon, pituitary, brain, and pancreas. ZAC1 binds *in vitro* and *in vivo* to the

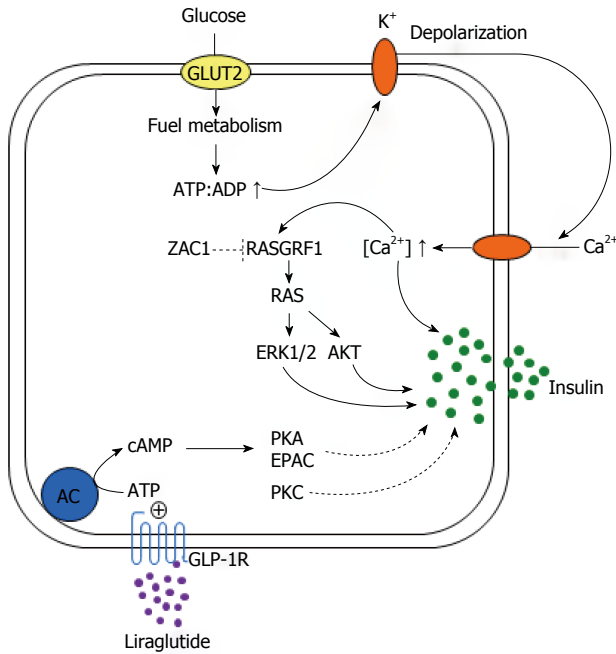


Figure 2 Roles for RAS protein-specific guanine nucleotide-releasing factor 1 in insulin secretion and transient neonatal diabetes mellitus type 1. Glucose is the main stimulus for insulin secretion. Once transported into pancreatic β -cells by glucose transporter 2 (GLUT2), metabolism of glucose results in the production of ATP. A subsequent rise in the ATP:ADP ratio drives closure of ATP-regulated K^+ channels and accumulation of intracellular K^+ . Heightened K^+ concentrations lead to depolarization of the plasma membrane and promote opening of voltage-dependent Ca^{2+} channels with subsequent influx of Ca^{2+} and increases in free cytoplasmic Ca^{2+} . This event stimulates exocytosis of insulin from the insulin-storing secretory granules through different routes including activation of the Ca^{2+} -calmodulin activated RAS protein-specific guanine nucleotide-releasing factor 1 (RASGRF1), a direct ZAC1 target gene. In its activated state the small G-protein RAS couples to ERK1/2 and PI3K/AKT signaling among other downstream effectors, which jointly enhance the exocytotic process of insulin secretion. ZAC1 overexpression in transient neonatal diabetes mellitus type 1 (TNDM1) is predicted to directly repress RASGRF1 and consequently GSIS. Contrarily, PKA signaling is undisturbed in TNDM1 as well as the potentiating effects of the GLP-1R agonist liraglutide on GSIS.

proximal promoter region of *PPAR γ 1* in various cell types (neural cells, pituitary cells, and colon cells among others) derived from rodent or men^[74]. Functionally, ZAC1 antiproliferation partly depends on *PPAR γ 1* expression and associates with the upregulation of *PPAR γ* target genes contributing to differentiation (*KER20* and *TSC22*), apoptosis (*POX*) or growth inhibition (*PTEN*).

Notably, *PPAR γ* overexpression has been suggested to attenuate glucose- and potassium-induced stimulated insulin secretion^[77,78]. This effect is thought to relate to *PPAR γ* 's upregulation of uncoupling protein-2 (*UCP-2*), which reduces the availability of ATP needed for the energy-consuming exocytotic process of insulin secretion.

On the contrary, β -cell specific deletion of *Ppar γ* causes islet hyperplasia with unaltered glucose homeostasis^[75]. A plausible *Ppar γ* target gene contributing to these effects is *Pten* (phosphatase and tensin homologue

deleted on chromosome 10), which diminishes intracellular levels of phosphatidylinositol *via* inhibition of phosphatidylinositol-3-kinase (PI3K). As a result, deletion of *Pten* enhances cell proliferation and survival without disrupting differentiation^[79].

Interestingly, *Pten*'s effects on β -cell proliferation extend to models of T2D and deteriorated insulin secretion in aged mice. In this regard, continuous β -cell specific deletion of *Pten* protects against T2D ensuing from high-fat diet or deficient leptin-signaling due to a gain in β -cell mass under basal and diabetic circumstances^[80]. Similarly, a knockout of *Pten* at adult age manifests an enhanced β -cell mass and proliferation as result of a sustained response to mitogenic stimulation during aging^[81].

In summary, these findings support that enhanced ZAC1 expression may impair insulin secretion and β -cell proliferation *via* induction of *PPAR γ* and its downstream targets *UCP-2* and *PTEN*. Because this pathway has not formally been proven to exist in pancreatic β -cells, further studies on this transcriptional network are looked for.

Rasgrf1

The small G-protein Ras integrates multiple upstream signaling pathways and connects them to different downstream effectors comprising the Raf-MEK-Erk kinase cascade, the PI3K-Akt pathway, and RalGDS family members among others^[82]. Ras alternates between an active GTP-bound and inactive GDP-bound state. This balance is tightly regulated by GTPase-activating proteins (GAPs) and guanine nucleotide-exchange factors (GEFs), respectively. Various GEF proteins have been isolated; among these RAS protein-specific guanine nucleotide-releasing factor 1 (RASGRF1) and RASGRF2 are stimulated by the G-protein subunits β and γ or by calmodulin-mediated Ca^{2+} influxes (Figure 2).

Genome-wide expression profiling for *Zac1* target genes in neural cells evidenced downregulation of *Rasgrf1*^[74]. Originally thought to be confined to postnatal brain, *Rasgrf1* expression has been also detected in adult mice islets^[83]. Consistent with this report, cytoplasmatic RASGRF1 expression colocalizes with nuclear ZAC1 expression in adult human insulin-positive cells with weak expression in the parenchyma^[42]. A similar expression pattern exists in mice with *Rasgrf1* and *Zac1* mRNA and protein expression correlating inversely with each other pre- and postnatally. Accordingly, *Rasgrf1* was weakly expressed at E18 when *Zac1* expression is high and rapidly increased postnatally when *Zac1* expression declined. Expression of both *Zac1* and *Rasgrf1* in two mouse β -cell models (Min6 and R7T1) was similar to P4 neonate islets from mice with a strong decrease of *Rasgrf1* expression following *Zac1* transfection. Chromatin-immunoprecipitation (ChIP) analysis evidenced that *Zac1* bound at multiple predicted DNA-binding sites at the *Rasgrf1* promoter

and associated with the presence of repressive histone marks^[42].

R7T1 β -cells were stably transfected to express double amounts of Zac1 as an *in vitro* model to simulate the effects of biallelic ZAC1 expression in TNDM1. Zac1 overexpressing β -cells showed moderately delayed differentiation with unaltered cell proliferation. Importantly, Zac1 overexpression impaired glucose-, insulin-, and potassium-induced depolarization and the subsequent activation of MAPK and PI3K pathways driving insulin secretion (Figure 2). Consistent with a crucial function of Rasgrf1 in Zac1-dependent insulin secretion, reinstatement of Rasgrf1 expression in Zac1 overexpressing R7T1 β -cells normalized insulin secretion^[42]. Moreover, subcutaneous transplantation of parent, but not of Zac1 overexpressing R7T1 β -cells ameliorated hyperglycemia in streptozotocin-treated diabetic mice.

Notably, activated Ras may additionally switch on the GEF function of RalGEF and Tiam1. Hereby, Tiam1 facilitates the action of Rac1 with a regulatory role in GSIS^[84] and RalGEF enhances the activity of the small G-protein Ral with a regulatory role in different steps of insulin exocytosis^[85,86]. Hence, Zac1-dependent Rasgrf1 repression may integrate additional pathways beyond canonical MAPK and PI3K signaling, which overall result in an impaired GSIS.

Activation of GPCRs leading to an increase in intracellular levels of cAMP (see above), are known to optimize the insulinotropic effects of glucose. In this regard, the glucagon-like peptide 1 receptor (GLP-1R) stimulates insulin secretion independent of Ras pathways. Indeed, liraglutide, a clinically used GLP-1 analog, undistinguishably stimulated MAPK and PI3K pathways and downstream insulin secretion in stable mock- and Zac1-transfected R7T1 β -cells and strongly reduced hyperglycemia following their subcutaneous transplantation in experimental diabetic mice.

Interestingly, the oral or intravenous glucose tolerance tests display a subnormal insulin response in adolescent TNDM1 patients whilst glucagon-stimulated insulin secretion is preserved^[87]. Hence, β -cells in TNDM1 patients seem to sustain the capability to secrete insulin in response to stimulatory GPCR pathways, but not to glucose stimulation.

In summary, this work evidences a critical role for the regulatory Ras/Rasgrf1 dyad in GSIS. Zac1-dependent repression of Rasgrf1 diminishes the activation of Ras' downstream effectors comprising canonical and non-canonical pathways in insulin secretion. On the contrary, GPCR's capability to potentiate GSIS seems to be spared from Zac1 overexpression in accord with findings from adolescent TNDM1 patients (Figure 2).

Socs3

The neural stem cell line (C17.2) has been used for additional genome-wide expression profiling and led to the identification of *Socs3* (suppressor of cytokine

signaling) as potential Zac1 target gene^[88]. SOCS proteins comprise a group of eight members (SOCS1 through SOCS7 and CIS) all of which encompass a COOH-terminal SOCS box and a characteristic central Src homology-2 (SH2) domain. Cytokine stimulation leads to the activation and nuclear translocation of the signal transducer and activator of transcription (STAT) proteins that in turn upregulate the expression of SOCS genes among others. These transcription factors recognize their DNA address code at regulatory regions of the SOCS genes, and once induced, CIS, SOCS1, SOCS2, and SOCS3 form a canonical negative feedback loop that serves to attenuate further cytokine signaling^[89]. Similarly, SOCS family members might also inhibit tyrosine kinase signaling, particularly by the insulin-like growth factor-1 (IGF-1) and insulin receptors (see below).

The *Socs3* gene contains multiple predicted Zac1 DNA-binding sites clustered at the regulatory upstream region and the neighboring first intron. Complementary experiments including different *Socs3* promoter reporter assays in conjunction with various Zac1 expression constructs as well as ChIP studies evidenced that Zac1 confers transactivation in an additive manner following binding to the *Socs3* promoter and first intron. Similarly, ZAC1 potently induced SOCS3 in human neuroblastoma cells indicating that regulation of this target gene is preserved in human cells^[88]. Functionally, Zac1 transiently induces *Socs3* in NSCs during the course of astroglial, but not of neural, differentiation. Zac1-dependent *Socs3* expression peaks at the astroglial transition to maturation and has been suggested to prevent precocious astroglial differentiation by inhibition of related signaling pathways independent of Zac1's cell cycle arrest function. In accord with this hypothesis, Zac1 knock-down advanced astroglial differentiation. Conversely, knock-down of *Socs3* in Zac1 overexpressing cells reinstated timely astroglial differentiation irrespective of a Zac1-driven sustained G1 arrest.

In sum, Zac1 transactivates in a lineage-specific manner *Socs3* in NSCs and thus fine tunes astroglial differentiation independent of Zac1's cell cycle arrest function^[88]. Moreover, these findings raise the possibility of a Zac1 role for *Socs3* regulation in TNDM1, particularly in pancreatic islets. Although there are no experimental data available yet, we will discuss in the following part the rationale for future studies to explore this topic.

Originally viewed as negative regulators of cytokine signaling, SOCS proteins have been more recently also suggested to attenuate insulin receptor signaling^[90]. Contrarily to cytokine receptors, insulin and IGF-1 receptors contain tyrosine kinase activity on their own. Once bound by its receptor, insulin triggers autophosphorylation and catalytic activation leading to the phosphorylation of various intracellular targets, including insulin receptor substrate (IRS) proteins. In

their phosphorylated state, IRSs associate with multiple SH2 domain-containing proteins and enable that multiple signaling proteins are subsequently recruited to the plasma membrane where they regulate various signal transduction pathways controlling translocation of glucose transporters from intracellular vesicles to the plasma membrane, protein and glycogen synthesis, and ultimately cell proliferation and survival^[90].

Insulin resistance denotes an impaired tissue response to insulin and presents an important pathogenic determinant in the manifestation of T2D. Heightened concentrations of proinflammatory cytokines comprising tumor necrosis factor- α (TNF α), interleukins (IL-1 β and IL-6), and growth hormone (GH) during injury, obesity, or infection, are considered to advance the onset of insulin resistance in peripheral tissues^[6]. In this context it is important to note that SOCS1 and SOCS3 proteins have been shown to disrupt signaling complex formation at insulin or IGF-1 receptors as a result of an enhanced degradation of the adaptor proteins IRS-1 and IRS-2. Consequently, insulin signaling declines in disease-relevant organs such as liver and muscle as well as in adipose tissue^[90]. Because both ZAC1 and SOCS3 are expressed in these tissues, ZAC1 overexpression in TNDM1 patients may contribute to relapse with T2D in adults *via* sustained upregulation of SOCS3 and subsequent impairments in insulin signaling.

In addition to an effect on peripheral insulin signaling, SOCS3 appears also to affect pancreatic β -cells themselves^[91]. Tyrosine phosphorylation of the insulin receptor and associated IRS proteins in response to insulin binding is inhibited by IL-1 β *via* upregulation of SOCS3 expression and the formation of SOCS3/insulin receptor complexes. Together, these events result in reduced PI3K signaling and impaired long term survival of β -cells^[91]. Relatedly, SOCS3 has been reported to attenuate growth factor signaling important to β -cell proliferation and cell mass^[92]. In this respect, overexpression of SOCS3 exclusively in β -cells caused a reduction in β -cell volume by one third in transgenic mice and reduced STAT5 phosphorylation upon GH treatment of transgenic islets when compared to wild-type ones. Similar results were obtained for adenovirus mediated SOCS3 overexpression in primary islets. Notably, SOCS3 overexpression spared the effects of GLP-1, mediated by PKA, on cell proliferation. Collectively, these results suggest that SOCS3 inhibits β -cell proliferation by counteracting growth factor receptor signaling.

On the other side, a number of reports evidenced for SOCS3 a role in β -cell survival following exposure to cytotoxic factors. As far back as 2001, Socs3 has been suggested to protect against interferon- γ and IL-1 β -mediated β -cell damage by reduced transcription of inducible nitric oxide synthase (iNOS)^[93]. Similarly, TNF α -induced apoptosis with a possible role in T1D is prevented by Socs3 overexpression in rat INS β -cells^[94]. In further support of these findings, ciliary

neurotrophic factor signaling (CNTF) increased STAT3 (cytoplasmatic and nuclear phosphorylated forms) as well as Socs3 expression and protected against cytokine- or streptozotocin-induced apoptosis^[95,96]. At the same time, increased Socs3 expression impaired GSIS in isolated rat neonatal islets.

Collectively, these results suggest that the effects from SOCS3 expression are context-dependent involving growth inhibition as well as improved survival following cytotoxic insults.

Overall, ZAC1-dependent SOCS3 upregulation may contribute to different aspects in TNDM1 by: (1) facilitating development of peripheral insulin resistance in adults; (2) inhibiting prenatal β -cell proliferation; and (3) impairing GSIS. Future experimental studies are looked for to address these topics.

p57^{Kip2}

The cyclin-dependent kinase inhibitor (CKI) p57^{KIP2} fulfils a critical task in cell cycle progression and differentiation^[97]. Cyclin-dependent kinases (CDK) represent an evolutionary conserved group of serine/threonine kinases whose catalytic activities are tightly controlled through the association with various cyclins. The expression levels of cyclins oscillate periodically across the cell cycle and stimulate the sequential formation of different CDK-cyclin complexes that drive the coordinate progression through the cell cycle.

Application of the neuropeptide PACAP (see above) rapidly increases p57^{KIP2} expression in embryonic cortical precursors and reduces the activity of CDK2, entry into S-phase, and the synthesis of DNA. At the same time PACAP enhanced the association of p57^{KIP2}, but not of p21^{Cip1} or p27^{Kip1}, with the kinase complex in accord with PACAP's antimitogenic function^[98]. Hence, Zac1-dependent induction of *Pac1* in neural cells, but not in β -cells where *Pac1* is repressed, may contribute *via* enhanced p57^{KIP2} expression to antiproliferation.

Relatedly, Zac1 can induce p57^{KIP2} *via* an alternative route involving the basic helix-loop-helix (bHLH) protein Tcf4. The family of bHLH proteins coordinate cell lineage decisions with proliferation and differentiation and the subsequent migration of progenitors during neurogenesis among other functions^[99]. These proteins share a bHLH domain enabling homo- or heterodimerization upon DNA-binding to E-box (CANNTG) motives. Two major groups can be distinguished: the spatiotemporally expressed specification factors (*e.g.*, NeuroD, neurogenin, Mash, and Math) and the ubiquitously expressed dimerization factors known as E-proteins. The latter group comprises the two splice variants of *E2A*, *E12* and *E47*, *HEB*, and *TCF4* (also called *E2-2*)^[100]. Among these genes, *Tcf4* has been recently identified as direct Zac1 target gene during early neurogenesis^[101]. Zac1 recognizes multiple GC-rich DNA-elements at both the promoter and first intron of *Tcf4* to confer synergistic transactivation as shown by reporter assays with various *Tcf4* promoter reporter plasmids and Zac1 constructs.

Moreover, sequential ChIP assays corroborated that Zac1 binding associates with the presence of active histone marks at *Tcf4*.

Interestingly, E-proteins share with Zac1 a cell cycle arrest function, which has been attributed to their induction of $p57^{Kip2}$ in neuroblastoma cells^[102]. Compatible with these findings several predicted E-box motifs exist in the distal $p57^{Kip2}$ promoter and confer Zac1 transactivation *via* Tcf4 upregulation^[101].

Collectively, these data suggest that $p57^{Kip2}$ is an indirect Zac1 target gene *via* transactivation of *Tcf4*. In this regard, a further investigation has pointed to a critical function of $p57^{Kip2}$ in β -cell replication^[103]. Hyperinsulinism of infancy presents a complex syndrome in which β -cells fail to suppress the secretion of insulin under hypoglycemic conditions^[104]. The majority of patients suffer from mutations of either in *KCNJ11* (encoding two subunits of the β -cell ATP-sensitive K^+ channel)^[105] or *ABCC8* (encoding a sulfonylurea receptor)^[106], both of which regulate the secretion of insulin.

Hyperinsulinism of infancy can be histologically distinguished into a diffuse form affecting all β -cells and a focal one in which a single cell population undergoes clonal expansion. Genetically, focal β -cell hyperplasia results from a duplication of the p terminus of paternal chromosome 11 at the cost of the maternal allele. This recombination event results in two mutated alleles at the *ABCC8/KCNJ11* loci and UPD for all genes telomeric to this site^[107]. The duplicated segment harbors several paternally silenced imprinted genes including $p57^{Kip2}$ whose expression is extinguished in mutated β -cell descendants^[108]. To evidence a role in β -cell proliferation, shRNA was used to silence $p57^{Kip2}$ in islets obtained from deceased donors free of pancreatic dysfunctions^[103]. Interestingly, such $p57^{Kip2}$ knock-down β -cells reassumed replication when transplanted under the kidney capsule of immunodeficient hyperglycemic mice and showed a normal glucose-induced calcium influx in support of an intact β -cell function.

In sum, this study identifies biallelic silencing of $p57^{Kip2}$ as a critical pathogenic factor for focal hyperinsulinism. Functionally, $p57^{Kip2}$ seems to act as a gatekeeper of mature β -cell replication. In this respect, ZAC1 overexpression in TNDM1 may upregulate $p57^{Kip2}$ *via* *TCF4* at crucial stages of prenatal and postnatal development. Transgenic ZAC1 mice provide a valuable model to explore this hypothesis in future studies.

CONCLUSION

The steady rise of the diabetic epidemic makes further progress on the genetic, cellular, and physiological basis of this multifactorial disease mandatory. Accidents of nature leading to well-defined genetic defects can provide valuable insights into β -cell function and glucose metabolism in general. TNDM1, a rare

monogenetic disease, appears of particular interest in this respect given its variable clinical course representing postnatally with severe hyperglycemia and insulin deficiency, remission within one and a half year, and frequent relapse with T2D in adolescence. Identification of the genetic defects at chromosome 6q24 (UPD6, duplication of the TNDM1 region, relaxation of genomic imprinting) point to altered dosage of monoallelically expressed ZAC1 as important pathogenic factor in TNDM1. Consistent with this view, ZAC1 overexpression in transgenic mice simulated major aspects of the human disease. A set of biochemical and molecular studies showed that Zac1 represents a zinc-finger protein with versatile regulatory functions dependent on DNA-binding to specific GC-rich motives and interconnected regulation of recruited coactivator activities. Additionally, Zac1 can serve as coactivator for the p53 and nuclear receptor families even so this function is presently largely unexplored with respect to TNDM1. Contrarily, the role as canonical transcription factor allowed gaining insight into ZAC1's role in TNDM1 through the identification of downstream target genes. Genome-wide expression profiling enabled the isolation of a set of direct target genes known to regulate various aspects of β -cell function and peripheral insulin sensitivity. Among these, Zac1-dependent upregulation of the transcription factors Ppar γ and Tcf4 may translate into an altered expression of downstream targets sharing a role in insulin secretion (*Ucp-2*) and β -cell proliferation (*Pten* and $p57^{Kip2}$) (Figure 3, middle). Similarly, Zac1-mediated transactivation of *Socs3* may inhibit β -cell proliferation and survival by attenuating growth factor signaling. Moreover, altered ZAC1 dosage in TNDM1 may also extend to *SOC3* upregulation in peripheral tissues and underlie impaired insulin signaling in relapsed patients with T2D (Figure 3, right).

Additionally, Zac1-dependent repression of downstream target genes may as well contribute to reduced insulin secretion and β -cell proliferation. In this regard, *Pac1* and *Rasgrf1* were repressed by Zac1 in β -cells, but not in neural cells, indicating that Zac1's transcriptional or coregulatory activities are tissue-specific^[42,53,59,61]. *Pac1* enhances the insulin-secretory response to insulin, GSIS, and β -cell proliferation (Figure 3, left). Similarly, *Rasgrf1* repression attenuated Ras-mediated activation of canonical and non-canonical pathways in GSIS. Even so, Zac1 overexpression in β -cells spared the effects of GPCR on GSIS. In any case, the concerted regulation of these target genes in β -cells and peripheral tissues may partly explain the clinical course of TNDM1 ranging from fatal neonatal hyperglycemia, subsequent remission, to adolescent relapse with T2D.

Interestingly, relapsed TNDM1 patients show in response to both the oral and intravenous glucose tolerance test a subnormal insulin secretion, whilst glucagon-stimulated insulin secretion is preserved^[87]. This raises the possibility that insulin secretion *via*

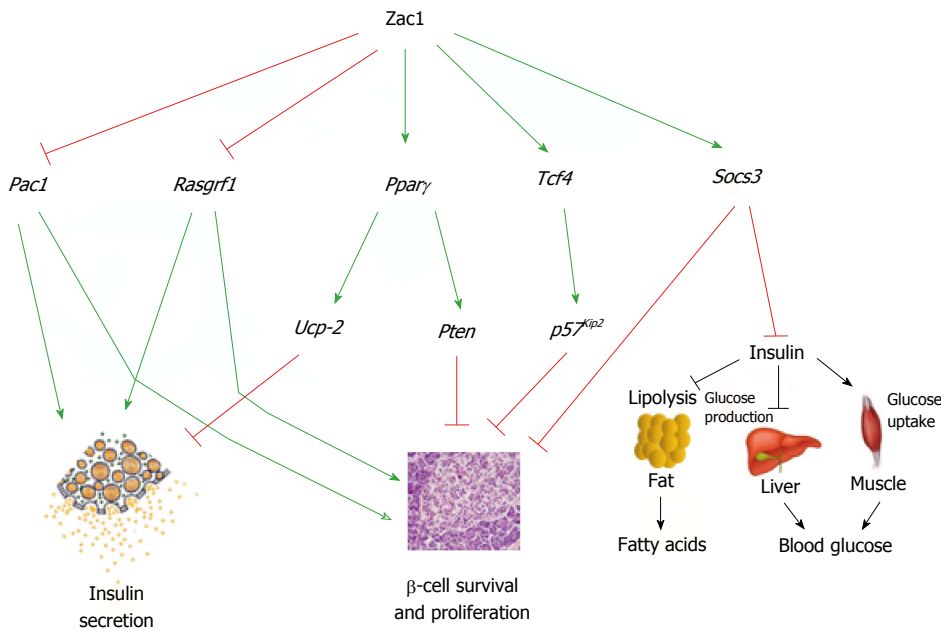


Figure 3 Integrated model of Zac1's role in β -cell function and glucose metabolism. Zac1 DNA-binding at downstream target genes confers transcriptional activation or repression in a partly tissue-specific manner in mice. In this respect Zac1 binding represses *Pac1* and *Rasgrf1* in pancreatic β -cells leading to an impaired insulin-secretory response and GSIS (left). Additionally, Zac1 upregulation of the transcription factors *Ppar γ* and *Tcf4* may translate in enhanced expression of their downstream targets *Ucp-2*, *Pten*, and *p57^{Kip2}* with an inhibitory role in GSIS and β -cell proliferation (middle). Moreover, Zac1-dependent transactivation of *Socs3* may attenuate growth factor and insulin signaling in β -cells and peripheral tissues, respectively (right).

stimulatory G-protein signaling is maintained in TNDM1 and thus partly restores β -cell function while being compromised for GSIS. Indeed, treatments targeting the GLP-1 pathway have been lately reported as an interesting therapeutic option instead of insulin substitution in relapsed TNDM1 patients^[109]. This finding corroborates previous work in experimental diabetic mice, where liraglutide treatment ameliorated hyperglycemia following subcutaneous transplantation of either parent or Zac1 overexpressing β -cells^[42].

Overall, these results demonstrate that elucidation of the molecular and cellular foundations of rare monogenetic diabetic diseases can substantially advance our understanding of β -cell function and glucose metabolism in general. The advent of precision medicine^[110] may not only offer access to personalized information we look for to stay healthy but may also lead to tailored treatments as suggested here for TNDM1 following ZAC1 overexpression.

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Biology of hyaluronan: Insights from genetic disorders of hyaluronan metabolism

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Abstract

Hyaluronan is a rapidly turned over component of the vertebrate extracellular matrix. Its levels are determined, in part, by the hyaluronan synthases, HAS1, HAS2, and HAS3, and three hyaluronidases, HYAL1, HYAL2 and HYAL3. Hyaluronan binding proteins also regulate hyaluronan levels although their involvement is less well understood. To date, two genetic disorders of hyaluronan metabolism have been reported in humans: HYAL1 deficiency (Mucopolysaccharidosis IX) in four individuals with joint pathology as the predominant phenotypic finding and HAS2 deficiency in a single person having cardiac pathology. However, inherited disorders and induced mutations affecting hyaluronan metabolism have been characterized in other species. Overproduction of hyaluronan by HAS2 results in skin folding and thickening in shar-pei dogs and the naked mole rat, whereas a complete deficiency of HAS2 causes embryonic lethality in mice due to cardiac defects. Deficiencies of murine HAS1 and HAS3 result in a predisposition to seizures. Like humans, mice with HYAL1 deficiency exhibit joint pathology. Mice lacking HYAL2 have variably penetrant developmental defects, including skeletal and cardiac anomalies. Thus, based on mutant animal models, a partial deficiency of HAS2 or HYAL2 might be compatible with survival in humans, while complete deficiencies of HAS1, HAS3, and HYAL3 may yet be recognized.

Key words: Hyaluronidase; Hyaluronan; Hyaluronidase 1; Mucopolysaccharidosis; Hyaluronidase 2; Hyaluronan synthase 2

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Core tip: This manuscript summarizes the phenotypes that have been associated with alterations in hyaluronan synthesis or degradation. It should serve as a reference

for those who are considering an alteration in hyaluronan metabolism as the cause of a genetic condition.

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INTRODUCTION

Hyaluronan (HA) is a straight chain polymer comprised of repeating disaccharide units of glucuronic acid and N-acetylglucosamine ($\rightarrow 4$)- β -D-GlcA-[$\rightarrow 3$]- β -D-GlcNAc-[$\rightarrow 1 \rightarrow$] $_n$, and has substantial size heterogeneity in different tissues, although in most tissues it is greater than 10^6 daltons^[1]. Although the presence of both uronic acid and an amino sugar define HA as a glycosaminoglycan (previously known as mucopolysaccharides), it differs from the other members of this family of compounds in that it is not sulfated or protein-linked, and rather than being synthesized in the Golgi, it is synthesized at the cell membrane and extruded into the extracellular space^[2]. HA can assemble with tissue-specific binding proteins known as hyaladherins to form large complexes. Within the extracellular matrix, many structural and biological functions have been attributed to HA of different sizes. Examination of the phenotypes associated with naturally-occurring and laboratory-induced alterations in HA metabolism has facilitated knowledge of HA metabolism and function.

DISTRIBUTION AND FUNCTION OF HA

It is estimated that HA accounts for 15 g of the "average" 70 kg human^[3]. Studies of adults have revealed the highest HA concentrations in soft connective tissues such as skin, synovial fluid, Wharton's jelly of the umbilical cord, and vitreous body^[4]. The concentration of HA is also very high during embryogenesis and wound healing^[5]. Low concentrations of HA are present in serum, where there is an average of 20-40 μ g per liter^[6,7], although there are many pathological states that are associated with and may cause increased levels of serum HA^[4,8]. Based on whole body studies in the rat and other species, it is estimated that 50% of the HA is in the skin, 25% in the skeleton and joints, and most of the remaining HA is in brain, kidney, lung and muscles^[4,9].

HA is critical to the assembly and structure of the extracellular matrix at both tissue and cellular levels. The large size of HA provides rigidity to some types of matrix and serves a scaffolding function for several constituents of the matrix. The hydrophilic groups of HA attract water to hydrate tissues, lubricate joints, and fill

space. A HA-enriched, hydrated matrix facilitates cellular movement and proliferation that is critical for cellular migrations during early developmental processes and in the regeneration and remodeling of tissues such as during wound healing. HA has also been shown to play a role in inflammation and ovulation^[10,11]. The functions of HA depend on its size; high molecular mass and low molecular mass HA typically have opposing effects on epithelial to mesenchymal transition, inflammation, and angiogenesis^[12,13].

HA TURNOVER

Among extracellular matrix molecules, HA has one of the fastest rates of turnover^[1,14,15], with an estimated one-third of the 15 g of HA in an average adult human turned over daily^[8]. The half-life of HA was initially estimated to be 2-4 d^[15], but it is now clear that the half-life varies among and within tissues because of differences in the rates of degradation or other forms of turnover. For example, HA has a half-life of about 2-5 min in blood^[16,17], whereas it is estimated to be 2.5 d in the skin^[18], and up to 18 d when bound to aggrecan in cartilage^[19]. Approximately 30% of HA degradation occurs locally, within the tissue in which it is synthesized^[18]. The remaining about 70% enters the lymphatic drainage where 90% is removed by the lymph nodes^[20] and the remainder is taken up from the blood by the liver^[16] and, to a lesser extent, the kidney and spleen^[21]. Sinusoidal endothelial cells are responsible for uptake by the liver^[22-24] via receptors with high affinity for HA and chondroitin sulfate^[25]. Only about 1% of blood HA is eliminated each day in the urine^[26], and this is limited to lower molecular mass HA (< 12000 Da) that can pass through the glomerular filtration barrier.

The internalization of HA is essential for its complete breakdown in cells, but identifying the receptor(s) responsible for uptake in each tissue has been challenging. Three receptors, HA receptor for endocytosis (HARE, also known as stabilin-2), cluster of differentiation antigen 44 (CD44), and lymphatic vessel endothelial receptor-1 (LYVE-1), are able to internalize HA for degradation *in vitro*, but their relevance *in vivo* is less clear. HARE localizes to endothelial cells of organs that internalize circulating HA including liver, lymph nodes and spleen, as well as to endothelial cells of the oviduct, corneal and lens epithelium, mesenchymal cells of the heart valve, ependymal cells of the brain, macrophages, and epithelial cells of the renal papillae^[27]. HA uptake via clathrin-coated pits of liver endothelial cells^[28] is inhibited by a HARE blocking antibody^[29,30], and an anti-cluster of differentiation associated antigen 44 (CD44) blocking antibody inhibits both endocytosis and cleavage of HA^[31]. The LYVE-1 receptor binds and internalizes HA in transfected human embryonic kidney cells^[32]; much remains to be learned about

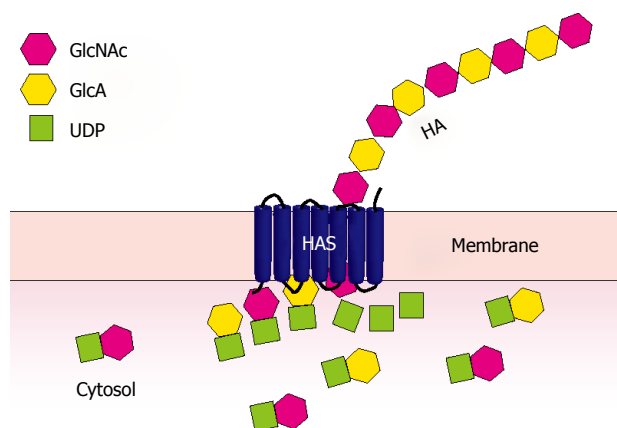


Figure 1 Hyaluronan synthesis. Uridine diphosphate (UDP)-linked sugar substrates, glucuronic acid (GlcA) and N-acetylglucosamine (GlcNAc), are sequentially added to the reducing end of the growing HA chain at the cytoplasmic aspect of the cell membrane through the action of hyaluronan synthase (HAS). The resulting sugar polymer is extruded into the extracellular matrix surrounding the cell.

its regulation^[33]. It seems likely that each of these receptors can internalize HA under specific circumstances, but that HARE appears to be most important in the internalization of circulating HA. Only HARE knock-out mice have elevated circulating HA^[30]; an increased concentration of serum HA was not found in mice lacking both the CD44 and LYVE-1 receptors^[34]. CD44 appears to function primarily in the uptake of HA during pathological processes such as inflammation^[35], although it may also have a role in local degradation of HA such as in cartilage^[36] or developing heart^[37].

Internalization of HA is likely preceded by its depolymerization. This concept was first proposed because the size of HA was found to be reduced after transit through lymph nodes^[38]. In cartilage explants, a decrease in the size of steady-state HA compared to newly synthesized HA, also suggested that extracellular depolymerization of HA was an initial step in HA degradation^[19]. A model whereby a cell surface hyaluronidase, putatively HYAL2 (described below), initiates the cleavage of HA before receptor-mediated internalization for intracellular degradation has been proposed^[39]. Other modes of HA depolymerization, such as by oxygen-derived radicals^[40], have also been suggested. The studies in cartilage explants also suggested that HA is internalized and degraded in conjunction with the binding domain of aggrecan^[19], a finding that has not been explored in the context of current degradation models.

Among the HA binding proteins that have been identified, the recently described KIAA1199 is unique because its presence facilitates the degradation of HA^[41]. Unlike CD44 which is concentrated in caveolin-rich lipid rafts, KIAA1199 appears to engage HA *via* a clathrin-coated pit pathway^[41]. KIAA1199 facilitates the degradation of HA in some tissues, although it does not have homology to the active sites of mammalian hyaluronidases and it is unclear how it directly or

indirectly depolymerizes HA. Variants of KIAA1199 that are associated with reduced *in vitro* HA degradative activity are described that have been found in several persons with non-syndromic hearing deficit^[41,42]. The mouse orthologue of KIAA1199 also has HA degradative activity^[43]. While the mode of action toward HA and the physiological role of KIAA1199 are not yet known, it has been proposed that KIAA1199 has a key role in HA catabolism in dermis and, possibly, brain^[41].

The rapid rate of HA turnover in many tissues requires high rates of both synthesis and degradation. Taken together with the important functional roles of HA, one might expect that defects in either HA synthesis or degradation could cause clinical phenotypes. However, for these same reasons, it has been speculated that defects in HA metabolism may be lethal. To date, only two genetic disorders of HA metabolism have been described in humans and studies in the mouse provide evidence to support the earlier contentions that complete deficiencies in at least some HA metabolizing enzymes may be lethal during embryonic or fetal development.

HA SYNTHESIS

HA synthesis at the inner aspect of the plasma membrane takes place through the sequential transfer of uridine diphosphate (UDP)-GlcA and UDP-GlcNAc to the reducing terminus of the growing polysaccharide chain^[44] (Figure 1). The first mammalian HA synthase to be identified, HAS1, was identified from cDNA libraries by functional expression; HAS2 and HAS3 were identified soon after by homology-based cloning^[2]. Genes encoding these three enzymes, HAS1, HAS2, and HAS3, were mapped to human chromosomes 19q13.3, 8q24.12, and 16q22.1, respectively^[45]. All of these enzymes are capable of HA synthesis, but of different average sizes and at different rates^[46,47]. The three HAS enzymes are expressed in both embryonic and adult tissues; HAS2 is most broadly expressed during development, while HAS3 appears to be most broadly expressed in adult tissues^[48,49]. The *in vivo* functions of the individual enzymes are becoming clearer through the characterization of mouse knockouts of these genes.

DEFICIENCIES IN HA SYNTHESIS

HAS1 is the least active of the synthases, requiring a very high concentration of UDP-GlcNAc^[46,47]. No abnormalities were detected in initial studies of mice with a targeted disruption of *Has1*^[50], although subsequent studies that focused on the brain revealed a mild increase in seizure activity^[51]. Accelerated wound closure and decreased HA synthesis was recently demonstrated in *Has1*^{-/-} *Has3*^{-/-} mice, but the involvement of HAS1 appears minimal in comparison to HAS3^[52]. Altered splicing of *HAS1* mRNA has

been reported in cases of malignant myeloma and Waldenström's macroglobulinemia^[53,54], and inherited and acquired *HAS1* mutations are found in increased frequency in these conditions^[55,56]. Despite this strong association, the relationship between this finding and the function of *HAS1* remains to be determined.

HAS2 is recognized as the synthase responsible for the majority of HA synthesis during development^[50]. *Has2*^{-/-} mouse embryos die at embryonic day (E) 9.5 due to a failure to form the HA-rich cardiac cushion and initiate the epithelial to mesenchymal transition (EMT) required to form the valves and septa^[57,58]. Exogenous HA could rescue the EMT defect in *ex vivo* explants from the developing cardiac cushion of *Has2*^{-/-} embryos^[58], providing evidence that HA regulates signal transduction pathways during development. The severity of the cardiac defect in *Has2*^{-/-} embryos required that conditional deletions of *Has2* be used to study its role in other aspects of development. A deletion of *Has2* limited to the limb bud mesoderm of mice demonstrated that HA synthesis is also essential for growth and patterning of the limb, maturation of developing chondrocytes, and joint formation^[59]. A nestin-cre driven deletion of *Has2* did not severely impact the developing brain, although the mice had increased seizure activity^[51]. The conditional deletion of *Has2* in other tissues will be necessary to determine its full role in development; given the high HA levels in many developing tissues^[60], other critical roles for *HAS2* in development will likely be forthcoming.

Alterations in HA synthesis might be expected to affect heart development in humans and this led investigators to screen the DNA from 100 children with a ventricular septal defect for *HAS2* mutations^[61]. In a single patient, a c.A1496T (p.Glu499Val) mutation was identified that reduced the activity of transiently expressed *HAS2* by 40%. This suggests that partial *HAS2* deficiency could be the cause of congenital heart defects in other patients. These results have not yet been replicated in independent cohorts.

The *HAS3* enzyme is the most active of the HA synthases and produces HA of lower molecular mass^[46]. Initial studies of *Has3*^{-/-} mice showed no obvious phenotype although they were more resistant to ventilator-induced lung injury^[62]. However, in recent studies focused on the brain, *Has3*^{-/-} mice were found to have reduced HA, crowding of neurons and reduced extracellular space in specific regions of the brain^[51]. These mice exhibited a significant increase in abnormal electrical activity and a substantial predisposition to seizure activity. The reduced extracellular space in the affected brain regions reduced the diffusion of molecules and provided evidence for a physiological role of HA in the regulation of brain extracellular space. Studies of the role of *HAS3* in human epilepsy have not yet been reported and there are only limited data regarding other clinical phenotypes^[51].

INCREASED HA SYNTHESIS

The first disorder of increased HA synthesis to be described in humans concerned an individual with thickened skin and excessive skin folding^[63]. The skin folding was particularly prominent in the face and limbs; skin of the trunk was thickened but not folded. That individual did not have other significant medical issues and the skin folds decreased with age. Histological studies demonstrated excessive extracellular HA in the skin. The concentration of serum HA was markedly increased, up to 1000 fold greater than normal. Although an underlying genetic cause was not reported, the authors speculated that a similar disorder affects the shar-pei dog.

The shar-pei dog was selectively bred to increase the presence and prominence of skin folds, resulting in the meat-mouth shar-pei, which has an extreme presentation of the trait and a condition termed hereditary cutaneous mucinosis. Demonstration that the material accumulating in the skin and serum is HA^[64] and that these dogs have elevated levels of *HAS2* mRNA^[65], led the condition to be renamed as hereditary cutaneous hyaluronosis^[66]. Mapping studies linked the severe HA accumulation to duplications upstream of *HAS2* and to a familial fever syndrome that is found in shar-pei dogs^[67]. However, the linkage between this duplication and the familial fever syndrome has recently been refuted^[68].

Another naturally occurring animal model with increased levels of HA in the skin is the naked mole rat, a rodent recognized for its long life span (about 30 years) and resistance to cancer. Investigators seeking to identify the basis of the resistance to cancer discovered that fibroblasts derived from the naked mole rat synthesize very large quantities of high molecular mass HA^[69]. The naked mole rat was found to express a reduced level of the HA-degrading enzyme, *HYAL2*, as well as a unique form of *HAS2*. *HAS2* from the naked mole rat contains substitutions of Ser for Asn at two different and highly conserved regions of the enzyme. Together, these changes in HA metabolism result in increased HA of higher molecular mass. To demonstrate the relationship between the excessive high molecular mass HA and resistance to cancer, the authors used transient expression approaches to alter the levels of *HAS2* or *HYAL2*. Either a reduction in *HAS2* by siRNA, or an increase in *HYAL2* by transient overexpression, resulted in a decrease in the extracellular HA and susceptibility to tumorigenesis^[69].

Thus, in contrast to the severe developmental abnormalities identified in mice with *HAS2* deficiency, an overexpression of *HAS2* results in a much milder phenotype in rodents and dogs. It is even possible that the excessive synthesis of HA has some protective roles although the studies in the naked mole rat

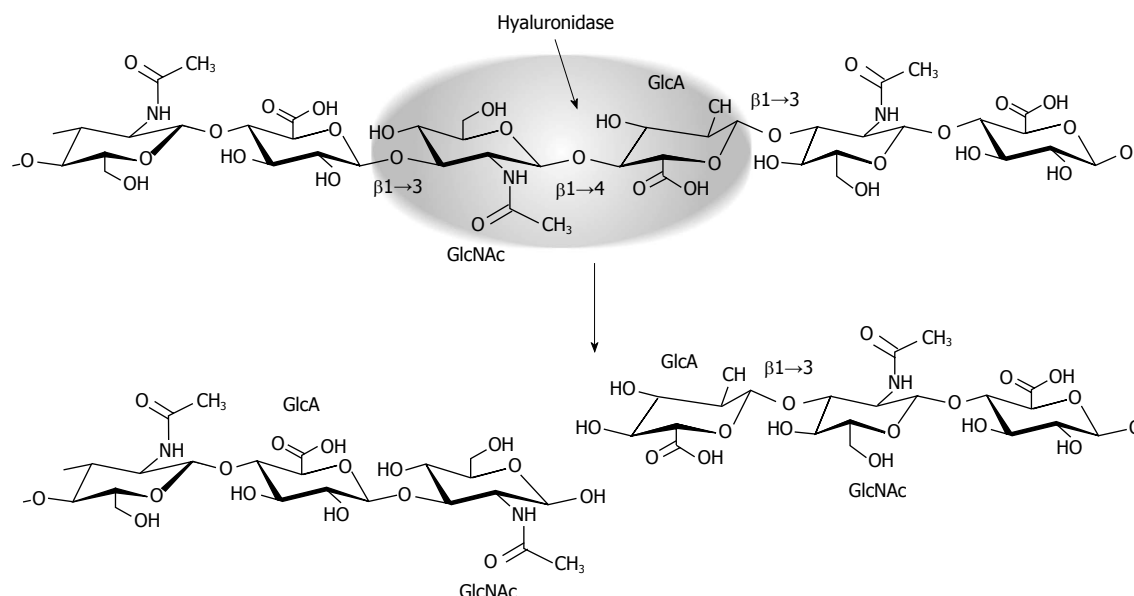


Figure 2 Hyaluronidase function. Mammalian hyaluronidases are endoglycosidases that hydrolyze the $\beta 1 \rightarrow 4$ linkage between the GlcNAc and GlcA disaccharide units that make up HA. This results in non-reducing and reducing termini; the non-reducing sugar becomes a substrate for the exoglycosidase β -glucuronidase.

indicated that other factors work in conjunction with HA to mediate the cancer resistance phenotype^[69]. In humans, a better understanding of the full range of phenotypes that might be associated with alterations in HAS2 expression awaits the identification of additional patients.

DEGRADATION OF HA

Within the cell, the complete degradation of HA to monosaccharides requires the concerted action of an endoglycosidase, hyaluronidase, and the exoglycosidases β -N-acetyl-D-hexosaminidase and β -D-glucuronidase^[70]. As an endoglycosidase, hyaluronidase cleaves the hyaluronan polymer internally at $\beta 1 \rightarrow 4$ linkages between β -N-acetylglucosamine and β -D-glucuronic acid (Figure 2). The resulting oligosaccharides become substrates for the exoglycosidase β -D-glucuronidase which hydrolyzes the terminal non-reducing glucuronic acid, leaving a non-reducing β -N-acetylglucosamine that can then be hydrolyzed by β -N-acetyl-D-hexosaminidase^[71].

Two forms of hyaluronidase were originally characterized in human tissues, one with a neutral pH optimum that was rich in testes, and a second with an acid pH optimum that was found in most solid tissues and serum^[72]. A glycosylphosphatidylinositol (GPI)-linked sperm protein important for fertilization, PH-20, was later found to have sequence similarity to bee venom hyaluronidase^[73], and was soon recognized as the neutrally active hyaluronidase^[74]; its gene, *SPAM1*, was mapped to human 7q31 (and mouse chromosome 6A2)^[75]. The acid-active hyaluronidase in human plasma, *HYAL1*, was isolated^[76], and mapped to human chromosome 3p21.3 (mouse chromosome

9F1-F2), a region previously characterized as having a tumor suppressor locus^[77]. The human genome sequence allowed additional putative hyaluronidases to be identified, including *HYAL2* which encodes an enzyme with weak activity toward high molecular mass HA^[78] and mapped in close proximity to *HYAL1*^[79]. Bioinformatic approaches revealed two tandemly repeated groups of three genes, which mapped to human chromosome 3p21.3 (centromere-*HYAL2-HYAL1-HYAL3*-telomere)^[80] and 7q31.3 (centromere-*SPAM1-HYAL4-HYALP1*-telomere)^[81,82]. An additional gene, *Hyal5*, is found downstream of *Spam1* in mice^[83]. These genes were identified as putative hyaluronidase homologues based on sequence conservation but further studies demonstrated that *HYALP1* is an expressed pseudogene in humans^[81], *HYAL4* encodes a chondroitinase^[84,85], and *HYAL3* has no detectable *in vitro* activity^[86,87]. Additional expression studies using human and mouse tissues showed that *HYAL1/Hyal1*, *HYAL2/Hyal2*, and *HYAL3/Hyal3* are broadly expressed in somatic tissues^[81,88], while the expression of *SPAM1* is limited primarily to the testes^[75] and *HYAL4* to skeletal muscle and placenta^[81]. The recently described HA binding/degradative protein, KIAA1199, is expressed in many tissues including brain, lung, pancreas, testis and ovary but is not expressed in liver, kidney or spleen^[89].

HYALURONIDASE DEFICIENCIES

The first individual to be identified with a hyaluronidase deficiency was a young girl who had mild short stature, mild facial dysmorphism including a flattened nasal bridge, a bifid uvula and a submucosal cleft palate, and a presenting complaint of multiple periarticular

soft tissue masses involving both large and small joints; there was no evidence of visceral or neurological involvement. Histological and ultrastructural analyses of biopsies showed the periarticular masses to have a synovium that was massively expanded with macrophages containing numerous lysosomes containing fibrillar storage material; the skin showed similar pathology with fibroblasts containing increased numbers of lysosomes with excess macromolecular substrate. This work suggested some type of lysosomal storage disorder. Early biochemical investigations disclosed markedly elevated levels of serum HA and a deficiency of acid pH optimum hyaluronidase (and chondroitinase) and the condition, a new lysosomal storage disorder, was termed mucopolysaccharidosis IX^[90]. This patient was subsequently found to have a 1361del37ins14 mutation in *HYAL1* that was predicted to result in a premature stop codon on one allele, and a c.G1412A (p.Glu268Lys) on the other allele; no *HYAL1* activity was detectable^[91]. Three additional patients with *HYAL1* deficiency have since been identified in a consanguineous family of Saudi Arabian origin^[92]. The proband in this family was initially characterized with idiopathic juvenile arthritis that did not respond to treatment with non-steroidal anti-inflammatory drugs. Two siblings of the proband were subsequently found to have joint abnormalities including joint effusions, synovial proliferation, and cysts. No serum *HYAL1* activity was detected in any of the patients and all of them were homozygous for a c.104delT (p.Val35AlafsX25) mutation. The phenotypes of all 4 individuals with *HYAL1* deficiency are less severe than that predicted for human hyaluronidase deficiency based on the broad distribution of HA in the human body and the significance of HA in many developmental and remodeling processes. Taken together, this information suggests that other enzymes (partially) compensate for the deficiency of *HYAL1*.

Consistent with the phenotype of persons with mucopolysaccharidosis IX, a *Hyal1*^{-/-} mouse model did not show a generalized accumulation of HA^[93]. The only detectable phenotype was a progressive loss of articular cartilage proteoglycan in the knee joint, starting as early as 3 mo of age. The mild phenotype in the mouse model is likely due, at least in part, to compensation by certain exoglycosidase activities, as mice deficient in both *HYAL1* and β -hexosaminidases A and B (*Hyal1*^{-/-}*Hexa*^{-/-}*Hexb*^{-/-}) showed global HA accumulation that was significantly higher than that in either *Hyal1*^{-/-} or *Hexa*^{-/-}*Hexb*^{-/-} mice^[94]. It seems likely that except in the joints the levels of β -hexosaminidase and β -glucuronidase are adequate to compensate for *HYAL1* deficiency in mice, and by extrapolation, in humans as well. Interestingly, female *Hyal1*^{-/-} mice had prolonged fertility; the basis for this is currently unclear^[95].

HYAL2 is a broadly expressed glycosylphosphatidylinositol-linked cell surface protein whose localization

and activity have been controversial. Its activity is weak, but has been successfully demonstrated toward high molecular mass HA which it appears to cleave to 20 kDa fragments^[86,96]. *HYAL2* interacts with CD44 and NHE1 proteins^[97,98], which has led to the model shown in Figure 3, proposing that *HYAL2* initiates the breakdown of extracellular HA, in partnership with the Na/H exchanger isoform 1 (NHE1) which acidifies the local environment^[39]. That model further proposes that fragments generated by *HYAL2* are internalized by receptor-mediated endocytosis. The generation of a *Hyal2*^{-/-} mouse which exhibited mild anemia, craniofacial abnormalities, and evidence of pre-weaning lethality, did not completely support this model, as generalized storage of HA was not detected^[99]. However, further studies demonstrated substantial (2/3) pre-weaning lethality in *Hyal2*^{-/-} mice and severe cardiopulmonary dysfunction leading to death in 54% of *Hyal2*^{-/-} mice by an average of 3.2 mo of age^[100]. Accumulating extracellular HA in the heart, lung, and serum of these mice was of higher than average molecular mass, consistent with the model that *HYAL2* may be important in initiating the depolymerization of HA in the extracellular matrix. The pre-weaning lethality in the mice suggests that a complete deficiency of *HYAL2* may cause a severe, if not lethal, condition in humans, although the genetic background may provide important modifying effects that might either mitigate or exacerbate any of the above mentioned monogenic biosynthetic or catabolic enzyme disorders.

HYAL3 is broadly but weakly expressed in somatic cells. Although it is homologous to the other hyaluronidases, no activity has been clearly associated with the enzyme^[86,87]. *Hyal3*^{-/-} mice did not accumulate HA, and the only detectable phenotype was a minor change in the histopathology of the lungs^[101]. However, the overexpression of *HYAL3* in cultured baby hamster kidney cells resulted in increased *HYAL1* activity, suggesting a role for *HYAL3* in HA metabolism^[87].

PH-20 (SPAM1) plays a role in both penetration of the cumulus barrier and binding of the zona pellucida during fertilization^[102,103]. When it was initially mapped to human chromosome 7q31 and shown to be specifically expressed in the testes, it was thought that mutations in the gene might be identified as a cause of infertility^[75], but no reports of mutations have been made. Studies in *Spam1*^{-/-} mice have not provided insights regarding phenotype(s) that might be associated with human SPAM1 mutations because *HYAL5* in mice can compensate for SPAM1 deficiency^[104-106].

CONCLUSION

It is clear that the range of conditions that result from abnormalities in HA metabolizing enzymes is only partially understood. Monogenic dysfunction of enzymes involved in HA synthesis and HA catabolism can have marked phenotypic effects that can involve many

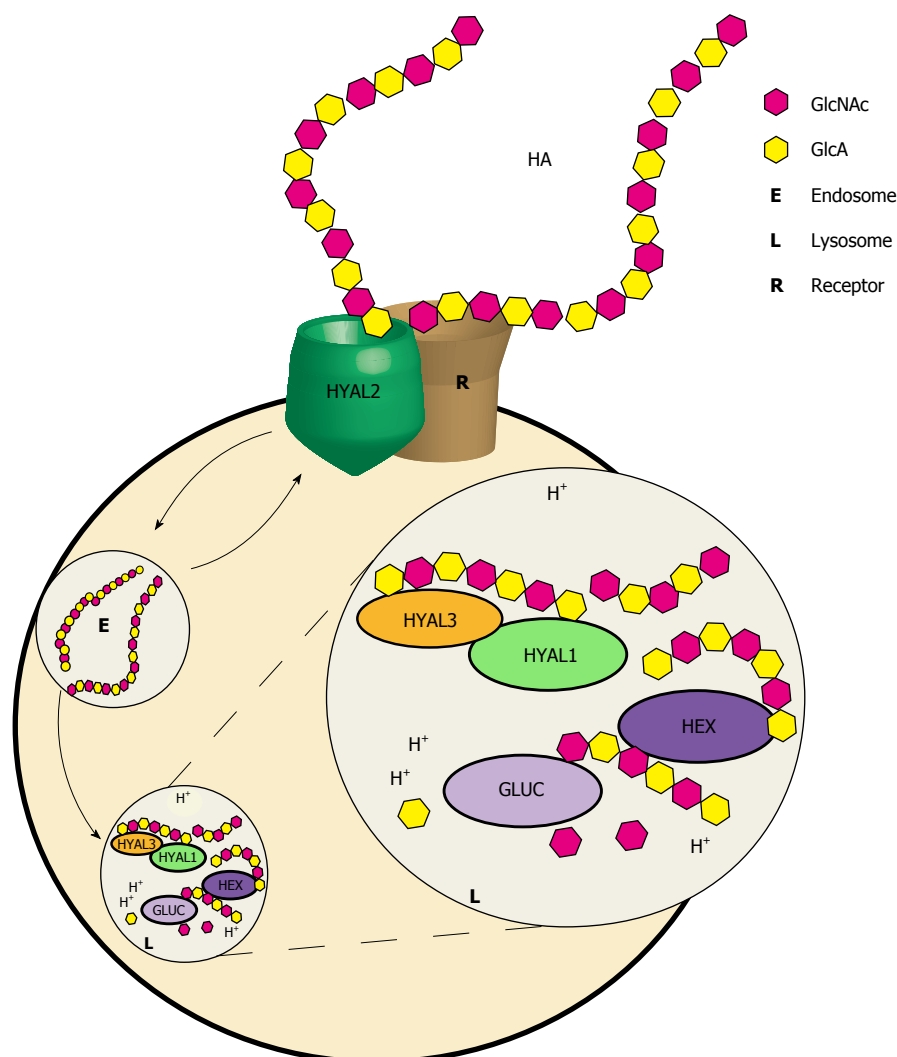


Figure 3 Proposed model for hyaluronan breakdown. Hyaluronan (HA) bound by a cell- or matrix-associated receptor such as CD44, HARE, or LYVE-1 is proposed to be hydrolyzed to intermediate-sized fragments by the GPI-linked HYAL2. The resulting fragments are then internalized by receptor-mediated endocytosis and transported to lysosomes. Once inside the lysosome, further degradation takes place through the action of the acid-active HYAL1. HYAL1 cleaves the intermediate-sized HA fragments to smaller fragments such that they become substrates for the sequential action of the exoglycosidases, β -glucuronidase (Gluc) and β -N-acetylhexosaminidase (Hex) which hydrolyze terminal GlcA and GlcNAc, respectively. The role of HYAL3 is unclear although its overexpression increases HYAL1 activity in cell culture based studies.

tissues and organ systems. It is likely that the ongoing broad application of next generation sequencing to identify the causes of unexplained phenotypes in humans will identify novel human conditions that result from variations in the HYAL and HAS enzymes and broaden the spectrum of already described conditions, furthering our understanding of their *in vivo* functions.

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Molecular basis of cleft palates in mice

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Abstract

Cleft palate, including complete or incomplete cleft palates, soft palate clefts, and submucosal cleft palates, is the most frequent congenital craniofacial anomaly in humans. Multifactorial conditions, including genetic and environmental factors, induce the formation of cleft palates. The process of palatogenesis is temporospatially regulated by transcription factors, growth factors, extracellular matrix proteins, and membranous molecules; a single ablation of these molecules can result in a cleft palate *in vivo*. Studies on knockout mice were reviewed in order to identify genetic errors that lead to cleft palates. In this review, we systematically describe these mutant mice and discuss the molecular mechanisms of palatogenesis.

Key words: Tbx1; Submucosal cleft palate; Incomplete cleft palate; Palatal shelf; Palatogenesis; Knockout mice

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Core tip: Cleft lip and/or palate is one of the most frequent congenital craniofacial anomalies observed. Multifactorial conditions, including genetic and environmental factors, induce the formation of cleft palates. We screened knockout mice with cleft palate phenotypes and observed approximately 180 mice with the anomaly. In order to understand the molecular regulatory mechanisms of palatogenesis and to identify genetic errors that lead to cleft palates, we aimed to review studies performed using knockout mice with cleft palates.

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INTRODUCTION

Cleft lip and/or palate (CL/P) is the most frequent congenital craniofacial anomaly observed in humans, with an incidence of 1 per 700 births worldwide^[1]. Furthermore, 55% of the patients with CL/P are reported to have a multiple malformation syndrome^[2]. CL/P involves a multifactorial etiology, both genetic and environmental. Teratogens that cause CL/P in humans include common environmental exposures, such as alcohol, smoking, infections, dioxin, estrogen, retinoic acid, and altitude (reviewed by Murray^[1]). The offspring of parents with CL/P present a higher incidence of CL/P than those without a family history^[1]. Gene-environment interactions for non-syndromic CL/P have also been reported^[1]. Cleft palate (CP) cases include complete CP, incomplete CP, and soft palate clefts. The mildest form of cleft palates is the soft palate cleft or bifid uvula because the initial palatal fusion occurs in the anterior region of secondary palatal shelves. Incomplete CP and soft palate clefts can manifest together with submucosal CP. This review focuses on studies performed using knockout mice with CP, aiming to clarify the molecular regulatory mechanisms of palatogenesis and to identify genetic errors underlying mammalian cleft palates.

MAMMALIAN PALATOGENESIS

The palate is formed with the primary and secondary palate. The primary palate is derived from the frontonasal prominence and becomes a small anterior part of the adult hard palate. The secondary palatal shelves extend bilaterally from the internal aspects of the maxillary prominences and will become the adult hard and soft palates. The process of palatogenesis consists of several stages: palatal shelf formation, elevation, and midline fusion of the palatal shelves (Figure 1). The secondary palatal shelves develop between embryonic day (E) 11.5 and 12.5 in the mouse embryo (Figure 1A). At E13.5, the palatal shelves grow downward on each side of the tongue (Figure 1B). As the jaws develop, the tongue descends and the palatal shelves elevate to a horizontal position above the dorsum of the tongue (E14). Continuing their growth, the bilateral palatal shelves meet at the midline and fuse between E14.5 and E15.5 (Figure 1C).

The palatal shelves are composed of the neural crest-derived mesenchyme and ectoderm-derived epithelia, which cover the palatal mesenchyme (Figure 1D). Both elevation and fusion of the secondary palatal shelves occur in the midline from anterior to posterior. The secondary palatal shelves also fuse with the primary palate, separating the oral and nasal cavities. The anterior two-thirds of the palate forms the hard palate with neural crest-derived palatal bones (Figure 2A). The posterior one-third of the palate forms the bone-free soft palate and is involved in the

palatopharyngeal sealing. Disruption at any stage of the formation, elevation, growth, or fusion of the secondary palatal shelves results in CP^[3].

MOUSE MODELS FOR STUDYING THE MOLECULAR MECHANISMS OF PALATAL DEVELOPMENT

Major advances have been achieved regarding the molecular mechanisms that regulate palatal development using genetically engineered mice. Deletions in many genes of mice result in CP and the most frequent phenotype seen is complete CP (Figure 2B). Uniquely, *Tbx1*^{-/-} mice present various phenotypes of CP^[4], including complete CP (Figure 2B), incomplete CP (Figure 2C), and anterior CP (Figure 2D). Bone staining showed that some mice potentially had a submucosal CP (Figure 2G). These observations are in agreement with various CP phenotypes in humans.

In order to elucidate the molecular pathogenesis of CL/P, we conducted a literature search on PubMed (<http://www.ncbi.nlm.nih.gov/pubmed>) and the Mouse Genome Informatics (MGI) from the Jackson Laboratory (<http://www.informatics.jax.org>). The search was limited to knockout mice with CP and excluded the teratogen-induced CP (Table 1). We also investigated diseases/syndromes using the Online Mendelian Inheritance in Man (OMIM) (<http://omim.org>). Not all the molecules involved in cleft palates in mice are correlated to CL/P in human (Table 1). When genes in Table 1 were analyzed by biological function using BioCarta (<http://www.biocarta.com>) and the Kyoto Encyclopedia of Genes and Genomes (KEGG) Pathway Database (<http://www.genome.jp/kegg/pathway.html>), the transforming growth factor (TGF), hedgehog, Wnt, fibroblast growth factor (FGF), and mitogen-activated protein kinase (MAPK) signaling pathways were found to be critical in palatogenesis (Table 2). When genes were analyzed by molecular function using the PANTHER (Protein ANALysis THrough Evolutionary Relationships) database (<http://pantherdb.org>)^[5], the most significantly enriched molecular function was the "transcription factor", especially the "homeobox transcription factors" (Table 2 and Figure 3).

To analyze mutant mice with cleft palates, the defects in palatal shelf development were divided into the following six categories (Table 3), which were modified from a previously published classification^[3]. The first category is the failure of the palatal shelf formation. The gene mutations affect the initial development of the palatal shelf. The second one is the abnormal fusion of the palatal shelves and the mandible or tongue. Oral fusions between the palatal shelves and the tongue or mandible are rare. In *Tbx1* (T-box 1) knockout mice, the posterior part of the palatal shelves fuse to the mandible, inhibiting the

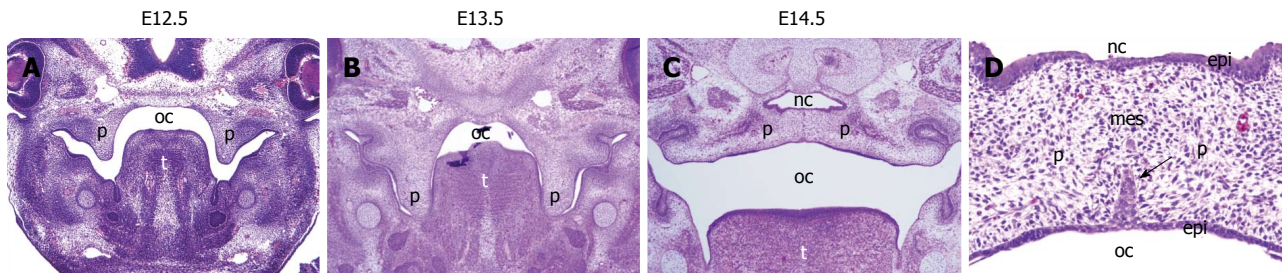


Figure 1 Palatogenesis in mice. Hematoxylin and eosin staining of coronal sections of the head of a wild-type mouse at embryonic day (E) 12.5 (A), E13.5 (B), and E14.5 (C, D). A: Mouse palatal shelves (p) develop from the maxillary prominences; B: By E13.5, the palatal shelves grow downward on each side of the tongue (t); C and D: At E14.5, the palatal shelves face each other along the midline above the tongue and fuse, separating the oral cavity (oc) from the nasal cavity (nc). The arrow in (D) indicates the medial edge epithelial (MEE) cells that constitute the midline epithelial seam. All animal experimental procedures were reviewed and approved by the Institutional Animal Care and Use Committees of the University of Texas Southwestern Medical Center and Tokyo Medical and Dental University. mes: Mesenchyme; epi: Epithelium.

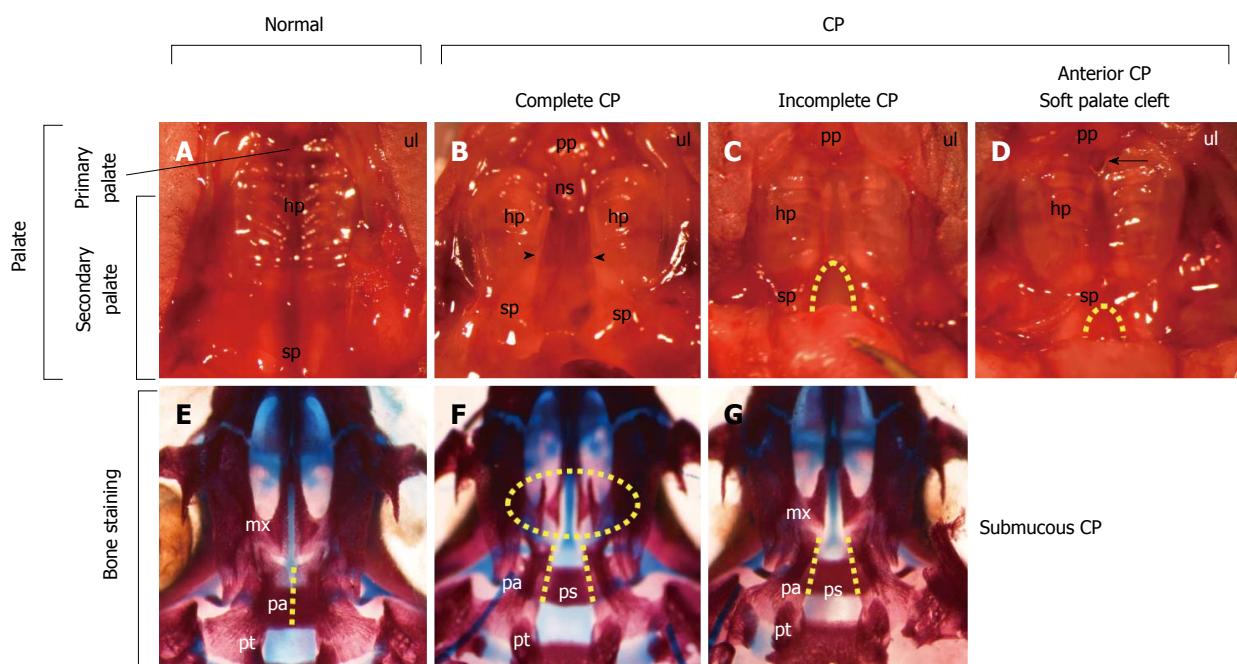


Figure 2 View of the palate from wild-type and *Tbx1*^{-/-} mice with cleft palates. A-D: Ventral view of the maxilla of newborn wild-type (A) and *Tbx1*^{-/-} mice with cleft palates (B-D). The palate consists of the primary palate (pp) and the secondary palate (sp), which consists of a hard palate (hp) and a soft palate (sp) (A). *Tbx1*^{-/-} mice show complete cleft palate (CP) (arrowheads in B), incomplete CP (dashed line in C), and soft palate clefts associated with anterior CP (dashed line in D). An anterior CP (an arrow in D) is present at the junction between the primary palate and secondary palate, while the posterior palate remains fused; E-G: Ventral view of the cranial base of newborn wild-type (E) and *Tbx1*^{-/-} mice (F, G) stained with alizarin red for mineralized bone and alcian blue for cartilage. Fusion of the bilateral palatal bones (pa) observed in the wild-type (dashed line in E) is absent in *Tbx1*^{-/-} mice (dashed lines in F, G). The palatal shelves in the maxilla (mx) of *Tbx1*^{-/-} mice with complete CP (oval dashed line in F) failed to grow toward the midline. Note the visible presphenoid bone (ps) associated with CP (F, G). Modified and used with permission from Funato *et al.*⁴¹. ns: Nasal septum; pt: Pterygoid bone.

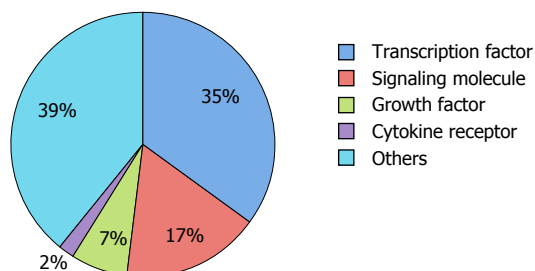


Figure 3 Gene ontology analysis of genes associated with cleft palate in mice. Gene ontology analysis of genes associated with cleft palate in mice was performed using the PANTHER classification system (<http://pantherdb.org>). The most significantly enriched molecular function was the "transcription factor" ($P = 1.2 \times 10^{-12}$). A P value less than 0.05 was considered statistically significant.

elevation of the palatal shelves^[4]. The third category is the failed or delayed palatal shelf elevation. Ablation of *Pax9* (paired box gene 9), *Pitx1* (paired-like homeodomain 1), *Gli2* (GLI family zinc finger 2), or *Osr2* (Odd-skipped related transcription factor 2) in the palatal mesenchyme results in the failed palatal shelf elevation^[6-9], suggesting crucial roles for these transcription factors in controlling the mesenchymal cells during palatal shelf elevation. The fourth one is the failure of the palatal shelf development after elevation. The loss of *Msx1* (msh homeobox 1) and *Lhx8* (LIM homeobox gene 8) and the conditional ablation of *Tgfbr2* (transforming growth factor,

Table 1 Molecules involved in cleft palate in mice

Knockout mice with cleft palates			Humans		
Gene/category	Protein	Ref.	OMIM	Syndrome	CL/P
Growth factors, antagonist, and receptors					
<i>Acrv1/Alk2</i> (<i>Wnt1-Cre</i> -mediated ablation)	Activin A receptor, type I	[33]	¹ 102576	Fibrodysplasia ossificans progressiva	nr
<i>Acrv2a</i>	Activin A receptor, type II A	[34]	¹ 102581	nr	nr
<i>Bmp4</i>	Bone morphogenetic protein 4	[35]	¹ 112262	Microphthalmia, syndromic 6 Orofacial cleft 11	r
<i>Bmp7</i>	Bone morphogenetic protein 7	[36]	¹ 112267	nr	nr
<i>Bmpr1a/Alk3</i> (<i>Nestin-Cre</i> -mediated ablation)	Bone morphogenetic protein receptor, type I A	[35]	¹ 601299	Juvenile polyposis syndrome, Polyposis syndrome	nr
<i>Chrd</i>	Chordin	[37]	¹ 603475	nr	nr
<i>Ctgf</i>	Connective tissue growth factor	[38]	¹ 121009	nr	nr
<i>Edn1</i>	Endothelin 1	[39]	² 131240	Auriculocondylar syndrome 3	r
<i>Egfr</i>	Epidermal growth factor receptor	[17]	¹ 131550	nr	nr
<i>Fgf9</i>	Fibroblast growth factor 9	[40]	¹ 600921	uc	nr
<i>Fgf10</i>	Fibroblast growth factor 10	[13,41]	¹ 602115	Aplasia of lacrimal and salivary glands LADD syndrome	nr
<i>Fgf18</i>	Fibroblast growth factor 18	[42,43]	¹ 603726	nr	nr
<i>Fgfr1</i>	Fibroblast growth factor receptor 1	[44]	¹ 136350	Nonsyndromic cleft lip/palate Hartsfield syndrome Hypogonadotropic hypogonadism 2 Pfeiffer syndrome Apert syndrome Crouzon syndrome Pfeiffer syndrome Saethre-Chotzen syndrome	r
<i>Fgfr2</i> (knockout) (<i>Krt14-Cre</i> -mediated ablation)	Fibroblast growth factor receptor 2	[13,45]	¹ 176943		r
<i>Fst</i>	Follistatin	[46]	¹ 136470	nr	nr
<i>Gabrb3</i>	Gamma-aminobutyric acid A receptor, beta 3	[47]	¹ 137192	Epilepsy, childhood absence, susceptibility to, 5	r
<i>Gdf11/Bmp11</i>	Growth differentiation factor 11	[48]	¹ 603936	nr	nr
<i>Gpr124</i>	G protein-coupled receptor 124	[49]	¹ 606823	nr	nr
<i>Inhba</i>	Inhibin, beta A/activin A	[50]	¹ 147290	nr	nr
<i>Pdgfc</i>	Platelet-derived growth factor C	[51]	¹ 608452	nr	r [52]
<i>Pdgfra</i> (knockout) (<i>Wnt1-Cre</i> -mediated ablation)	Platelet-derived growth factor receptor, alpha polypeptide	[53,54]	¹ 173490	Gastrointestinal stromal tumor, somatic Hypereosinophilic syndrome, idiopathic, resistant to imatinib	r
<i>Tgfb2</i>	Transforming growth factor, beta 2	[55]	¹ 190220	Loeys-Dietz syndrome, type 4	r
<i>Tgfb3</i>	Transforming growth factor, beta 3	[15,16,18]	¹ 190230	Arrhythmogenic right ventricular dysplasia 1	r
<i>Tgfb1/Alk5</i> (<i>Wnt1-Cre</i> -, and <i>Nestin-Cre</i> -mediated ablation)	Transforming growth factor, beta receptor I	[56,57]	¹ 190181	Loeys-Dietz syndrome, type 1	r
<i>Tgfb2</i> (<i>Wnt1-Cre</i> -, and <i>KRT14-Cre</i> -mediated ablation)	Transforming growth factor, beta receptor II	[12,58]	¹ 190182	Loeys-Dietz syndrome, type 2	r
<i>Vegfa</i>	Vascular endothelial growth factor A	[59]	² 192240	nr	nr
Membrane proteins					
<i>Ceacam1</i>	Carcinoembryonic antigen-related cell adhesion molecule 1	[60]	¹ 109770	nr	nr
<i>Efna5</i>	Ephrin A5	[61]	¹ 601535	nr	nr
<i>Efnb1</i>	Ephrin B1	[62]	¹ 300035	Craniofrontonasal dysplasia	r
<i>Efnb2</i>	Ephrin B2	[63]	¹ 600527	nr	nr
<i>Fzd2</i>	Frizzled class receptor 2	[64]	¹ 600667	nr	nr
<i>Itga5</i> (knockout) (<i>Mesp1-Cre</i> -mediated ablation)	Integrin alpha 5	[65,66]	¹ 135620	nr	nr
<i>Itgb1</i> (<i>Col2a1-Cre</i> -mediated ablation)	Integrin beta 1	[67]	¹ 135630	nr	nr
<i>Itgb8</i>	Integrin beta 8	[68]	¹ 604160	nr	nr
<i>Jag1</i> (<i>Wnt1-Cre</i> -mediated ablation)	Jagged1	[69]	² 601920	Alagille syndrome	nr
<i>Jag2</i>	Jagged2	[70]	¹ 602570	nr	nr
<i>Kcnj2</i>	Potassium inwardly-rectifying channel, subfamily J, member 2	[71]	¹ 600681	Andersen syndrome Atrial fibrillation, familial, 9 Short QT syndrome 3	r

<i>Lrp6</i>	Low density lipoprotein receptor-related protein 6	[72]	¹ 603507	nr	nr
<i>Ror2</i>	Receptor tyrosine kinase-like orphan receptor 2	[73]	¹ 602337	Robinow syndrome, autosomal recessive Brachydactyly, type B1	r
<i>Ryk</i>	Receptor-like tyrosine kinase	[74]	¹ 600524	nr	nr
<i>Ryr1</i>	Ryanodine receptor 1, skeletal muscle	[75]	¹ 180901	Central core disease King-Denborough syndrome Minicore myopathy with external ophthalmoplegia	nr
<i>Sc5d/Sc5dl</i>	Sterol-C5-desaturase (fungal ERG3, delta-5-desaturase) homolog (<i>S. cerevisiae</i>)	[76]	¹ 602286	Lathosterolosis	nr
<i>Shh</i> (<i>KRT14-Cre-</i> , and <i>Sox2-Cre-</i> mediated ablation)	Sonic hedgehog	[13,77]	¹ 600725	Holoprosencephaly-3 Microphthalmia with coloboma 5 Single median maxillary central incisor	r
<i>Smo/Smoh</i> (<i>Wnt1-Cre-</i> mediated ablation)	Smoothed, frizzled class receptor	[78]	¹ 601500	Basal cell carcinoma, somatic	nr
<i>Tctn2</i>	Tectonic family member 2	[79]	¹ 613846	Meckel syndrome 8	r
<i>Wls/Gpr177</i> (<i>Wnt1-Cre-</i> mediated ablation)	Wntless homolog (<i>Drosophila</i>)	[80]	¹ 611514	nr	nr
<i>Wnt5a</i>	Wingless-type MMTV integration site family, member 5A	[81]	¹ 164975	Robinow syndrome, autosomal dominant	r
<i>Wnt9b</i> (knockout) (<i>Foxg1-Cre-</i> mediated ablation)	Wingless-type MMTV integration site family, member 9B	[82,83]	¹ 602864	nr	nr
Transcription and nucleolar factors					
<i>Alx1</i>	Aristaless-like homeobox 1	[84]	¹ 601527	Frontonasal dysplasia 3	r
<i>Alx3</i>	Aristaless-like homeobox 3	[85]	¹ 606014	Frontonasal dysplasia 1	r
<i>Alx4</i>	Aristaless-like homeobox 4	[85]	¹ 605420	Frontonasal dysplasia 2 Parietal foramina 2 Craniosynostosis 5	Cleft alae nasi
<i>Anp32b</i>	Acidic (leucine-rich) nuclear phosphoprotein 32 family, member B	[86]	nr	nr	nr
<i>Arid5</i>	AT-rich interaction domain-containing protein 5A	[87]	¹ 611583	nr	nr
<i>Asxl1</i>	Additional sex combs like 1	[88]	¹ 612990	Bohring-Opitz syndrome Myelodysplastic syndrome, somatic	r
<i>Barx1</i>	BarH-like homeobox 1	[89]	¹ 603260	nr	nr
<i>Cdc42</i> (<i>Prrx1-Cre-</i> mediated ablation)	Cell division cycle 42	[90]	¹ 116952	nr	nr
<i>Chd7</i> (heterozygotes) (<i>Wnt1-Cre-</i> mediated ablation)	Chromodomain helicase DNA binding protein 7	[91,92]	¹ 608892	CHARGE syndrome Hypogonadotropic hypogonadism 5 with or without anosmia	r
<i>Cited2</i>	CBP/p300-interacting transactivator, with Glu/Asp-rich C-terminal domain, 2	[93]	¹ 602937	Atrial septal defect 8 Ventricular septal defect 2	nr
<i>Crebbp/Cbp</i>	CREB binding protein	[94]	¹ 600140	Rubinstein-Taybi syndrome	nr
<i>Dlx1</i>	Distal-less homeobox 1	[95]	¹ 600029	nr	nr
<i>Dlx2</i>	Distal-less homeobox 2	[95]	¹ 126255	nr	nr
<i>Dlx5</i>	Distal-less homeobox 5	[96,97]	¹ 600028	Split-hand/foot malformation 1 with sensorineural hearing loss	r
<i>Dph1/Ovca1</i>	DPH1 homolog (<i>S. cerevisiae</i>)	[98]	¹ 603527	nr	nr
<i>Eya1</i>	Eyes absent 1 homolog (<i>Drosophila</i>)	[99]	¹ 601653	Branchiootic syndrome 1 Branchiootorenal syndrome 1, with or without cataracts Anterior segment anomalies with or without cataract	r
<i>Foxc2/Mfh1</i>	Forkhead box C2	[100]	¹ 602402	Lymphedema-distichiasis syndrome	r
<i>Foxd3</i> (<i>Wnt1-Cre-</i> mediated ablation)	Forkhead box D3	[101]	¹ 611539	uc	nr
<i>Foxe1/Titf2/Fkhl15</i>	Forkhead box E1	[102]	¹ 602617	Bamforth-Lazarus syndrome Nonsyndromic orofacial clefting	r
<i>Foxf2</i>	Forkhead box F2	[103]	¹ 603250	nr	nr
<i>Gbx2</i>	Gastrulation brain homeobox 2	[104]	¹ 601135	nr	nr
<i>Gli2</i>	GLI family zinc finger 2	[8]	¹ 165230	Culler-Jones syndrome Holoprosencephaly-9	r
<i>Gli3</i>	GLI family zinc finger 3	[105]	¹ 165240	Greig cephalopolysyndactyly syndrome Pallister-Hall syndrome	r

<i>Gsc</i>	Goosecoid homeobox	[106]	¹ 138890	Short stature, auditory canal atresia, mandibular hypoplasia, skeletal abnormalities	nr
<i>Hand2/dHand</i>	Heart and neural crest derivatives expressed 2	[107]	¹ 602407	nr	nr
<i>Hic1</i>	Hypermethylated in cancer 1	[108]	¹ 603825	nr	nr
<i>Hoxa2</i>	Homeobox A2	[19]	¹ 604685	Microtia with or without hearing impairment (AD)	r
				Microtia, hearing impairment, and cleft palate (AR)	
<i>Irf6</i>	Interferon regulatory factor 6	[109,110]	¹ 607199	van der Woude syndrome	r
				Orofacial cleft 6	
				Popliteal pterygium syndrome 1	
<i>Jmjd6/Ptdsr</i>	Jumonji domain containing 6	[111]	¹ 604914	nr	nr
<i>Kat6a/Moz/Myst3</i>	K (lysine) Acetyltransferase 6A	[112]	¹ 601408	nr	nr
<i>Lhx7</i>	LIM homeobox gene 7	[113]	nr	nr	nr
<i>Lhx8</i>	LIM homeobox gene 8	[11]	¹ 604425	nr	r
<i>Luzp1</i>	Leucine zipper protein 1	[114]	¹ 601422	nr	nr
<i>Mef2c</i> (<i>Wnt1-Cre</i> -mediated ablation)	MADS box transcription enhancer factor 2	[115]	¹ 600662	Chromosome 5q14.3 deletion syndrome	nr
				Mental retardation, stereotypic movements, epilepsy, and/or cerebral malformations	
<i>Meox2</i>	Mesenchyme homeobox 2	[116]	¹ 600535	nr	nr
<i>Mn1</i>	Meningioma 1	[117]	¹ 156100	Meningioma	nr
<i>Mnt</i>	Max binding protein	[118]	¹ 603039	nr	nr
<i>Msx1</i>	Msh homeobox 1	[10,23]	¹ 142983	Ectodermal dysplasia 3, Witkop type	r
				Orofacial cleft 5	
				Tooth agenesis, selective, 1, with or without orofacial cleft	
<i>Msx2</i> (missense mutation)	Msh homeobox 2	[119]	¹ 123101	Craniosynostosis, type 2	r
				Parietal foramina 1	
				Parietal foramina with cleidocranial dysplasia	
<i>Nabp2/Obfc2b/hSSB1</i>	Nucleic acid binding protein 2	[120,121]	¹ 612104	nr	nr
<i>Osr2</i>	Odd-skipped related transcription factor 2	[9]	¹ 611297	nr	r
<i>Pak1ip1</i>	PAK1 interacting protein 1	[122]	¹ 607811	nr	nr
<i>Pax9</i>	Paired box gene 9	[6]	¹ 167416	Tooth agenesis, selective, 3	nr
<i>Pbx1</i>	Pre B cell leukemia homeobox 1	[83]	¹ 176310	Leukemia, acute pre-B-cell	nr
<i>Pds5a</i>	PDS5, regulator of cohesion maintenance, homolog A (<i>S. cerevisiae</i>)	[123]	¹ 613200	nr	nr
<i>Phc1/Rae28</i>	Polyhomeotic homolog 1	[124]	¹ 602978	uc	nr
<i>Pitx1</i>	Paired-like homeodomain 1	[7,125]	¹ 602149	Clubfoot, congenital, with or without deficiency of long bones and/or mirror-image polydactyly	r
				Liebenberg syndrome	
<i>Pitx2</i>	Paired-like homeodomain 2	[126]	¹ 601542	Axenfeld-Rieger syndrome, type 1	nr
				Iridogoniodysgenesis, type 2	
				Peters anomaly	
<i>Pnn</i>	Pinin	[127]	¹ 603154	nr	nr
<i>Prdm16</i>	PR domain containing 16	[128]	¹ 605557	Cardiomyopathy, dilated, 1LL	nr
				Left ventricular noncompaction 8	
<i>Prrx1/Prx1/Mhox</i>	Paired related homeobox 1	[129]	¹ 167420	Agnathia-otocephaly complex	r
<i>Ptch1/Ptc1</i> (<i>Wnt1-Cre</i> -mediated ablation)	Patched 1	[130]	¹ 601309	Basal cell nevus syndrome (Gorlin syndrome)	r
				Holoprosencephaly type 7	
<i>Pygo2</i> (<i>CMV-Cre</i> -mediated ablation)	Pygopus 2	[131]	¹ 606903	nr	nr
<i>Rax</i>	Retina and anterior neural fold homeobox	[132]	¹ 601881	Microphthalmia, isolated 3	nr
<i>Recql4</i>	RecQ protein-like 4	[133]	¹ 603780	Baller-Gerold syndrome	r
				RAPADILINO syndrome	
				Rothmund-Thomson syndrome	
<i>Runx2</i>	Runt-related transcription factor 2	[134]	¹ 600211	Cleidocranial dysplasia	r
<i>Sall3</i>	Spalt-like transcription factor 3	[24]	¹ 605079	nr	nr
<i>Satb2</i>	SATB homeobox 2	[135,136]	¹ 608148	Glass syndrome	r
<i>Shox2</i>	Short stature homeobox 2	[22]	¹ 602504	nr	nr
<i>Sim2</i>	Single-minded family bHLH transcription factor 2	[137]	¹ 600892	nr	nr

<i>Smad4</i> (<i>Osr2-Cre</i> -mediated ablation)	SMAD family member 4	[138]	¹ 600993	Juvenile polyposis/hereditary hemorrhagic telangiectasia syndrome Myhre syndrome	nr
<i>Smad7</i>	SMAD family member 7	[139]	¹ 602932	uc	nr
<i>Snai2</i>	Snail family zinc finger 2	[140]	¹ 602150	Piebaldism	nr
<i>Sox5</i>	SRY (sex determining region Y)-box 5	[141]	¹ 604975	Waardenburg syndrome, type 2D	nr
<i>Sox9</i> (heterozygous) (<i>Wnt1-Cre</i> -mediated ablation)	SRY (sex determining region Y)-box 9	[142,143]	¹ 608160	Acampomelic campomelic dysplasia	r
<i>Sox11</i>	SRY (sex determining region Y)-box 11	[144]	¹ 600898	Mental retardation, autosomal dominant, 27	nr
<i>Sp8</i>	Sp8 transcription factor	[145]	¹ 608306	nr	nr
<i>Tshz1</i>	Teashirt zinc finger family member 1	[146]	¹ 614427	Aural atresia, congenital	nr
<i>Tbx1</i> (knockout) (<i>KRT14-Cre</i> -mediated ablation)	T-box 1	[4,147]	¹ 602054	DiGeorge syndrome Velocardiofacial syndrome Conotruncal anomaly face syndrome Tetralogy of Fallot	r
<i>Tbx2</i>	T-box 2	[148]	¹ 600747	nr	nr
<i>Tbx22</i>	T-box 22	[149]	¹ 300307	Cleft palate with ankyloglossia submucous cleft palate (SMCP)	r
<i>Tcof1</i> (heterozygous)	Treacher Collins-Franceschetti syndrome 1	[150]	¹ 606847	Treacher-Collins syndrome	r
<i>Tfap2A</i> (<i>Wnt1-Cre</i> -mediated ablation)	Transcription factor AP-2 alpha	[151]	¹ 107580	Branchio-oculo-facial syndrome	r
<i>Trp63/Trp63</i>	Transformation related protein p63	[152]	¹ 603273	Ectrodactyly, ectodermal dysplasia, and cleft lip/palate syndrome 3 Orofacial cleft 8 Hay-Wells syndrome Limb-mammary syndrome	r
<i>Vax1</i>	Ventral anterior homeobox 1	[153]	¹ 604294	Microphthalmia, syndromic 11	r
<i>Whsc1</i>	Wolf-Hirschhorn syndrome candidate 1	[154]	¹ 602952	nr	nr
<i>Zeb1</i>	Zinc finger E-box binding homeobox 1	[155]	¹ 189909	Corneal dystrophy	nr
<i>Zfp640/Mzf6d</i>	Zinc finger protein 640	[87]	nr	nr	nr
<i>Zic3</i>	Zinc finger protein of the cerebellum 3	[156]	¹ 300265	Congenital heart defects, nonsyndromic Heterotaxy, visceral, 1 VACTERL association	r
Cytoplasmic proteins					
<i>Akap8/Akap95</i>	A kinase (PRKA) anchor protein 8	[157]	¹ 604692	nr	nr
<i>Apaf1</i>	Apoptotic peptidase activating factor 1	[158]	¹ 602233	nr	nr
<i>B9d1</i>	B9 protein domain 1	[159]	¹ 614144	Meckel syndrome 9	nr
<i>Cask</i>	Calcium/calmodulin-dependent serine protein kinase	[160]	¹ 300172	FG syndrome 4 Mental retardation and microcephaly with pontine and cerebellar hypoplasia	r
<i>Cdkn1c/p57kip2</i>	Cyclin-dependent kinase inhibitor 1C	[161,162]	¹ 600856	Beckwith-Wiedemann syndrome IMAGe syndrome	r
<i>Chuk/Ikk1/Tcf16</i>	Conserved helix-loop-helix ubiquitous kinase	[163]	¹ 600664	Cocoon syndrome	nr
<i>Crk</i>	v-crk sarcoma virus CT10 oncogene homolog	[164]	¹ 164762	nr	nr
<i>Ctnnb1</i> (<i>KRT14-Cre</i> -mediated ablation)	Catenin (cadherin-associated protein), beta 1,	[165,166]	¹ 116806	Mental retardation, autosomal dominant 19	nr
<i>Cyp26B1</i>	Cytochrome P450, family 26, subfamily b, polypeptide 1	[167]	¹ 605207	Craniosynostosis with radiohumeral fusions and other skeletal and craniofacial anomalies	nr
<i>Cyp51</i>	Cytochrome P450, family 51	[168]	¹ 601637	nr	nr
<i>Dhcr7</i>	7-dehydrocholesterol reductase	[169,170]	¹ 602858	Smith-Lemli-Opitz syndrome	r
<i>Dhrs3</i>	Dehydrogenase/reductase (SDR family) member 3	[171,172]	¹ 612830	nr	nr
<i>Dicer1</i> (<i>Pax2-Cre</i> -mediated ablation)	Dicer 1, ribonuclease type III	[29]	¹ 606241	Rhabdomyosarcoma, embryonal, 2 Goiter, multinodular 1 Pleuropulmonary blastoma	nr
<i>Dlg1/Dlg1/Sap97</i>	Discs large 1	[173]	¹ 601014	nr	nr
<i>Fuz</i>	Fuzzy planar cell polarity protein	[174]	¹ 610622	Neural tube defects	nr
<i>Gab1</i>	Growth factor receptor bound protein 2-associated protein 1	[175]	¹ 604439	nr	nr

<i>Gad1/Gad67</i>	Glutamate decarboxylase 1	[176,177]	¹ 605363	Cerebral palsy, spastic quadriplegic, 1	r
<i>Gfce</i>	Glucuronyl C5-epimerase	[178]	¹ 612134	nr	nr
<i>Glg1</i>	Golgi apparatus protein 1	[179]	¹ 600753	nr	nr
<i>Grb2</i>	Growth factor receptor bound protein 2	[180]	¹ 108355	nr	nr
<i>Gsk3b</i>	Glycogen synthase kinase 3 beta	[181]	¹ 605004	nr	nr
<i>Hs2st1</i>	Heparan sulfate 2-O-sulfotransferase 1	[182]	¹ 604844	nr	nr
<i>Hspb11/lft25</i>	Heat shock protein family B (small), member 11	[183]	¹ 604844	nr	nr
<i>Ilk</i>	Integrin linked kinase	[184]	¹ 602366	nr	nr
(<i>Col2a1-Cre</i> -mediated ablation)					
<i>Impad1/jaws</i>	Inositol monophosphatase domain containing 1	[185]	¹ 614010	Chondrodysplasia with joint dislocations, GRAPP type	r
<i>Inpp5e</i>	Inositol polyphosphate-5-phosphatase E	[186]	¹ 613037	Joubert syndrome 1	nr
				Mental retardation, truncal obesity, retinal dystrophy, and micropenis	
<i>Kif3a</i>	Kinesin family member 3A	[187]	¹ 604683	nr	nr
(<i>Wnt1-Cre</i> -mediated ablation)					
<i>Map3k7/Tak1</i>	Mitogen-activated protein kinase kinase kinase 7	[188,189]	¹ 602614	nr	nr
(<i>Wnt1-Cre</i> -mediated ablation)					
<i>Npr13</i>	Nitrogen permease regulator-like 3	[190]	¹ 600928	nr	nr
<i>Ofd1</i>	Oral-facial-digital syndrome 1 gene homolog (human)	[191]	¹ 300170	Joubert syndrome 10	r
(<i>CAG-Cre</i> -mediated ablation)				Orofaciodigital syndrome I	
				Simpson-Golabi-Behmel syndrome, type 2	
<i>Pdss2</i>	Prenyl (solanesyl) diphosphate synthase, subunit 2	[192]	¹ 610564	Coenzyme Q10 deficiency, primary, 3	nr
(<i>Pax2-Cre</i> -mediated ablation)					
<i>Piga</i>	Phosphatidylinositol glycan anchor biosynthesis, class A	[193]	¹ 311770	Multiple congenital anomalies-hypotonia-seizures syndrome 2	nr
(<i>Ella-Cre</i> -mediated ablation)				Paroxysmal nocturnal hemoglobinuria, somatic	
<i>Pkdcc/Vlk</i>	Protein kinase domain containing, cytoplasmic	[194,195]	¹ 614150	nr	nr
(<i>Sox2-Cre</i> -mediated ablation)					
<i>Prickle1</i>	Prickle homolog 1	[196]	¹ 608500	Epilepsy, progressive myoclonic 1B	nr
<i>Rad23b</i>	RAD23b homolog (<i>S. cerevisiae</i>)	[197]	¹ 600062	nr	nr
<i>Rspo2</i>	R-spondin 2 homolog (<i>Xenopus laevis</i>)	[198,199]	¹ 610575	nr	nr
<i>Schip1</i>	Schwannomin interacting protein 1	[87]	nr	nr	nr
<i>Sdccag8</i>	Serologically defined colon cancer antigen 8	[200]	¹ 613524	Bardet-Biedl syndrome 16	nr
				Senior-Loken syndrome 7	
<i>Slc32a1/Viaat</i>	Solute carrier family 32, member 1	[201,202]	nr	nr	nr
<i>Spry1</i>	Sprouty homolog 1	[203]	¹ 602465	nr	nr
(<i>Wnt1-Cre</i> -mediated ablation)					
<i>Spry2</i>	Sprouty homolog 2	[204]	¹ 602466	nr	nr
<i>Sumo1</i>	SMT3 suppressor of mif two 3 homolog 1 (yeast)	[205]	¹ 601912	Orofacial cleft 10	r
(heterozygous)					
<i>Ugdh</i>	UDP-glucose dehydrogenase	[206]	¹ 603370	nr	nr
(<i>Wnt1-Cre</i> -mediated ablation)					
<i>Wdpcp</i>	WD repeat containing planar cell polarity effector	[207]	¹ 613580	uc	nr
Extracellular proteins					
<i>Col2a1</i>	Collagen, type II, alpha 1	[208]	² 120140	Achondrogenesis, type II	r
				Stickler syndrome, type I	
				Kniest dysplasia	
<i>Hspg2</i>	Heparan sulfate proteoglycan 2, perlecan	[209,210]	¹ 142461	Dyssegmental dysplasia	nr
<i>Serpinh1/Hsp47</i>	Serpine peptidase inhibitor, clade H, member 1	[211]	¹ 600943	Schwartz-Jampel syndrome, type 1	nr
(<i>Col2a1-Cre</i> -mediated ablation)				Osteogenesis imperfecta, type X	
<i>Smoc1</i>	SPARC related modular calcium binding 1	[212]	¹ 608488	Microphthalmia with limb anomalies	r

¹Before an OMIM entry number indicates a gene; ²Before an OMIM entry number indicates that the entry includes a description of a gene of known sequence and a phenotype. OMIM: Online Mendelian Inheritance in Man (<http://omim.org>); CL/P: Cleft lip and/or palate; r: Reported; nr: Not reported; uc: Unclassified.

beta receptor II) in the neural crest or *Shh* (sonic hedgehog) in the epithelium result in failure of the palatal shelf development^[10-13]. The fifth category is the

persistence of medial edge epithelial (MEE) cells. The palatal epithelia are regionally divided into three parts: oral, nasal, and MEE. The MEE cells are removed from

Table 2 Classification of genes associated with cleft palate in mice

	Genes
Signaling pathway	
TGF-beta signaling pathway	<i>Acor1/Alk2, Acor2a, Bmp4¹, Bmp7, Bmpr1a/Alk3, Cdc42, Chrd, Crebbp/Cbp, Cited2, Foxc2/Mfh1¹, Foxd3, Foxe1/Titf2/Fkhl15¹, Foxf2, Fst, Inhba, Gdf11/Bmp11, Map3k7/Tak1, Pitx2, Smad4, Smad7, Tgfb2¹, Tgfb3¹, Tgfb1/Alk5¹, Tgfb2¹</i>
Hedgehog signaling pathway	<i>Bmp4¹, Bmp7, Crebbp/Cbp, Gli2¹, Gli3¹, Gsk3b, Ptch1/Ptc1¹, Shh¹, Smo/Smoh, Wnt5a¹, Wnt9b</i>
Wnt signaling pathway	<i>Acor1/Alk2, Ctnnb1, Crebbp/Cbp, Edn1¹, Fzd2, Gsk3b, Lrp6, Map3k7/Tak1, Prickle1, Smad4, Smo/Smoh, Wnt5a¹, Wnt9b</i>
FGF signaling pathway	<i>Fgf10, Fgf18, Fgf9, Fgfr1¹, Fgfr2¹, Grb2, Spry1, Spry2</i>
MAPK signaling pathway	<i>Cdc42, Chuk/Ikk1/Tcf16, Egfr, Fgf10, Fgf18, Fgf9, Fgfr1¹, Fgfr2¹, Grb2, Map3k7/Tak1, Pdgfra¹, Tgfb2¹, Tgfb3¹, Tgfb1/Alk5¹, Tgfb2¹, Crk, Itgb1</i>
Cytokine-cytokine receptor interaction	<i>Acor1/Alk2, Acor2a, Bmp7, Bmpr1a/Alk3, Egfr, Inhba, Pdgfra¹, Pdgfc¹, Tgfb2¹, Tgfb3¹, Tgfb1/Alk5¹, Tgfb2¹, Vegfa</i>
CBL mediated ligand-induced downregulation of EGF receptors	<i>Egfr, Grb2, Pdgfra¹</i>
Sprouty regulation of tyrosine kinase signals	<i>Egfr, Grb2, Spry2, Spry1</i>
NFkB activation	<i>Crebbp/Cbp, Chuk/Ikk1/Tcf16, Map3k7/Tak1, Smad4, Tgfb1/Alk5¹, Tgfb2¹</i>
Adherens junction	<i>Crebbp/Cbp, Ctnnb1, Cdc42, Egfr, Fgfr1¹, Map3k7/Tak1, Smad4, Snai2, Tgfb1/Alk5¹, Tgfb2¹</i>
Focal adhesion	<i>Ctnnb1, Cdc42, Col2a1¹, Crk, Egfr, Gsk3b, Grb2, Itga5, Itgb1, Itgb8, Ilk, Pdgfra¹, Pdgfc¹, Vegfa</i>
Steroid biosynthesis	<i>Cyp51, Dhcr7¹, Sc5d/Sc5dl</i>
Cell cycle	<i>Crebbp/Cbp, Cdkn1c/p57kip2¹, Gsk3b, Smad4, Tgfb2¹, Tgfb3¹</i>
Regulation of actin cytoskeleton	<i>Cdc42, Crk, Egfr, Fgf9, Fgf10, Fgf18, Fgfr1¹, Fgfr2¹, Itga5, Itgb1, Itgb8, Pdgfra¹, Pdgfc¹</i>
Axon guidance	<i>Cdc42, Efn5, Efnb1¹, Efnb2, Gsk3b, Itgb1</i>
Endocytosis	<i>Cdc42, Egfr, Fgfr2¹, Pdgfra¹, Tgfb1/Alk5¹, Tgfb2¹</i>
Angiogenesis	<i>Ctnnb1, Crk, Efnb1¹, Efnb2, Fgfr1¹, Fgfr2¹, Fzd2, Gsk3b, Grb2, Jag1, Jag2, Pdgfra¹, Pdgfc¹, Vegfa, Wnt5a¹</i>
Family	
Homeobox protein	<i>Alx1¹, Barx1, Alx3¹, Alx4¹, Dlx1, Dlx2, Dlx5¹, Gbx2, Gsc, Hoxa2¹, Msx1¹, Msx2¹, Pax9, Prrx1¹, Pitx1¹, Pitx2, Rax, Shox2, Vax1¹</i>
Tgf-beta receptor type I and II	<i>Acor1/Alk2, Acor2a, Bmpr1a, Tgfb1/Alk5¹, Tgfb2¹</i>
Tgf-beta family	<i>Bmp4¹, Bmp7, Gdf11, Inhba, Tgfb2¹, Tgfb3¹</i>
Tyrosine protein kinase	<i>Egfr, Fgfr1¹, Fgfr2¹, Pdgfra¹, Ror2¹, Ryk</i>
Ephrin	<i>Efn5, Efnb1¹, Efnb2</i>
Zinc finger protein	<i>Gli2¹, Gli3¹, Zic3¹, Hic1, Snai2</i>
Forkhead protein	<i>Foxc2¹, Foxd3, Foxe1¹, Foxf2</i>
T-box protein	<i>Tbx1¹, Tbx2, Tbx22¹</i>
Sox transcription factor	<i>Sox5, Sox9¹, Sox11</i>
Heparin-binding growth factor family member/FGF	<i>Fgf9, Fgf10, Fgf18</i>
Sprouty	<i>Spry1, Spry2</i>
Smad	<i>Smad4, Smad7</i>
Integrin beta subunit	<i>Itgb1, Itgb8</i>
Frizzled	<i>Fzd2, Smo</i>
Wnt related	<i>Wnt5a¹, Wnt9b</i>
Serine-threonine protein kinase	<i>Ilk, Mpa3k7/Tak1</i>
LIM domain containing protein	<i>Lhx8¹, Lhx7, Prickle1</i>
EGF-like domain protein	<i>Jag1, Jag2</i>

¹Genes involved in human cleft lip and/or palate. TGF: Transforming growth factor; FGF: Fibroblast growth factor; MAPK: Mitogen-activated protein kinase; CBL: Casitas B-lineage lymphoma; EGF: Epidermal growth factor.

the fusion line by epithelial cell migration, apoptosis, and epithelial-mesenchymal transdifferentiation^[14]. *Tgfb3* (transforming growth factor, beta 3) or *Egfr* (epidermal growth factor receptor) knockout mice lack the adhesive interactions between the palatal shelves because the fate of MEE cells is altered^[15-18]. In the last category, the cleft palate arises as a secondary defect, due to tongue or bone anomalies during development. For example, *Hoxa2* (homeobox A2) knockout mice exhibit CP, because depression of

the tongue is inhibited by the abnormal attachment of the hyoglossus muscle to the greater horn of the hyoid^[19,20].

There is molecular heterogeneity along the medial-lateral and anterior-posterior axes of palatal shelves. Regionally restricted expression of molecules provides distinct regulatory mechanisms for the development of palatal shelves. For instance, *Msx1*, *Shox2* (short stature homeobox 2), *Fgf10* (fibroblast growth factor 10), *Bmp2* (bone morphogenetic protein 2), and *Bmp4* (bone

Table 3 Six categories of defects that result in cleft palate in mutant mice

Defects		Knockout mice
(1)	Failure of the palatal shelf formation (small palatal shelves)	<i>Acr2a</i> ^[34,50] , <i>Fgfr2</i> ^[13] , <i>Lhx8</i> ^[11] , <i>Pitx2</i> ^[126] , <i>Itga5</i> ^[65] , <i>Fst</i> ^[46]
(2)	Abnormal fusion of palatal shelves and tongue or the mandible	<i>Jag2</i> ^[70] , <i>Irff</i> ^[109,110] , <i>Tbx1</i> ^[4] , <i>Egfr</i> ^[41]
(3)	Failure or delayed palatal shelf elevation	<i>Pax9</i> ^[6] , <i>Pitx1</i> ^[7] , <i>Osr2</i> ^[9] , <i>Gli2</i> ^[8] , <i>Tgfb2</i> ^[55] , <i>Pdgfr</i> ^[51] , <i>Dhrs3</i> ^[172]
(4)	Failure of the palatal shelf development after the elevation	<i>Msx1</i> ^[10] , <i>Lhx8</i> ^[11] , <i>Tgfr2</i> (<i>Wnt1-Cre</i> -mediated ablation) ^[12]
(5)	Persistence of medial edge epithelial cells	<i>Apafl</i> ^[158] , <i>Tgfb3</i> ^[18] , <i>Egfr</i> ^[17] , <i>Ctnnb1</i> (<i>K14-Cre</i> -mediated ablation) ^[166]
(6)	Secondary defect	<i>Hoxa2</i> ^[19,20] , <i>Satb2</i> ^[135] , <i>Acr1/Alk2</i> (<i>Wnt1-Cre</i> -mediated ablation) ^[33]

¹Genes involved in human cleft lip and/or palate.

morphogenetic protein 4) are exclusively expressed in the anterior region of the palatal shelves^[4,13,21,22]. The ablation of *Msx1* in mice results in cell proliferation alterations in the anterior palatal mesenchyme and cleft palate^[23]. *Shox2* shows restricted expression patterns in the anterior palatal mesenchyme and the ablation of *Shox2* in mice results in anterior cleft palates^[22]. *Fgf10* is also expressed in the anterior palatal mesenchymal cells and induces *Shh* expression through its receptor *Fgfr2* (fibroblast growth factor receptor 2) in the palatal epithelium^[13]. On the other hand, *Pax9* is expressed in the posterior palatal shelves. Ablation of *Pax9* results in cleft palates because of a palatal shelf development defect^[6,21]. Even though it is known that *Tbx1* induces the expression of *Pax9* in the posterior part of palatal shelves^[4], the mechanism of *Tbx1*-induced *Pax9* expression during palatogenesis remains unknown. There is also molecular heterogeneity along the medial-lateral axis of the palatal shelf. For instance, *Osr2* expression in the palatal shelf is characterized by a medial-lateral gradient. Loss of *Osr2* results in the failure of palatal shelf elevation because of the delayed development of the medial part of palatal shelf^[9].

MOLECULAR PATHOGENESIS OF CLEFT PALATES

Since most of the studies in mice focus on complete CP, the pathogenesis of other CP phenotypes is not well understood. *Tbx1* is expressed in the developing palatal shelves in mice^[4], highlighting the crucial function of *Tbx1* in regulating palatal development. Loss of *Tbx1* results in the abnormal fusion of the oral epithelia, which induces CP by preventing the elevation of palatal shelves^[4]. The phenotypic variation in the *Tbx1*^{-/-} palates strongly suggests that *Tbx1* is involved in modifier genes and/or stochastic factors. *Tgfb3*^{-/-} mice also exhibit either incomplete or complete CP^[15,16]. Ablation of *Shox2* results in anterior cleft palates^[22]. Knockout mice of *Sall3* (spalt-like transcription factor 3), which is expressed in the palatal mesenchyme, show hypoplasia of the soft palate and epiglottis^[24]. These mice are unique models for studying the etiopathogenesis underlying the variety of CP phenotypes in humans.

A comprehensive list of molecules associated with CL/P in mice and their classification should provide

insights into the genetic etiology of CL/P; however, the phenotype of knockout mice does not always recapitulate the phenotype in humans (Table 1). Since Table 1 includes the genes associated with tissue-specific conditional knockout mice, mutations of these genes may induce the phenotype of embryonic lethality in humans. Haploinsufficiency mutations of the *TBX1* mutation are associated with CP^[25]; however, heterozygous mice with *Tbx1* are phenotypically normal, and *Tbx1*^{-/-} mice have CP phenotypes^[4], thereby suggesting a species-specific requirement for *Tbx1* dosage. Mutations of the *PVR1* (poliovirus receptor-related 1 or Nectin 1) cause CL/P-ectodermal dysplasia syndrome and nonsyndromic CL/P (OMIM #225060), whereas *Pvr1*^{-/-} mice do not develop CP^[26]. Lack of palatal phenotypes in mice may be a consequence of functional redundancy of *Pvr1* genes. Interestingly, *Smad4*, *Smad7*, *Fgf9*, *Fgf10*, and *Fgf18* are involved in CP in mice (Table 1), whereas *SMAD3* (OMIM #613795), *FGF8* (OMIM #600483), and *FGF17* (OMIM #603725) are involved in CP in humans.

Candidate genes for nonsyndromic CP in human must show a relevant spatio-temporal gene expression pattern in the developing palatal shelves, and induce a specific cleft palate phenotype when deleted^[1]. Disease genes responsible for Mendelian forms of syndromic CP are also important in the etiology of nonsyndromic CP^[27]. *TBX1* mutations have been found in patients with incomplete CP without clinical diagnosis of del22q11.2 syndrome^[25]. *TBX1* is also one of the disease genes of conotruncal anomaly face syndrome (OMIM #217095), which is often associated with cleft palates, particularly submucosal CP, and bifid uvula. *Tbx1*^{-/-} palatal phenotype in mice makes *Tbx1* a potential candidate gene for nonsyndromic CP, especially submucosal CP and incomplete CP in humans.

RECENT ADVANCES IN PALATOGENESIS

Even though many genes associated with CP have been identified, little is known about how the environment influences gene expression in palatogenesis, and palatal phenotype. Epigenetics, such as DNA methylation and chromatin remodeling, and the microRNA (miRNA) regulation could change gene expression profiles and phenotypes. Hundreds of miRNAs, small non-

coding RNAs that modulate gene expression at the post-transcriptional level are expressed in murine embryonic craniofacial tissue^[28]. Conditional knockout mice of *Dicer1* (dicer 1, ribonuclease type III), which regulates the generation of miRNA, resulted in disrupted palatogenesis^[29], suggesting that the miRNA function may be important in mammalian palatogenesis. miR-140, which modulates BMP signaling, regulates palatogenesis in mice^[30] and miR-17-92 modulates Tbx1 and Tbx3 (T-box 3) activity, resulting in orofacial clefting^[31]. Interestingly, transcription of *Dicer1* is regulated by TP63 (transformation related protein p63)^[32], whose mutations are associated with cleft palate phenotypes (Table 1). Since genes involved in miRNA generation and individual CP genes can both be modulated by several miRNAs, it is conceivable that complex gene-miRNA interactions exist during palatogenesis. Genetically engineered mice with miRNAs, which modulate CP genes, may provide new information on the gene interactions underlying the palatogenesis. Further studies on miRNA and methylated genes involved in palatogenesis are necessary to understand the environmental factors contributing to CP.

CONCLUSION

Studies with genetically engineered mice with CP reveal the importance of regulated molecular functions in palatogenesis and provide the opportunity to discover new genes implicated in palatogenesis. However, there is still much to learn about transcriptional regulation and molecular networks in palatogenesis. The interactions between environmental/stochastic factors and genes in the etiopathogenesis of CL/P require further studies. Teratogenic effects of dioxins and retinoic acid have been reported in mice^[1]. Mutant mice with CP can also be used as models to assess environmental effects or gene-environment interactions. Epithelial abnormal fusion could be one of the stochastic causes that induce a variety of CP phenotypes in mice. Understanding the palatal epithelial functions during palatogenesis may also lead to the discovery of novel therapeutic methods for CL/P.

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SGF29 and Sry pathway in hepatocarcinogenesis

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Abstract

Deregulated c-Myc expression is a hallmark of many human cancers. We have recently identified a role of mammalian homolog of yeast SPT-ADA-GCN5-acetyltransferas (SAGA) complex component, SAGA-associated factor 29 (SGF29), in regulating the c-Myc overexpression. Here, we discuss the molecular nature of SFG29 in SPT3-TAF9-GCN5-acetyltransferase complex, a counterpart of yeast SAGA complex, and the mechanism through which the elevated SGF29 expression contribute to oncogenic potential of c-Myc in hepatocellularcarcinoma (HCC). We propose that the upstream regulation of SGF29 elicited by sex-determining region Y (Sry) is also augmented in HCC. We hypothesize that c-Myc elevation driven by the deregulated Sry and SGF29 pathway is implicated in the male specific acquisition of human HCCs.

Key words: c-Myc; SPT3-TAF9-GCN5-acetyltransferase complex; SGF29; Sry; Hepatocarcinogenesis

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Core tip: Deregulated c-Myc expression is a hallmark of many human cancers. We have recently identified a role of mammalian homolog of yeast SPT-ADA-GCN5-acetyltransferas (SAGA) complex component, SAGA-associated factor 29 (SGF29), in regulating the c-Myc overexpression. We propose that the upstream regulation of SGF29 elicited by sex-determining region Y (Sry) is also augmented in hepatocellularcarcinoma (HCC). We hypothesize that c-Myc elevation driven by the deregulated Sry and SGF29 pathway is implicated in the male specific acquisition of human HCCs.

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C-MYC AND HEPATOCARCINOGENESIS

c-myc is a protooncogene of the viral homolog of *v-myc* which causes myelocytomatosis^[1-4]. The expression of *c-Myc* is tightly regulated by the many ligand-stimulated receptor signaling under normal condition^[5]. The deregulated expression level of *c-Myc* contributes many aspects of cancer development including proliferation, growth, DNA replication, protein synthesis, metabolism, cell adhesion, angiogenesis, metastasis and immune responses by regulating the transcription of its target genes^[6-11]. Only two-fold increase in the expression level of *c-Myc* can affect cell cycle progression^[12]. In over 50% of human cancers, *c-Myc* is deregulated and/or activated^[13-18]. In Burkitt lymphoma, the translocation of *c-myc* gene to the immunoglobulin gene causes the overexpression of *c-Myc*^[19,20]. *c-Myc* is also highly expressed by the gene amplification in many human and rodent cancers including hepatocellular carcinoma (HCC)^[21-23]. The 8q22-24 region including *c-myc* locus is involved in the early onset of HCC and represent the frequent amplification in early HCC^[24,25]. In mice models, the expression level of *c-Myc* is also associated with the development of HCC^[26-28]. In colon cancer and T cell leukemia, WNT and Notch signaling pathways are involved in the upregulation of *c-Myc*, respectively^[29-32]. Conversely, suppression of *c-Myc* expression can cause tumor regression by inducing cell cycle arrest, differentiation, or senescence depending on the cell contexts in mice models. The effect of *c-Myc* downregulation on tumor regression is permanent in some tumors such as lymphoma and osteosarcoma, whereas in hepatocellular or breast cancers this effect is reversible^[33-37]. Thus, inhibiting the *c-Myc* expression could be a promising therapeutic strategy^[38-44].

TRANSCRIPTIONAL REGULATION OF C-MYC VIA SPT3-TAF9-GCN5-ACETYLTRANSFERASE

c-Myc has N-terminal transcriptional regulatory domain consisting of Myc boxes (Mb) I, II, III, IV and nuclear localization signal. The basic Helix-Loop-Helix leucine-Zipper (bHLHZ) domains encompasses C-terminal region of *c-Myc*^[45-50]. In the nucleus, *c-Myc* heterodimerizes with Max through their bHLHZ domains to bind to the E-box sequence (5'-CACGTG-3') in the gene regulatory region^[51-54]. The E-box sequences mainly exist in the promoter or intron 1 of the target genes. Although E-boxes are occupied with other E-box-binding transcription factors including ChREBP, SREBP, HIF-1, NRF1, USF, TFE3, Clock, and Bmal to regulate cellular homeostasis in quiescent state, *c-Myc* is re-

placed with these factors upon its induction by ligand stimulation^[11]. Genome-wide screening for *c-Myc*-bound target genes using chromatin immunoprecipitation (ChIP)-sequencing reveal that around 6000 genes can be bound with *c-Myc* in human genome^[55]. Of these genes, a set of 300 genes are actually inducible by *c-Myc* and are involved in nucleotide metabolism, ribosome biogenesis, RNA processing, and DNA replication^[56]. These genes are also suggested to be induced in *c-Myc*-overexpressing transformed cells. *c-Myc* can recruit some cofactor complexes including histone acetyltransferases, ubiquitin ligases, and kinases, and the N-terminal region of MbI and MbII domains are required for the transcriptional activation and malignant transformation activities^[57-59]. These domains can recruit three classes of histone acetyltransferase (HAT) complexes including SPT3-TAF9-GCN5-acetyltransferase (STAGA) complex^[60], p300/CBP-associated factor (PCAF) complex^[61,62] and the TIP60-containing complex^[63] to activate the adjacent gene transcription. Of these three classes of HAT complexes, STAGA complex is thought to be responsible for the transactivation of *c-Myc*, because the only direct interaction between STAGA and *c-Myc* is demonstrated^[64]. The *Saccharomyces cerevisiae* SPT-ADA-GCN5-acetyltransferase (SAGA) complex is the 1.8 MDa transcriptional coactivator that is highly conserved counterpart of STAGA complex consisting of 18 to 20 proteins^[65-71]. The detailed information about these complexes is derived from the studies of yeast SAGA complex. SAGA is essential for about 10% of whole gene transcription^[65,66,70,72]. The SAGA complex subunits are classified into four functional groups. The first group includes GCN5 acetyltransferase, ADA2B, ADA3 and SAGA-associated factor 29 (SGF29) regulating the acetylation of multiple lysine residues of histone H3 including H3K9, H3K14, H3K18 and H3K23^[73-76]. The acetylation of histones is significantly implicated in the activation state of transcription^[77-79]. Moreover, GCN5 can bind to the acetylation mark of histone H3 by its bromo domain^[80]. The second group constitutes the ubiquitin-specific protease Ubp8, SGF11, SGF73 and SUS1 to perform the deubiquitination of H2B^[81,82]. Deubiquitination of Lys123 of histone H2B eventually induce the phosphorylation of the C-terminal domain of RNA polymerase II (Pol II) to facilitate release of Pol II into transcription elongation^[83,84]. The third group contains TATA-binding protein (TBP)-associated factor (TAF) proteins that are also incorporated in the general transcription factor TFIID^[67,68]. Since a complex consisting of the histone-fold domains of TAF6, TAF9, TAF12 and ADA1 have structural resemblance to the histone octamer, TAF proteins make the structural backbone of the SAGA complex^[85]. The last group consists of Tra1 and SPT proteins to tether the SAGA complex to the specific transcriptional activators^[67,86-91]. SPT proteins are also reported to be required for the recruitment of TBP to the target gene promoters^[69,92-94]. These subunits cooperatively recruit the Pol II and assemble the preinitiation complex to induce the target

gene transcription. Of these components, Tra1 and TAF proteins are essential for cellular viability in yeast^[95,96]. Most of these components are highly conserved between yeast and human. The human orthologue of Tra1, Transactivation/transformation-associated protein (TRRAP), can interact with the Mb I and Mb II domains of c-Myc, which is essential for malignant transformation and for the hyperacetylation of histone H3 and H4^[64,97-102]. In addition, TRRAP is implicated in the transactivation and transformation activity of E2F1, E2F4, p53, and E1A^[98,103-105]. The acetyltransferase activity of human GCN5 is also reported to be involved in the malignant transformation potential of c-Myc^[99]. Thus, STAGA complex has significant role in the c-Myc-mediated onset of many human malignancies.

INVOLVEMENT OF SGF29 IN HEPATOCARCINOGENESIS

The critical factor in transformation activity of c-Myc other than TRRAP and GCN5 is SGF29 which is also conserved between yeast and mammals. SGF29 is originally identified using the mass spectrometric analysis of yeast SAGA^[81]. By directly interacting with ADA3, SGF29 is incorporated into the STAGA complex^[106]. The structural domains of SGF29 are N-terminal coiled-coil domain and C-terminal double Tudor domains^[107,108]. Tudor domain is originally cloned in *Drosophila*, and to date, about 30 Tudor-containing proteins have been identified in mammals such as SGF29, 53BP1, Spindlin1 and UHRF1 most of which can bind to a methylated lysine^[75,109-115]. The Tudor domain-containing proteins belong to so-called histone reader proteins that can recognize the various sites for post-translational histone modifications including methylation, acetylation, ubiquitination, and phosphorylation^[116]. The histone readers recruit specific protein complexes at the site of histone modifications. These modifications are referred to as histone code to regulate chromatin organization and gene transcription. Many human disorders are associated with the misreading of histone codes^[117-119]. The Tudor domains of SGF29 specifically recognize the histone H3K4me3/2 sites in a manner of preference to tri-methylation in yeast and human cells^[75,110]. Because tri-methylation of histone H3K4 is prerequisite for subsequent acetylation of histone H3^[120] and no other subunits of SAGA complex harbor the Tudor domain, only SGF29 mediates a direct connection between histone H3K4me3/2 and acetylation of histone H3. Knocking down the yeast SGF29 or its mutated form of it decrease the acetylation levels of histone H3 without disturbing the integrity of SAGA complex because of the loss of its ability to bind histone H3K4me3/2 and to load SAGA complex at target gene promoters^[67,75,121]. The histone H3K4me3/2 is frequently observed in the active promoter region of genes, and the histone H3 in the E-box sequences are also reported to be highly methylated^[122-126]. These methylation of histone H3K4

in E-box occur prior to the induction of acetylation of H3 by c-Myc expression^[127]. Because c-Myc is not thought to directly recognize these histone markers, SGF29 in the STAGA complex may help to recruit c-Myc to the E-box in cooperation with TRRAP^[106]. The loaded c-Myc induces the local histone acetylation at the site of E-box leading to histone unwinding and significant induction of downstream target gene transcription^[128]. In normal organs, SGF29 is robustly expressed in testis and modestly in thymus, spleen and lung. In rat hepatomas, three out of five cell lines overexpress SGF29 together with c-Myc^[106]. In the presence of c-Myc, SGF29 enhance the transcriptional activating activity of the promoter of a c-Myc target gene, ornithine decarboxylase (ODC)^[129,130], *in vitro*. Conversely, the deletion of SGF29 expression causes the decrease in this promoter activity in rat hepatoma K2 cells. The downregulation of SGF29 also abrogates the colony forming ability of K2 cells in soft agar and tumorigenicity in nude mice concomitant with the decreased expression of c-Myc target genes such as ODC, lactate dehydrogenase-A (LDH-A)^[131,132] and metastasis-associated protein 1 (MTA1)^[133], whereas growth rate in monolayer culture is not affected. Moreover, the lowered expression level of SGF29 suppresses the metastatic ability of K2 cells to lung^[106]. Taken together, the increased SGF29 expression is closely associated with the oncogenic potential of c-Myc by controlling its target gene expression.

MALE SPECIFIC HEPATOCARCINOGENESIS AND SRY

How could the factor(s) cause the deregulated expression of SGF29? The *in silico* search for the transcription factor binding site in the promoter region of rat SGF29 gene predicts that ten transcriptional factors could bind this region^[134]. These factors contain c-Myc, sex-determining region Y (Sry), AML-1a and GATA-1. Given that there are seven high-mobility group (HMG)-boxes (5'-AACAAAG-3'), Sry-binding DNA element, and high-expression level of SGF29 in testis, Sry may be the most potent regulator of SGF29 overexpression. Indeed, SGF29 is shown to be a novel Sry target gene because Sry can directly bind the proximal promoter region of rat SGF29 gene and increase SGF29 transcript^[134]. Sry is originally identified as the male sex-determining gene and is located in the male-specific region of the Y chromosome^[135-137]. Induction of Sry expression in the genital ridges drives the testis differentiation and causes the activation of Sox9 to induce the downstream target genes required for male development^[138-140]. Sry is not so highly conserved in its N-terminal region among mammals, but these proteins harbor the same ability to control the sex determination in developmental process in a spatiotemporally regulated manner^[141-143]. The highly conserved HMG DNA-binding domain of Sry elicits the crucial function in developmental process^[139,144,145]. In general, transcription factor have

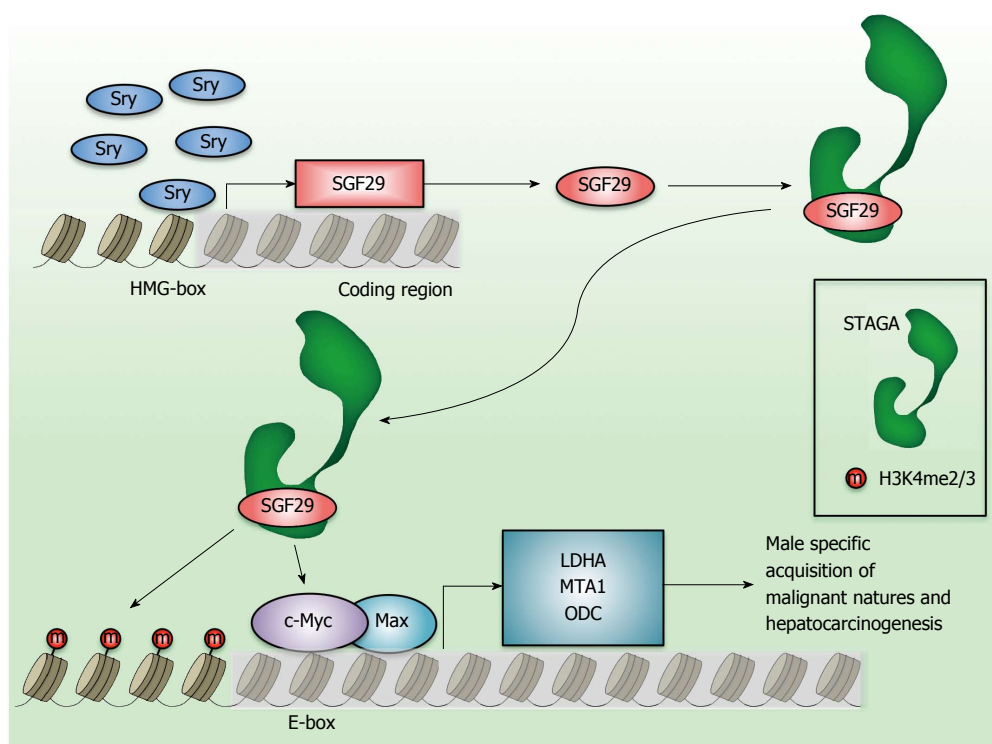


Figure 1 SAGA-associated factor 29 and Sry pathway in hepatocarcinogenesis. Deregulated c-Myc expression is a hallmark of many human cancers. The transcriptionally active marker of histone H3, H3K4me3/2 (red small circles depicted in left bottom of the figure), is frequently observed in the active promoter region of the E-box sequences in the c-Myc target genes. SGF29, a component of STAGA complex, which can interact with H3K4me2/3 induce the acetylation of histone at the sites of c-Myc loaded E-box leading to histone unwinding and significant induction of downstream target gene transcription. The upstream regulation of SGF29 elicited by male specific transcription factor, Sry, is also deregulated in HCC. Eventually, c-Myc target genes such as LDHA, ODC and MTA1 expression are induced, which could account for a molecular insight into the male specific acquisition of human HCC driven by the deregulated Sry and SGF29 pathway. SGF29: SAGA-associated factor 29; STAGA: SPT3-TAF9-GCN5-acetyltransferase; Sry: Sex-determining region Y; HCC: Hepatocellular carcinoma; LDH-A: Lactate dehydrogenase-A; ODC: Ornithine decarboxylase; MTA1: Metastasis-associated protein 1.

the DNA-binding and transactivation domains, but most of Sry proteins from different species except for mouse and rat do not have an obvious transactivation domain^[145]. Although many Sry-binding partner proteins are reported to bind to the non-conserved region of Sry^[146-149], physiological relevance between these interactions and testis differentiation remains obscure^[150,151]. Although Sry expression is limited in brain, thymus and testis in adulthood, some HCC cell lines such as K2 cells overexpress this gene^[134]. Moreover, the copy number gain or amplification of this gene locus occur in 11.8% (8/68) of human male HCC cases, which may reflect the fact that Sry is deregulated in some human HCC^[152]. The deletion of Sry expression causes the lowered SGF29 expression in K2 cells together with the decreased level of LDHA and MTA1^[134]. Like SGF29, the reduced Sry expression leads to the diminished colony forming ability in soft agar and tumorigenicity *in vivo*. The Sry expression solely does not bypass the malignant phenotype when SGF29 is deleted, which reveal that the induction of SGF29 brought by Sry is necessary for the acquisition of c-Myc-dependent transformation activity. Taken together, these findings suggest a hypothetical model of Sry and SGF29 pathway in male specific malignancy of HCC (Figure 1). The aberrant expression of Sry causes the elevation

of SGF29, which is integrated into STAGA complex to augment the c-Myc target genes' expression. Because Sry is expressed only in male, this scheme may be the explanation of male-specific acquisition of malignancy and hepatocarcinogenesis.

CONCLUSION

STAGA is one of the histone acetyltransferase complex and crucial for malignant transformation activity of c-Myc. STAGA complex can bind to c-Myc, and the deletion of key components of STAGA suppress the c-Myc target genes' transcription. Thus, uncovering the regulatory mechanism of STAGA complex can sheds light on the oncogenesis driven by c-Myc. As a component of STAGA, SGF29 physically interact with transcriptionally active histone marker H3K4me2/3. Whether SGF29 simultaneously associates with both STAGA complex and histone H3K4me2/3 is unclear, SGF29 might facilitate the efficient recruitment of Pol II to the promoter region of c-Myc target genes. We have shown that SGF29 expression is deregulated in some rodent HCCs and is important for tumorigenic activity of c-Myc. Furthermore, because Sry is shown to be directly upregulate the SGF29 transcription and the amplification of Sry gene is observed in human

male HCCs, Sry may be an attractive target for c-Myc-involved HCCs as well as SGF29. Given that c-Myc is deregulated in many human cancer types, the Sry-SGF29-c-Myc axis might be implicated in the onset of these cancers. However, not having any information about the expression level of SGF29 in cancers other than HCC, more detailed survey for this point will be required. On the other hand, in addition to Sry, c-Myc, AML-1a and GATA-1 may also bind the promoter region of SGF29 gene in *in silico* data analysis. Whether these factors will be the real upstream regulator(s) of SGF29 gene or not should be revealed. Although there are several protein complexes which are recruited to c-Myc, implications of these complexes in malignant transformations are unclear. Moreover, because the relationships between c-Myc and STAGA components other than SGF29, GCN5, and TRRAP are unknown, deubiquitination or TBP recruiting activities of STAGA toward c-Myc should be also clarified.

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Metabolic interplay between glycolysis and mitochondrial oxidation: The reverse Warburg effect and its therapeutic implication

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Abstract

Aerobic glycolysis, *i.e.*, the Warburg effect, may contribute to the aggressive phenotype of hepatocellular carcinoma. However, increasing evidence highlights the limitations of the Warburg effect, such as high mitochondrial respiration and low glycolysis rates in cancer cells. To explain such contradictory phenomena with regard to the Warburg effect, a metabolic interplay between glycolytic and oxidative cells was proposed, *i.e.*, the "reverse Warburg effect". Aerobic glycolysis may also occur in the stromal compartment that surrounds the tumor; thus, the stromal cells feed the cancer cells with lactate and this interaction prevents the creation of an acidic condition in the tumor microenvironment. This concept provides great heterogeneity in tumors, which makes the disease difficult to cure using a single agent. Understanding metabolic flexibility by lactate shuttles offers new perspectives to develop treatments that target the hypoxic tumor microenvironment and overcome the limitations of glycolytic inhibitors.

Key words: Hepatocellular carcinoma; Oxidative stress; Metabolic interventions; Aerobic glycolysis; Lactate

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Core tip: The Warburg effect plays a vital role in cancer cell proliferation and survival, and contributes to the initiation of tumor metastasis. To adapt to rapidly changing microenvironment for survival such as from normoxia to hypoxia, cancer cells vary in metabolic phenotype; "metabolic flexibility". Even in a hypoxic condition, oxidative cancer cells and/or stromal cells should theoretically exist to support the metabolic fuel for glycolytic cancer cells and handle lactate *via*

the dynamic shuttle; “the reverse Warburg effect”. Treatments against tumor metabolism may aim to target two distinct metabolic pathways of glycolysis and mitochondrial oxidative phosphorylation.

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INTRODUCTION

Hepatocellular carcinoma (HCC) is the seventh most common cancer and the third cause of cancer-related mortality all over the world^[1]. Despite the recent development of various types of targeted agents, minimal improvements have been identified in the survival of patients with advanced HCC since the introduction of sorafenib 10 years ago^[2]. To overcome the limitations of current anticancer agents, new strategies must be developed.

Most previous studies have strongly suggested the metabolic reprogramming of cancer cells into aerobic glycolysis, *i.e.*, the Warburg effect, in the process of carcinogenesis and adjustment for the hypoxic tumor microenvironment. Based on this concept, the development of anticancer agents that target the enzymes involved in glycolysis appears to be promising. 3-bromopyruvate (3-BP), is a potent anticancer agent that inhibits the glycolytic pathway primarily leads to a depletion of energy reserves. Previous studies have demonstrated that 3-BP could have strong anticancer effects in various cancer types. However, in a case study performed in Egypt, the killing effect of 3-BP was not as potent as expected in the treatment of a 28-year-old man who presented with stage IV metastatic melanoma. The patient died of cancer progression even though he had received 3-BP treatment^[3].

Based on the cancer progression despite 3-BP treatment in this case, mechanisms beyond the Warburg effect may contribute to cancer cell survival, *i.e.*, evading the glycolytic pathway or attenuating the 3-BP anticancer effect. One potential explanation of the mechanism is that cancer cells might have a preference to produce energy reserves *via* mitochondrial oxidative phosphorylation (OXPHOS) rather than high glycolysis according to their surrounding conditions. The cells use lactate from tumor stromal cells, which is the end product of glycolysis and can fuel mitochondrial OXPHOS after conversion to pyruvate. This phenomenon has been referred to as the “reverse Warburg effect”, which indicates increased aerobic glycolysis of stromal cells adjacent to tumor cells^[4]. Another potential reason for 3-BP resistance might be the capability of tumor

cells to balance the redox potentials, which play a key role in drug detoxification and cellular protection from oxidative injury by free radicals and peroxides, *i.e.*, “chemoresistance” to 3-BP^[5].

In this review, we describe the clinical implication of the Warburg effect with a high redox potential, and the roles of mitochondrial OXPHOS with a focus on lactate shuttles beyond the Warburg effect. Given the importance as modulators of tumor cell metabolism, these approaches may represent promising therapeutic targets to potentiate the anticancer effect of 3-BP, which has been well-known as the strongest inhibitor of glycolysis in cancer.

WARBURG EFFECT: CURRENT CLINICAL IMPLICATIONS IN CANCER

Warburg first reported an anomalous characteristic of energy metabolism in cancer cells^[6,7], even in the presence of oxygen, cancer cells can accelerate glycolysis rather than mitochondrial OXPHOS; “aerobic glycolysis”. Aerobic glycolysis is seemingly contradictory phenomena because cancer cells must compensate for the 18-fold lower efficiency of ATP production afforded by glycolysis as compared to mitochondrial OXPHOS. To compensate this lower efficiency, the cells upregulate glucose transporters such as GLUT1^[8-10]. This inefficient energy metabolism provides cancer cells with several advantages: (1) balancing the redox potential inner cell; and (2) increased biosynthesis of intermediate macromolecules, anti-apoptosis, and efficient signaling through metabolites as compared with mitochondrial OXPHOS^[9].

In hypoxic tumor cells, the overexpression of glucose transporters and glycolytic enzymes such as hexokinase II (HK II), phosphofructokinase (PFK), phosphoglycerate kinase, and lactate dehydrogenase (LDH), has been investigated^[11]. It was reported that high serum levels of glucose transporter (GLUT) 1, GLUT 3, aldolase-B, and HK II have been significantly associated with poor prognosis in various types of malignancies^[12]. Among them, HK II, involved in the first rate-limiting step of glycolysis, has been related to anti-apoptosis. A predominant fraction of HK II was bound to the voltage-dependent anion channel (VDAC) at the outer membrane of the mitochondria. Among the proposed mechanism of chemoresistance in cancer cells was the decreased availability of free VDAC sites that interact with pro-apoptotic proteins (Bax). HK II binds to the VDAC site and this interaction prevents the activation of Bax^[13]. In other words, HK II play a key role for prevention of chemotherapy-induced, mitochondria-mediated, tumor cell apoptosis. In addition, this glycolysis pathway can be further accentuated under the hypoxic conditions in many tumors: the hypoxic condition can upregulate glucose transporters and induce multiple enzymes of the

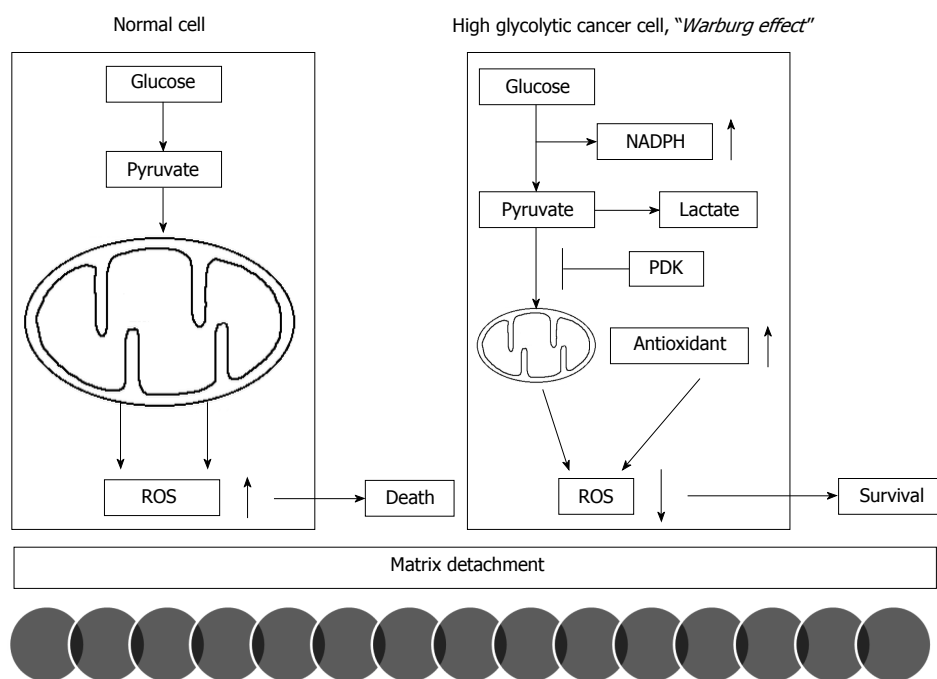


Figure 1 The Warburg effect contributes to the initiation of tumor metastasis by lowering intracellular reactive oxygen species levels. After matrix detachment, cancer cells decrease intracellular ROS levels through inducing enzymes involved in the glycolysis and antioxidant systems for their survival. The Warburg effect is strongly linked with modulation of ROS system. PDK: Pyruvate dehydrogenase kinase; ROS: Reactive oxygen species.

glycolytic pathway^[10,14,15]. For example, both Ras oncoprotein and hypoxia can independently increase HIF1a and HIF2a transcription factor levels which upregulate glycolysis^[14,16,17]. Our group demonstrated that hypoxia stimulates HCC cellular growth through hexokinase II induction, which thereby may participate in HCC progression, and the blockage of this enzyme may be therapeutically efficacious in human HCCs^[18,19]. Although tumor cells can redirect energy metabolism of “aerobic glycolysis”, it has become apparent that oxygenation, which ranges from normoxia to hypoxia, is not always static in tumor microenvironment, but instead dynamically fluctuates as a result of the dynamic changes of the tumor-associated neovasculature^[20].

WARBURG EFFECT WITH ROS: INITIATION OF METASTASIS

The acceleration of glycolysis in cancer cell energy metabolism could reduce the production of reactive oxygen species (ROS) by less reliance on mitochondrial OXPHOS and simultaneously enhance the redox potential *via* an increase in NADPH in the pentose phosphate pathway as byproducts for biosynthetic pathways of proliferation. This contribution of the Warburg effect to the balance of redox potential plays a pivotal role in the initiation of metastasis; matrix detachment. While normal cells attenuate mitochondrial OXPHOS in response to matrix detachment for their survival, many cancer cells already limit mitochondrial OXPHOS before detachment because of the Warburg effect. Normal cells activate PDK4 to inhibit PDH

following detachment to upregulate glycolytic pathway. However, cancer cells already express high levels of PDK1 under attached conditions^[21]. PDK1 and PDK3 expression in various cancers significantly correlates with patients’ prognosis: tumor histological grades and disease-free survival^[22,23]. PDK inhibition or PDH activation in cancer cells stimulates mitochondrial OXPHOS and thereby increase ROS production. Excess production of intracellular ROS levels increase their susceptibility to cell death after matrix detachment, which leads to a decreased metastatic potential^[21]. Therefore, the Warburg effect allows cancer cells to evade cellular oxidative stress that would be produced by mitochondrial OXPHOS for glucose metabolism^[24]. Thus, the reduction of ROS levels, which promotes metastasis, may represent an advantage given by the Warburg effect (Figure 1). Increased glucose consumption diverts more glucose carbon into the oxidative branch of the pentose phosphate pathway, which represents a major source to generate NADPH^[25]. NADPH is a critical cofactor for the replenishment of reduced glutathione (GSH) in a cell. Cancer cells can further enhance this antioxidant generation pathway *via* PKM inhibition when oxidative stress increases^[26].

In addition to the Warburg effect, cancer cells also potentiate antioxidant systems to cope with increased oxidative stress^[25]. For example, while MnSOD is induced following matrix detachment in normal cells^[27], MnSOD is constitutively overexpressed in cancer cells. Furthermore, increased MnSOD expression in cancer is significantly associated with poor prognosis^[27-29]. An enhanced antioxidant capacity allows cancer cells to

better survive detachment-induced oxidative stress and initiate to metastasize. In a lung cancer mouse model, antioxidant treatments have consistently reduced oxidative stress and accelerated lung cancer progression^[30].

LIMITATIONS OF THE WARBURG EFFECT AND GLYCOLYTIC INHIBITORS

To adapt to rapidly changing microenvironment for their survival such as from normoxia to hypoxia^[31,32], each cancer cells may vary in metabolic phenotype even in a single tumor mass; "metabolic flexibility"^[33]. Because total ATP production *via* the glycolytic pathway does not generally exceed 50%-60%^[34], mitochondrial OXPHOS still, to a certain extent, contributes to ATP generation in cancer cells, *i.e.*, a mixture of glycolysis and mitochondrial OXPHOS^[35]. The ratio of contribution to ATP production in cancer cells can be rapidly changed to maintain pace with the alteration of the tumor microenvironment. Herst *et al.*^[36] reported that energy production for tumor cell growth was altered from mitochondrial OXPHOS to accelerated glycolysis according to changes from normoxia to hypoxia: mitochondrial OXPHOS contributes to total ATP production, which was 91% in normoxia and reduced to 36% in hypoxia.

Given this flexibility, all cancer cells did not completely depend on accelerated glycolysis. Previous studies reported that mitochondrial OXPHOS in many cancers can be well-functioned to produce ATPs^[9,37-40]. These authors have concluded that the Warburg effect is a result of accelerated glycolysis which suppressed mitochondrial OXPHOS rather than the initial impairments in mitochondrial OXPHOS. If glycolysis is suppressed in cancer cells, the mitochondrial OXPHOS function can be recovered^[35,39,41,42]. In a noticeable investigation, Fantin *et al.*^[39] demonstrated that when glycolysis was inhibited by suppression of LDH-A in cancer cells, mitochondrial OXPHOS could be restored to compensate for energy production. This study reported that both LDH-A and mitochondrial function were modulated by the metabolite level such as pyruvate and the NADH/NAD⁺ ratio. This result indicates that cancer cells have the capacity of regulating ATP production by mitochondrial OXPHOS to adapt to the rapidly changing microenvironment.

According to a proposal by Smolková *et al.*^[41], metabolic phenotypes in cancer cells can be simply divided into two subgroups with each condition of the tumor microenvironment: (1) enhanced glycolysis and suppressed mitochondrial OXPHOS with a hypoxic condition; and (2) relatively suppressed glycolysis and restoration of mitochondrial OXPHOS with nutrient shortage because of high proliferation rates. This proposal explained that the Warburg phenotype is

not a universal finding, and mitochondrial respiration impairment is not a fixed feature of cancer cells^[41].

Although the glycolytic inhibitors targeting the Warburg effect have been investigated in various cancer types, the glycolytic inhibitors with the exception of 3-BP (a lactate analog)^[18,19,43-70], 3-BrOP (a 3-bromopyruvate derivative)^[71-74], and dichloroacetate (DCA)^[75-98] have demonstrated low efficacy in arresting tumor growth when used alone^[99]; these inhibitors include 2-deoxy-D-glucose (a glucose analog)^[70,100-112], lonidamine (a derivative of indazole-3-carboxylic acid)^[113-132], methyl jasmonate on HK^[133-161], 3-(3-pyridinyl)-1-(4-pyridinyl)-2-propen-1-one on PFK^[162-166], and iodoacetate on glyceraldehydes-3-phosphate dehydrogenase (GAPDH)^[167-170].

Among various glycolytic inhibitors, 3-BP could only target two energy pathways of glycolysis and mitochondrial OXPHOS. The mechanism for 3-BP action suggested that mitochondrial HK II is essential for the high glycolytic capacity *via* the utilization of mitochondrial ATP rather than cytosolic ATP, and the lowering of mitochondrial OXPHOS capacity by limiting Pi and ADP delivery to the mitochondria^[171,172]. In contrast, DCA would affect only in cancer cells having impaired mitochondria function by PDK inhibition: otherwise alternative energy sources could compensate for the inhibited glycolysis through the competent mitochondria^[173]. No phase III randomized clinical trial of glycolytic inhibitors has exhibited satisfactory clinical outcomes^[174].

Of note, the previously described studies that have reported the anticancer effects of these glycolytic inhibitors, with the exception of 3-BP, have exhibited common characteristics: (1) a low efficacy for anticancer effects when used alone; (2) capable of sensitizing cancer cells to conventional chemotherapy drugs, such as 5-fluorouracil, cisplatin, doxorubicin, and sorafenib; (3) the role of a sensitizer to make cancer cells vulnerable to radiotherapy and photodynamic therapy; and (4) an increase in the intracellular ROS levels as an important mechanism to induce their apoptosis.

PARADIGM SHIFT FROM THE WARBURG EFFECT TO THE REVERSE WARBURG EFFECT BASED ON LACTATE SHUTTLE

To explain metabolic flexibility, *i.e.*, mitochondrial OXPHOS, the reverse Warburg effect was suggested^[175]; cancer cells educate carcinoma-associated fibroblasts (CAFs) to enhance aerobic glycolysis, and CAFs thereby produce lactate, which was converted to pyruvate and utilized for mitochondrial OXPHOS in cancer cells^[175,176]. Tumor cells and CAFs influence each other in energy metabolites for co-evolution in cancer progression. A growing body of evidence indicates that lactate as an end product of glycolysis in hypoxic cancer cells and/or CAFs is not a waste product. Lactate can be used as

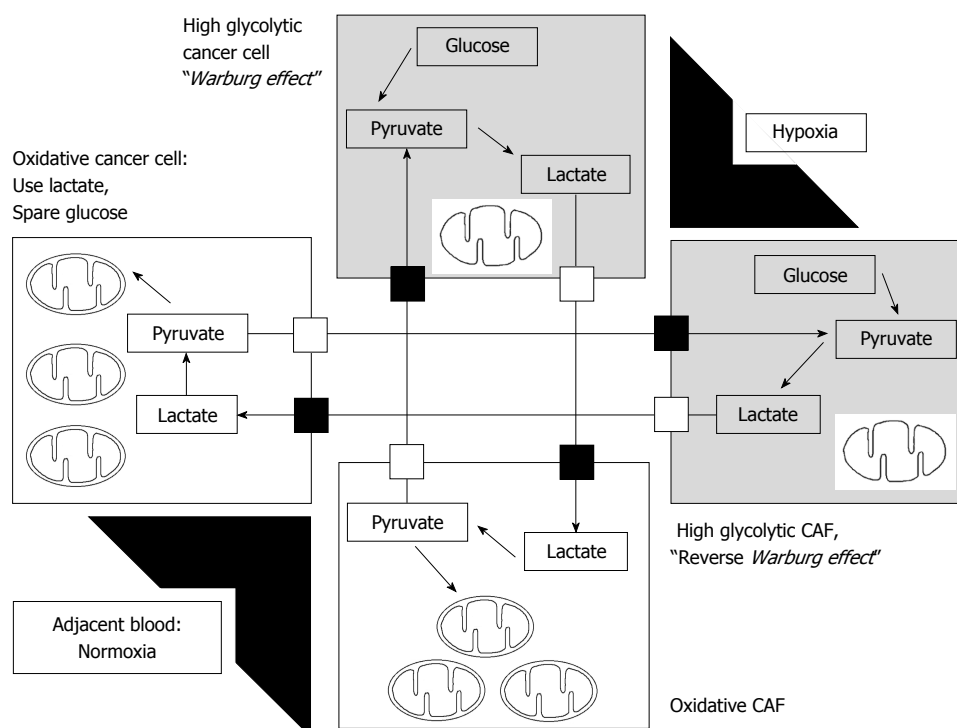


Figure 2 Metabolic interplay between high glycolytic cells and mitochondrial OXPHOS cells *via* lactate shuttle: the black box indicates monocarboxylate transporter 1, and the white box indicates monocarboxylate transporter 4: The gray zone means the hypoxic condition. Through MCT1 and 4, energy metabolites such pyruvate and lactate go in and out between glycolytic and oxidative cells: two roles of energy fuel and maintenance for acid-base balance in tumor microenvironment. MCT: Monocarboxylate transporters; CAF: Carcinoma associated fibroblast.

energy fuel for oxygenated tumor cells and/or oxidative CAFs as shown in Figure 2. Lactate is converted to pyruvate by LDH-B, which can enter the mitochondria to produce ATP in the cells with restored mitochondrial OXPHOS^[31,40,176-181]. Particularly, oxidative tumor cells in normoxic microenvironment use mitochondrial OXPHOS to spare glucose, which can be utilized by glycolytic tumor cells located in hypoxic microenvironment.

During communication between glycolytic and oxidative cells, MCT1 and MCT4 are key players in this metabolic cross-talk. Influx of lactate by oxidative cancer cells occurs through MCT1, whereas lactate is released through MCT4^[177]. MCT1 inhibition can make a metabolic shift from mitochondrial oxidation to glycolysis, which thereby induce glucose consumption. MCT4 inhibition can directly induce cell death *via* accumulation of intracellular lactic acid in hypoxic tumor cells^[177]. Clinical phase I trials investigating MCT1 inhibitors are ongoing (<http://clinicaltrials.gov/show/NCT01791595>).

In addition to metabolic fuel by lactate shuttle, this phenomenon can maintain an acid-base balance *via* the prevention of the development of a fatal acidic environment in cancer cells^[181,182]. Koukourakis *et al.*^[181,182] reported that increased expression of MCT1, LDH, and PDH in CAFs metabolically utilize lactate produced by tumor cells. In the other way, previous studies reported that some CAFs can undergo aerobic glycolysis and provide nearby oxidative cancer cells

with the released lactate^[176,182,183] (Figure 2).

Furthermore, accumulation of lactic acid, which represents high glycolysis rates in hypoxic conditions, reflects poor vascularity caused by rapid tumor growth. Previous studies demonstrated that lactate released from glycolytic tumor cells through MCT4 can stimulate angiogenesis and tumor growth *via* IL-8 dependent pathway^[184]. Taken together, these findings establish important roles for lactate shuttles in tumors: (1) it acts as both a metabolic fuel; and (2) maintains an acid-base balance in cancer cells; and (3) signals for angiogenesis in hypoxic microenvironment. Finally, cancer cells can adapt to rapid changes in the tumor microenvironment through the metabolic interplay between oxidative and glycolytic cells, such as glycolytic and oxidative tumor cells and glycolytic and oxidative stromal cells, through lactate shuttle: "tumor heterogeneity and metabolic flexibility" (Figure 2).

THERAPEUTIC IMPLICATIONS OF TARGETING THE METABOLIC INTERACTION BETWEEN HIGH GLYCOLYTIC CELLS AND MITOCHONDRIAL OXPHOS CELLS

Although increasing evidence has recently indicated that mitochondrial OXPHOS and lactate shuttle may

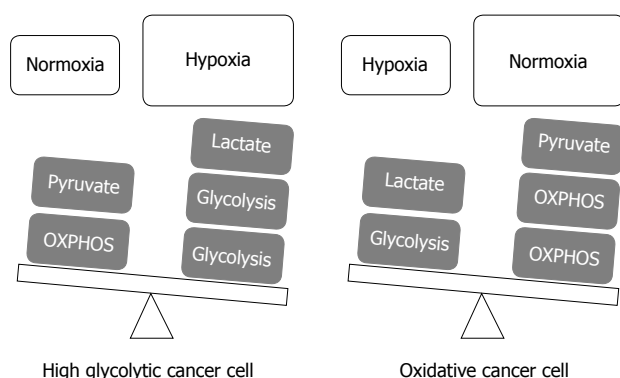


Figure 3 Metabolic flexibility of cancer cells depends on the tumor microenvironment: metabolic changes according to oxygen gradients. In hypoxic condition, glycolysis mainly contributes to produce ATPs with lowering reactive oxygen species production, thereby closely links with initiation of metastasis. In normoxic condition, mitochondrial oxidation more contributes for energy production than glycolysis. For survival, cancer cells adjust to dynamic changes between hypoxia and normoxia in tumor microenvironment. OXPHOS: Oxidative phosphorylation.

contribute to cancer cell survival and progression, the Warburg effect still plays a pivotal role in cancer cell metabolism and the initiation of metastasis in hypoxic conditions as previously discussed. Therefore, therapeutic strategies should focus on both targets, glycolysis and mitochondrial OXPHOS. Even though some studies reported that mitochondrial OXPHOS is also one of 3-BP targets^[185], 3-BP has been mainly demonstrated to be the most potent glycolytic inhibitor among various types of inhibitors. However, including our studies, the results of *in vivo* studies that have used human HCC cell lines did not exhibit complete remission, but showed only partial remission after 3-BP treatment^[18,19,43-70]. One reason why 3-BP did not completely suppress tumor growth might be the low efficiency to suppress mitochondrial OXPHOS, the lactate shuttle, and high redox potential in cancer cells. To overcome the weakness of glycolytic inhibitors, the inhibitors to target mitochondrial OXPHOS and other involved mechanisms such as the suppression of ROS production, might be effective when used simultaneously with glycolytic inhibitors as a combination treatment.

The metabolic interplay (lactate shuttle) between glycolytic and oxidative cells in tumors is modulated by two simple compartments for ATP production; glycolytic pathway and mitochondrial OXPHOS. Therefore, a combined treatment targeting both glycolysis and mitochondrial OXPHOS, could potentially be effective in suppression of tumor growth. Although various agents have been introduced to suppress mitochondrial metabolism, two agents, including metformin and glutamate dehydrogenase 1, were noticeable regarding their safety and potency. The metformin is widely used for Type II diabetes in practical fields and has anti-cancer effects in animal models. Previous studies demonstrated that metformin inhibits complex I *via* the inhibition of

ubiquinone reduction and independently stimulates ROS production by the complex I flavin^[186-188]. It might be more clinically meaningful in terms of tolerable safety for a long period as demonstrated in diabetic patients.

Another promising agent that targets mitochondrial metabolism is the mitochondrial enzyme glutamate dehydrogenase 1 (GDH1). Previous reports demonstrated that glutamine may be utilized as the energy fuel for mitochondrial OXPHOS in cancer cells^[189-192]. GDH1 is upregulated in human cancers and important for redox homeostasis by controlling the intracellular levels of its product alpha-ketoglutarate and subsequent metabolite fumarate, which subsequently activates glutathione peroxidase 1, *i.e.*, the antioxidant system. Targeting GDH1 by a small molecule inhibitor, the purpurin analog R162, resulted in an imbalanced redox homeostasis, which led to suppress tumor growth^[193].

As described above, the common characteristics of glycolytic inhibitors were to increase ROS levels in cancer cells. Thus, a further increase in ROS stress using exogenous ROS enhancers combined with glycolytic inhibitors might effectively increase ROS levels above the threshold stimulating cell death pathways. The vulnerability for ROS stress in normal and cancer cells is quite different. Normal cells have tolerability for a certain level of exogenous ROS stress because of their high antioxidant capacity for lowering the ROS level and thereby prevent to reach the cell-death threshold^[194,195]. In cancer cells, the increased ROS production from metabolic disarrangement and rapid proliferation may induce an upregulation of antioxidant capacity, *i.e.*, vulnerable redox equilibrium with high ROS production and elimination to maintain the ROS levels below the threshold for cell death^[196,197]. Thus, cancer cells would be more vulnerable to increased oxidative stress induced by exogenous ROS enhancers that directly or indirectly suppress the antioxidant system^[198-201]. These characteristics may provide a biochemical basis for a combination treatment of glycolytic inhibitors and ROS enhancers.

CONCLUSION

There have been a lot of advances to understand the importance of the Warburg effect and the metabolic interplay between glycolytic and oxidative cells in terms of lactate shuttle in previous decades. As previously discussed, the Warburg effect plays a vital role in cancer cell proliferation and survival in hypoxia, and also contributes to the initiation of tumor metastasis as matrix detachment. This effect is also highly linked to lowering the ROS levels to remain away from the cell death threshold. However, to survive under hypoxic conditions, a small portion of cancer cells and/or stromal cells should potentiate their mitochondrial OXPHOS rather than glycolysis to prevent a hostile acidic environment of lactate accumulation, which is the end product of the gly-

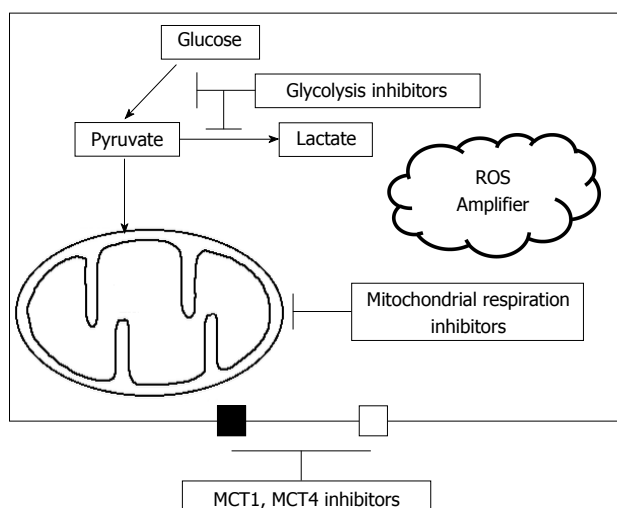


Figure 4 Potential therapeutic targets in metabolic interactions are suggested. A combination therapy might be promising; two targets that block the glycolysis and mitochondrial oxidative phosphorylation or block the glycolysis pathway and amplify ROS levels. Combination treatments targeting both the glycolysis and mitochondrial oxidation, or antioxidant systems can efficiently suppress energy production, or induce ROS-mediated apoptosis. ROS: Reactive oxygen species; MCT: Monocarboxylate transporters.

colytic pathway in hypoxic cancer cells. Thus, even in a hypoxic condition, oxidative cancer cells and/or stromal cells should theoretically exist to support the metabolic fuel for glycolytic cancer cells and handle their waste of lactate *via* the dynamic shuttle of lactate. Tumor heterogeneity exists in tumor mass under hypoxia, and metabolic flexibility is one adaptation mechanism to oxygen gradients (Figure 3).

Treatments against tumor metabolism may aim to target two distinct metabolic pathways of glycolysis and mitochondrial OXPHOS as a combination treatment (Figure 4). Although targeting one specific element of the tumor metabolism could often be ineffective because of the dynamic changes between glycolysis and mitochondrial OXPHOS, targeted treatments against glycolytic-oxidative cell interactions through the inhibition of glycolysis and mitochondrial metabolism might become a promising treatment for advanced stage HCC in clinical practice.

Given the unsatisfactory results of tyrosine kinase inhibitors in HCC treatment, a better understanding of the dynamic interactions between intracellular interactions, such as glycolysis and sensitivity of ROS amplification, and the intercellular interplay, such as glycolytic and mitochondrial OXPHOS cells in cancers, is critical to elucidate the heterogeneous biological features of HCC and identify effective strategies.

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Roles of the canonical myomiRs miR-1, -133 and -206 in cell development and disease

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Abstract

MicroRNAs are small non-coding RNAs that participate

in different biological processes, providing subtle combinational regulation of cellular pathways, often by regulating components of signalling pathways. Aberrant expression of miRNAs is an important factor in the development and progression of disease. The canonical myomiRs (miR-1, -133 and -206) are central to the development and health of mammalian skeletal and cardiac muscles, but new findings show they have regulatory roles in the development of other mammalian non-muscle tissues, including nerve, brain structures, adipose and some specialised immunological cells. Moreover, the deregulation of myomiR expression is associated with a variety of different cancers, where typically they have tumor suppressor functions, although examples of an oncogenic role illustrate their diverse function in different cell environments. This review examines the involvement of the related myomiRs at the crossroads between cell development/tissue regeneration/tissue inflammation responses, and cancer development.

Key words: Muscle microRNAs; MiR-1; MiR-206; MiR-133a; MiR-133b; Cell development; Cancer

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Core tip: The roles of the canonical muscle-associated microRNAs are reviewed, including microRNA families miR-1 and miR-133, and single miR-206, which are collectively known as the "myomiRs". The myomiRs act at the crossroads of the molecular regulation of muscle cells, linking between pathways for cell differentiation, development and maintenance, but also potentiate aberrant cell growth in numerous non-muscle cancers. Typically myomiRs are downregulated in cancers, but some myomiRs are upregulated in a few cancers, yet each dysregulation event advances tumor progression. The review examines normal and disease-linked molecular changes associated with the myomiRs, and collates the extensive literature into accessible tables.

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INTRODUCTION

MicroRNAs (miRs) are short single strand RNA molecules (typically 22 nt) which interact in a semi-complementary manner with numerous target gene mRNAs, directed by a short "seed sequence", destined the targeted mRNA for degradation or for translational inhibition, and thus an miR can downregulate the functional expression of the target gene. In this manner a single miR can influence the abundance of numerous independent gene targets, and aid in the co-ordinate regulation of members of diverse cell signalling pathways, as well as metabolic pathways and basic cell proliferation or developmental processes. Three miR families, miR-1, miR-133 and miR-206 constitute the original (canonical) myomiRs and were considered muscle specific because of their prevalence in skeletal and cardiac muscle^[1-5] and for their central roles in the regulation of myogenesis, muscle development and muscle remodelling^[6-8]. Although other muscle enriched miRs such as miR-499 and -208, and others with key roles in cardiac muscle development have been identified, and although the term "myomiR" is now often used to denote several miRs encoded within myosin genes, for brevity this review is restricted to discussion of the three canonical myomiRs.

In man the genes encoding the canonical myomiR are organized into three cistrons encoding partners (miR-1-2, miR-133a-1), (miR-1-1, miR-133a-2) and (miR-133b, miR-206) and are located on chromosomes 18q11.2, 20q13.33 and 6p12.2, respectively. In this review we examine the roles of the myomiRs in normal tissue development and their emerging functions in various non-muscle tissues and their influence on the progression of cancers. The dysregulation of expression of the myomiRs in cancers is often related to a significant worsening patient prognosis, *via* the deregulation of a variety of validated gene targets.

The two mature miR-1 isomers have identical sequence, as have the two miR-133a isomers. The mature miR-133 isomers are also highly similar, differing only at the 3'-terminal base, with miR-133a_{1/2} terminating G-3' and miR-133b with A-3', respectively. Independent upstream enhancers have been identified for the cistronic *miR-1-2 -133a-1* genes, as well as for the cistronic *miR-1-1/-133a-2* genes which are intronic to the *C20orf166* gene^[9]. These independent enhancers allow the different isomer genes to be independently expressed under cell specific regulation.

DIFFERENT ROLES OF MYOMIRS IN MUSCLE

MicroRNA-1 and -133 were initially identified during the development and differentiation of skeletal muscle^[7] and cardiac muscle^[2,6]. Both *miR-1/-133a* gene cistrons are canonically expressed in skeletal and cardiac muscle^[5,9], whilst the *miR-133b/-206* gene cluster is expressed in developing skeletal muscle^[5] but not (significantly) in cardiac muscle, defining seminal roles of miR-1 and miR-133a in muscle biogenesis, and specifically in cardiac biogenesis^[2,6]. A cartoon illustrating some of the major effects of myomiRs during differentiation of embryonic tissue and during tissue regeneration is shown in Figure 1.

MiR-133a has a regulatory role from the earliest differentiation of myogenic stem cells into myoblasts^[7,10] continuing throughout the growth of structurally complex muscle tissues^[7,11], and has homeostatic functions for muscle maintenance and protection in mature muscle, or in muscle regeneration from muscle progenitor cells after skeletal muscle stress or injury^[5]. Key studies show miR-1, -133b and -206 acting during early development of skeletal myocytes through to the homeostatic maintenance of skeletal muscle^[3,4,8], with miR-133b/-206 also having functions in neuromuscular synapse development and maintenance^[12], as detailed in Tables 1 and 2.

Others have noted that the canonical myomiRs act as balanced regulators, often specifying broadly opposing functions. The miRs-1 and -206 are semi-homologous with closely similar mature sequences (and identical seed sequences), and target some genes in common, as well as independent targets. The identical mature seed sequences of miRs-133a and -133b implies they would share many targets in common, yet each of these miRs appear to have distinct cellular functions, with miR-133a expression common to all muscle and miR-133b abundant in all muscle types, except cardiac muscle. Loosely, the cell signalling pathways targeted by miR-1/-206 tend to have opposing functions to the regulatory pathways targeted by miR-133a/-133b. Both miR-1/-206 act to promote myogenic differentiation, while the miR-133 isomers maintain the undifferentiated state and promote cell growth; hence co-expression of the myomiRs likely aids maintenance of homeostasis under normal cellular conditions.

This difference in expression of the related myomiR members in cardiac muscle compared to skeletal muscle may be associated with the physiological specialization of cardiac muscle, or its greater constancy of fibre type and function. In contrast, skeletal muscles constitute a variety of differentiated fibre types and are more plastic, capable of undergoing marked changes in myofibre content and physiology related to the level of use and workload^[1,3]. As understanding of the molecular

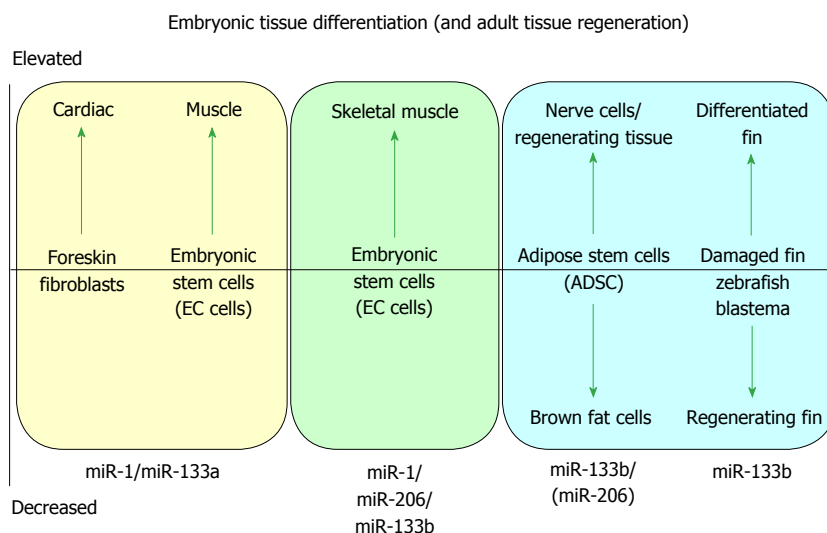


Figure 1 The roles of the myomiRs during embryonic tissue differentiation and adult tissue regeneration. Elevated levels of miR-1 and miR-133a are essential for differentiation of cardiac muscle^[10,15], whilst miR-1, miR-206 and miR-133b are required for skeletal muscle differentiation^[7,13]. Elevation of miR-133b levels in adipose stem cells leads to differentiation to a nerve-cell like fate^[7], whilst reduction in miR-133b leads to brown fat cell differentiation^[74]. Strong depletion of miR-133b and elevated Fgf allows regeneration of damaged zebrafish appendages^[67,69].

regulation of muscle types have deepened, it is clear that the physiological and functional specializations are also reflected in the functions of the myomiRs.

CARDIAC MYOGENESIS

Studies with mammalian stem cells reveal broad functions for the myomiRs in the definition of primary differentiation pathways. Both miR-133 and miR-1 have roles in early cell programs leading to differentiation of muscle^[2,10,13]. Pluripotent mammalian embryonic stem (ES) cells undertake cell fate decisions controlled by activation and repression of lineage-specific gene sets. These decisions are dictated by signalling networks which progressively narrow and specify the potential of ES cells as differentiation progresses. Muscle specific miR-133(a) and miR-1 both promote mesoderm formation from ES cells and suppress ectoderm and endoderm fates^[2], but later during further differentiation into cardiac muscle progenitors, these miRs appear to have opposing regulatory functions^[11,13]. Many non-muscle cell genes are repressed by miR-1 and miR-133 during this early ES cell differentiation program, suggesting that these two miRNAs may have general roles to regulate early ES cell-fate decisions from pluripotent cells^[13], with miR-1 specifically targeting the translational repression of Dll-1 and Cdk9^[10].

In vivo, the deletion of both *miR-133a1/2* genes causes lethal cardiac (ventricular-septal) abnormalities in about half of the mouse embryos or neonates, while mice deficient in only one of either miR-133a-1 or -133a-2 have phenotypically normal hearts^[14]. Skeletal muscles are normal in both double and single mutant miR-133a mice (dead and surviving), implying that miR-133b can replace the absent miR-133a species in

skeletal muscle and continue the regulation of normal development. In double mutant mice lacking all miR-133a, smooth muscle gene expression was activated 2–4 × and cardiomyocytes (but not cardio-fibroblasts) proliferated 2.5 × faster than normal, accompanied by increased expression of miR-133a targets, including PTBP2, CDC42, cell cycle control factors and cyclins D1, D2 and B1^[14]. Recently, both adult and neonatal human foreskin fibroblasts were found capable of being reprogrammed towards cardiac muscle by exogenous expression of only several factors, myocardin, HAND2, T-box-5, GATA4, and miR-1, miR-133a and miR-499^[15]. These stimulated cells expressed cardiac specific proteins and showed spontaneous contractility, emphasizing the role of these miRs in the control of specific cell development programs *via* the modulation of specific factor targets. Further, both human and mouse fibroblasts can be reprogrammed to form cardiomyocyte-like cells by overexpression of cardiac transcription factors (Gata4, Mef2c, and Tbx5 (GMT) or GMT plus Mesp1 and Myocd) along with miR-133a, which directly represses *Snai1* which normally regulates EMT processes^[16]. Interestingly, exogenous miR-133b can also downregulate *Snai1* expression, suppressing fibroblast genes and upregulate the expression of a number of characteristic cardiac cell genes *in vitro*, yet it cannot replace miR-133a during normal heart development *in vivo*.

CARDIAC MUSCLE INJURY

Heart contractility and heart rate are stimulated during chronic pressure overload by activation of the sympathetic nervous system causing catecholamine release. The catecholamines activate β-adrenergic receptors and overstimulation is a component of heart

disease. MiR-133 directly targets adenylate cyclase VI and the catalytic subunit of PKA, both elements of the β 1AR signal transduction cascade, reducing signalling^[17]. Similarly carvedilol, an *in vivo* β -adrenergic blocker, improves the cardiac function in infarcted rats by restoring miR-133 expression, resulting in reduced cardiomyocyte apoptosis^[18]. *In vitro* overexpression of miR-133a in cardiac cells has similar effects to carvedilol by downregulating caspase-9 and caspase-3 expression in the presence of H₂O₂. Overexpression of miR-133a also reduces ROS and malondialdehyde content, and increases SOD activity and GPx levels, protecting cardiomyocytes from apoptosis. Studies in mouse by Caré *et al.*^[6] also demonstrated that downregulation of both miR-133 and miR-1 are involved in cardiac hypertrophy. Specific targets of miR-133 such as RhoA, Cdc42 and Nelf-A/ WHSC2 can accumulate and contribute to the hypertrophy of cardiac myocytes during infarction.

SKELETAL MUSCLE MYOGENESIS

Liu *et al.*^[2,9] (2007, 2010) also established a fundamental model of differential expression of cisgenic *miR-1* and *miR-133a* genes during myogenesis and differentiation of skeletal muscle, smooth muscle and cardiac muscle. The factor MEF2 controls expression of the *miR-1-2/-133a-1* cisron *via* at least two MEF2 enhancer loci: one MEF2 enhancer located 3' upstream of the *miR-1-2* gene, a second intragenic MEF2 enhancer located upstream of the *miR-133a-1* gene and a third (less defined) locus far upstream that requires MyoD for expression^[19]. Transcripts of *pri-miR-1-2/-133a-1* (bi-cisron), *pri-miR-1* and *pri-miR-133a-1* genes indicate that both proximal enhancers are functional^[1]. Others have emphasized the distribution of these regulatory enhancers^[2] drawing attention to the differential expression of these cisgenic miRs which the regulatory factor binding sites provide. The regulation of expression of the cisgenic miRs by these key muscle development regulatory factors, which are themselves targets of repression by these self-same miRs, also emphasizes the precise inter-regulatory control of each of the various developmental program factors.

Notably, the 2,6-disubstituted purine reversine can induce differentiation reversal (de-differentiation) of C2C12 murine myoblast cells back into multipotent progenitor cells^[20]. This occurs by inhibition of Aurora A and B protein kinases, reducing histone H3 phosphorylation, which in turn induces chromatin remodelling and restores cell multipotency. Reversine treatment also stimulates expression of polycomb genes, *Phc1* and *Ezh2*, leading to inhibition of expression of the muscle-specific transcription factors, myogenin, MyoD, and Myf5^[21]. Concomitantly, reversine strongly inhibits miR-133a expression in C2C12 cells through the reduced expression of SRF

transcription factor and by reduction of its binding to the miR-133a enhancer and by reduced epigenetic histone modifications on the miR-133a promoter, including reduced trimethylation, phosphorylation, and acetylation^[22]. The co-overexpression of a miR-133a mimic along with reversine treatment prevents C2C12 myoblast de-differentiation, indicating the central role of the inhibition of miR-133a expression to the de-differentiation process. Significantly, reversine induced de-differentiation of committed cells is not limited to myoblasts, and reversine treatment can transform primary murine dermal fibroblasts into myogenic-competent cells within regenerating muscle *in vivo*^[23].

Skeletal muscle myogenesis also involves the IGF signalling pathway^[24], which activates muscle proliferation and differentiation *via* the PI3K/AKT pathway. The IGF pathway is regulated by miR-133-a1 which directly inhibits translation of IGF-1R protein, resulting in repression of PI3K/AKT pathway activity. IGF-1, which increases and activates IGF-1R during myogenesis by binding and inducing its phosphorylation, also indirectly activates myogenin, which in turn activates miR-133 activity. Thus miR-133 provides a negative regulation loop to monitor and control PI3K/AKT pathway activity. Similarly, miR-1 targets and reduces the activity of IGF-1 in differentiating C2C12 skeletal muscle cells and in heart muscle during cardiac failure states^[25], meanwhile active IGF-1 signalling pathway downregulates miR-1 *via* repression of FoxO3a transcription factor. Thus, miR-1 also mediates the activity of the IGF-1 signal pathway and is itself feed-back regulated by the IGF-1 signal transduction cascade. Significantly, IGF-1 signalling (IGF-1 and IGF-1R) has key roles in the growth and development of many tissues^[26], and also in the progression of many cancers (see later).

SKELETAL MUSCLE PLASTICITY

Skeletal muscles are plastic tissues in which the ratios of muscle fibre type (slow or fast twitch, smooth muscle, etc.) are to some degree responsive to remodelling through environmental input and nerve control. The muscle fibre type is maintained by the type of nerve signals received by the muscle, and transition between fast and slow twitch fibres can occur over time if nerve signals are changed from slow to fast type, and *vice versa*^[27]. Similarly, prolonged workload or exercise can alter muscle fibre type and its metabolism to allow it to better respond before exhaustion. Muscle development programs regulated by miR-1 and miR-133a play important roles in muscle remodelling^[4], and in hypertrophic skeletal muscle miR-1 and -133 levels are decreased^[28], indicating that functional overload of muscle induces regulatory alterations which are in part influenced *via* altered miR activities.

REGULATORS OF MYOMIR EXPRESSION

Myostatin is a repressor of myogenesis, and its down-regulation allows increase of miR-1, -133a, -133b and -206 expression, activating muscle cell proliferation^[29]. In contrast, myogenic factors myogenin and MyoD are well known positive regulators of myomiR expression in muscle that bind upstream of *miR-1/-133a* genes at defined enhancer regions^[2,11]. SRF, MyoD and MEF2 are also direct transcriptional activators of myogenesis-related miR-1 expression in cardiac muscle^[2,30]. Since downregulation of myostatin permits expression of miR-133b/-206, and MyoD and myogenin also binds the miR-206 promoter^[11], suggesting that miR-206/-133b expression in muscle may also be in part controlled by MyoD/ myogenin.

The ERK1/2 signalling pathway also regulates expression of miR-133 during myogenesis in the C2C12 cell model^[31], and its activity is also feedback influenced by miR-133. During myogenesis both miR-133a and -133b are upregulated, and both FGFR1 and PP2AC which function in the ERK1/2 pathway signal transduction are negatively regulated post-transcriptionally by both miRs. Inhibition of ERK1/2 pathway signalling inhibits C2C12 cell proliferation, stimulating initiation of differentiation and forming small truncated myotubes. Importantly, ERK1/2 signalling pathway activity negatively regulates expression of miR-133, providing a feedback loop between miR-133 levels and ERK1/2 signalling activity, forming an additional reciprocal mechanism for regulating myogenesis.

Recently other cellular factors have been identified that influence post-transcriptional maturation or bio-availability of myomiRs in muscle. mTOR regulates miR-1 indirectly in regenerating mouse skeletal muscle and differentiating myoblasts^[32]. mTOR most likely affects MyoD protein stability, which then alters miR-1 expression through the availability of MyoD to bind its upstream enhancer. A pathway downstream of mTOR also operates in which miR-1 suppression of HDAC4 results in production of follistatin, which subsequently activates myocyte fusion. This suggests that an mTOR-miR-1-HDAC4-follistatin pathway regulates myocyte fusion during myoblast differentiation and in regenerating skeletal muscle.

King *et al*^[33] (2014) demonstrated that the RNA-binding TDP-43 protein interacts with miR-1/-206 family (but not the miR-133 family) in skeletal myoblast cells, limiting their bioavailability by preventing interaction with the RISC silencing complex, noting this is the first observation of a mechanism differentiating between mature bicistronically encoded miRs, which selectively modulates the bioactivity of downstream targets of the sequestered miRs. TDP-43 accumulates in motor neurons during ALS, a neuromuscular wasting disease. Two miR-1/-206 targets, IGF-1 and HDAC-4 are elevated in both ALS-model transgenic mouse muscle

and in cells modified to overexpress TDP-43. The authors suggest the decreased miR-1 (-206) activity in ALS affected muscle could alter retrograde signalling at the NMJ through the dysregulation of both HDAC-4 and MEF-2, whereby miR-1 refines synaptic function by coupling changes in muscle activity to changes in presynaptic function^[34].

Factors KSRP^[35], MBNL1 and RNA binding protein LIN28^[36] also positively and negatively regulate miR-1 biogenesis respectively. Additionally, miR-206 binds to 3'-UTR sites of KSRP transcript to inhibit KSRP expression in skeletal muscle^[37]. Independently, miR-206 and KSRP are negative regulators of utrophin A, but unexpectedly, overexpression of miR-206 in both normal and dystrophic muscle cells promotes upregulation of utrophin A, *via* the downregulation of KSRP. Thus, miR-206 appears capable of switching between direct repression of utrophin A expression and the activation of its expression through decreased KSRP, the two molecular mechanisms providing close counter-regulation of utrophin A expression.

CISTRON MIR-206 AND MIR-133B

Although the microRNA-206 and -133b are thought typical of muscle specific miRs, little is known explicitly of their functions in skeletal muscle. Cesana *et al*^[38] (2011) showed that *miR-133b* gene transcript in mouse is located within the precursor of the long (spliced) non-coding RNA linc-MD1 which is expressed under the control of an upstream distal (DIST) cistronic promoter^[12] (Figure 2A), while the *miR-206* gene, which is located within the intron of linc-MD1, is transcribed autonomously under control of its own proximal (PROX) promoter. In proliferating myoblasts, only primary miR-206 transcript is expressed strongly, initiated from the PROX promoter. During mouse muscle differentiation, long distance interactions bring the DIST promoter into conjunction with PROX and the polyA addition regions of linc-MD1, facilitating the co-expression of linc-MD1 RNA, as well as the primary miR-206 transcript, with the PROX promoter activated by both MyoD and myogenin binding^[11]. Notably, mature linc-MD1 RNA contains binding sites for miR-133 (and miR-135) acting as a binding "sponge" to downregulate their free abundance, in turn contributing to the expression regulation of the targets of these miRs, which include key muscle transcription factors^[38]. In mouse muscle, the expression of mature linc-MD1 RNA mutually excludes the expression of miR-133b, which must be excised from the linc-MD1 pre-transcript. In the rat genome, a distinct ncRNA 7H4, covering less than half of the mouse linc-MD1 precursor transcript, but closely similar to a mouse RNA AK132542 transcript, also encodes the *miR-133b* gene, suggesting a similar function to linc-MD1 may occur in rat^[39].

Figure 2A uses information from Cesana *et al*^[38]

[illegible]

B

Genomic map of Human chromosome 9 showing the location of miR-133b and miR-206. The map includes tracks for Human miRs, Human lnc RNA RP11-771D21.2/ Hs.582788, Human homology to rat nc 7H4 RNA, and Human homology to mouse linc-MD1. A scale bar at the bottom indicates positions from 52008000 to 52018000. A legend identifies symbols for Exon, Intron, Homologous region, Promoter, and E-box enhancer, MyoD/myogenin binding sites.



(2011) and Rosenberg *et al.*^[40] (2006) to illustrate the aligned transcript regions of the mouse pri-linc-MD-1, the 5.3 kb random cloned mouse transcript cDNA (GenBank sequence) AK132542 and the 5.2 kb rat 7H4 ncRNA transcript. The independent pre-miR-206 transcript overlaps almost completely with the AK132542 transcript which is likely the mouse homolog of the expressed rat ncRNA 7H4. The 7H4 RNA gene is almost fully conserved in the mouse genome, with the 7H4 RNA overlapping the 3' exon of *linc-MD1* gene (containing *miR-133b* gene) almost to the 3' terminus of the *miR-206* gene. Velleca *et al.*^[39] (1994) found two major transcripts of 7H4 RNA, a long 5.2 kb molecule, and a short 1.6 kb molecule coincident with the 3'-terminal region of the 5.2 kb transcript. The 1.6 kb fragment is much more abundant than the full length molecule, suggesting it is a product from the excision of *miR-133b* from the full length primary transcript. Notably, both long and short ncRNA 7H4 transcripts contain the entire *miR-133b* gene locus, suggesting they may function in rat similar to mature linc-MD1 in mouse, to bind complementary miRNAs, including *miR-133*. Similarly in man, ncRNA RP11-771D21.2 may represent the functional human homologue of mouse linc-MD1 RNA (Figure 2B).

Recently Legnini *et al.*^[41] (2014) reported that the mutually alternative synthesis of linc-MD1 and *miR-133b* is controlled by the pleiotropic mRNA regulator protein, HuR. In developing skeletal muscle, HuR favors accumulation of mature linc-MD1 by binding to it and repressing cleavage that would release pre-miR-133b. The level of HuR protein expression is also under the repressive negative control by *miR-133* targetting, yet the sponging-up of *miR-133* by the linc-MD1 helps consolidate HuR expression by forward positive control. Muscle developmental progression to later differentiation stages may involve overcoming this HuR-lincMD1 repression of *miR-133b* expression by the independent *miR-133a1/2* isomers which could downregulate HuR expression, allowing *miR-133b* excision, permitting developing muscle to exit from the control circuit. The level of linc-MD1 correlates inversely with the level of *miR-135/-133b*, which in turn control the expression of transcription factors MAML1 and MEF2C which are necessary for specific muscle gene expression. Thus, linc-MD1 activity provides another mechanism for pleiotropic regulation, slowing or activating muscle differentiation. Other evidence suggests the influence of HuR on many gene mRNA transcripts depends on the interplay of HuR with particular regulatory miRs that target and control the expression of the self-same mRNAs.

In sum, because the myomiRs target components of key signalling pathways and processes that control muscle cell development and maintenance, expression of the myomiRs is tightly regulated, often *via* feedback and feedforward circuits that provide both tight regulatory control and the ability to amplify myomiR

expression. In muscle the myomiRs display high interconnectedness in terms of the regulation of their expression and the complementary processes that their regulatory functions influence. *MyomiR* genes are apparently cistronically encoded, yet expression of each of *myomiR* genes can be individually controlled by various transcription regulatory factors and other interactions such as RNA-RNA binding, such that expression of particular *myomiR* genes can be selectively enhanced under cellular conditions in which particular transcriptional regulatory factors are available.

ESTABLISHMENT OF NEUROMUSCULAR JUNCTIONS

MicroRNA *miR-206* is expressed (virtually) exclusively in (developing) skeletal muscle^[8], contributing to muscle differentiation programs through repression of *Idl-3* protein expression, a downregulator of MyoD activity, as well as repressing the p180 subunit of DNA polymerase alpha, essential for DNA synthesis which occurs during differentiation^[42]. MyoD itself promotes the expression of the *miR-133* cistrons^[11]. Further, in fast twitch muscle of mouse^[12] and rat^[43] *miR-206* has been found to promote formation of new neuromuscular junctions following peripheral nerve denervation (scission). The expression of *miR-206* and *miR-133b* are both upregulated strongly in muscle after denervation (as is 7H4 ncRNA), whereas *miR-1* and *miR-133a* are downregulated. Four months after nerve scission, the re-innervated muscle was predominantly type II glycolytic fibres, suggesting that *miR-206* may aid the determination of fibre type *via* down-regulation of MEF2 transcription factor activity^[43]. Valdez *et al.*^[44] (2014) also examined the role of *miR-133b* and *miR-206* on neuromuscular junction repair in injured mice. In *miR-206* null mice, re-innervation was impaired following nerve injury, and in mice null for *-133b* and *-206* genes the same impaired neuromuscular repair was seen as in single gene *miR-206* null mice, whilst in single gene *miR-133b* null mice development and re-innervation proceeds normally following nerve injury. Together, these findings imply that *miR-206* is the major regulator of nerve repair and reconnection to muscle following injury. In support, in *miR-133b* null mice *Pitx3* levels were normal and impairment of locomotion was not detectable, controversially implying that *miR-133b* has no significant roles in neuron development, neuron maintenance and function *in vivo*^[45]. In contrast, other studies with *miR-206* null mice show no obvious phenotypic effects, muscles develop normally and mouse physiology appears normal, suggesting that other factors (including *miR-133b*) can replace *miR-206* during development^[46]. However, if the *miR-206* null mice are then denervated, about 90% of both wt and *miR-206* null mice recover and re-innervate after about

8 wk. This strongly suggests that other factors (including miR-133b) can provide redundant functions for the absent miR-206, including promoting compensatory peripheral nerve regeneration. Furthermore, miR-133b directly stimulates neurite outgrowth following nerve damage in rat brain after treatment with multipotent MSC cells^[47], suggesting that elevation of levels of muscle miR-133b after muscle denervation is related to nerve regeneration, and that miR-133b may suffice in miR-206 null mice to replace absent functions. These various observations imply the likelihood that both miR-206 and -133b have functions in the recovery and maintenance of nerve-muscle signalling.

RETROGRADE SIGNALLING BETWEEN MUSCLE AND NERVE

Additionally, miR-206 targets BDNF which promotes efficient skeletal muscle regeneration following damage^[48]. BDNF also controls the initiation and maintenance of the differentiated state of muscle cells, potentially *via* the regulation of retrograde signalling at the neuromuscular junction. The loss of neural input to muscle also causes HDAC4 to accumulate, reducing MEF2-regulated gene expression. Importantly, miR-206 targets HDAC4 and fibroblast growth factor signalling pathways in muscle. HDAC4 regulates neuromuscular-related gene expression and acts in the regulation of muscle remodelling, influencing the formation of appropriate nerve types which connect to the muscle^[49]. Significantly, it has been shown that expression of miR-1/miR-133a is also regulated by an intragenic MEF2-enhancer^[9], and miR-1 also regulates a MEF-2 dependent retrograde signal at the neuromuscular junction, suggesting that members of both myomiR cistons act to maintain neuromuscular homeostasis^[34].

In mouse, members of the MyoD muscle transcription factor family, myf-4 and myogenin, are progressively downregulated during maturation from embryonic day 15 to the first postnatal weeks (weeks 1-3), coinciding with induction of muscle innervation^[50,51]. In contrast, muscle denervation results in strong expression of MyoD and myogenin, preceding the accumulation of nAChR, α -subunit^[39]. Additionally during myogenic differentiation, acetylcholinesterase transcript levels increase dramatically (5 ×), principally due to its stabilization by binding with HuR protein^[52], consistent with a regulatory role of HuR in neuron excitability. Normally the expression of MyoD and myogenin is suppressed by activated nerve signal pathways, including by electrical conduction *per se*, and sets of muscle genes regulated by the MyoD family and myogenin are downregulated by increasing electrical activity and other nerve-derived signals. Thus again, a pronounced neuromuscular maintenance function for miR-206/-133b can be implied from interplay of signalling control between skeletal muscle and nerve.

Both myogenin and MyoD induce the expression of miR-133b and -206, while repression of these factors inhibits their expression. On balance it appears that cistronic miR-206 and -133b and linc-MD1 homologues may contribute to programs of regulatory developmental gene expression in growing muscle and peripheral nerve, facilitating programs to interregulate the developing nerve connections with muscle, and speculatively aid in coordinating appropriate nerve and muscle gene expression programs, establishing interactions between skeletal muscles and their appropriate innervating nerves to maintain muscle fibre type and their correct neuromuscular junction associations.

MUSCLE MITOCHONDRIAL FUNCTION

Zhang *et al.*^[53] (2014) reported that miR-1 enters skeletal muscle mitochondria efficiently during muscle development whereby it stimulates the translation of specific mitochondrial genome-encoded transcripts, contributing positive regulation to muscle development. This stimulation of translation requires specific base-pairing between miR and its target mtRNA as well as interactions with mt-located Ago2 protein. These observations contrast earlier findings of Das *et al.*^[54] (2012) who showed that the mature miR-181c translocates into rat cardiac muscle mitochondria, reducing mitochondrial cytochrome oxygenase 1 (mt-COX1) compared to mt-COX2 and mt-COX3 proteins. The reduced mt-COX1 causes mitochondrial complex IV remodelling, resulting in increased mt respiration and increased ROS generation. Recently, Das *et al.*^[55] (2014) used cationic nanoparticles to deliver miR-181c into rat cardiac mitochondria *in vivo*, causing cardiac dysfunction and a tendency to develop heart failure. Taken together, these studies reveal important new miR-mediated regulatory pathways in muscle mitochondria involving direct manipulation of mitochondrial gene expression by cytosolic miRNAs, including by a myomiR.

Importantly in both cardiac and skeletal muscle, mitochondrial UPC2/UCP3 uncoupling proteins regulate energy homeostasis and the rate of development and differentiation, with UPC2 repressing differentiation and promoting cell proliferation. However, MyoD activates miR-133a expression which in turn directly downregulates UCP2 mRNA to alleviate the developmental repression, suggesting a feedback network involving MyoD-miR-133a-UCP2^[56]. Additionally, overexpression of myogenin and MyoD in mouse C2C12 myoblasts^[57] increase expression strongly from the UCP3 promoter, but act weakly at the UCP2 promoter. Together these observations suggest UCPs helps maintain balance between muscle differentiation and proliferation during myogenesis, regulated by a MyoD-miR-133a-UCP2 feedback network and by differential responsiveness of UCP2 and UCP3 promoters to activation by myogenin and MyoD.

Furthermore, a downregulation of mitochondrial function is associated with skeletal muscle injury, including increased ROS and reduced cellular ATP generation. However, the recovery and regeneration of post-injury skeletal muscle involves the activation and proliferation of resident stem cells, including satellite cells and endothelial precursor cells followed by their differentiation into myocytes. Jash *et al.*^[58] (2014) showed during recovery from muscle injury that the AMPK-CRTC2-CREB and Raptor-mTORC-4EBP1 pathways are activated in satellite cells. mTORC1 positively regulated Ccnd1 translation, yet destabilized Ccnd1 mRNA. These opposing effects of mTORC1 were mediated by two miRs which target the 3'-UTR of Ccnd1 mRNA: one being miR-1 which in mTORC-knockdown muscle was downregulated, allowing Ccnd1 mRNA to accumulate. The authors suggest that mTORC may act to coordinate satellite cell proliferation during the activation of myogenesis.

MUSCLE DEGENERATION AND INFLAMMATION

During conditions of skeletal muscle atrophy and wasting, the cytokine TWEAK and its binding receptor Fn14 are elevated, activating catabolic and pro-inflammatory processes^[59]. TWEAK inhibits expression of myoD, MEF2C and myogenin which in turn inhibits expression of miR-1, -133, and -206, suppressing differentiation of progenitor cells into myocytes. HMOX1, another factor associated with inflammation^[60], also inhibits myoblast differentiation and myotube formation, by inhibition of expression of each of the myomiRs, again by limiting their transcription factors, MyoD and myogenin. Thus, both HMOX1 and TWEAK may be potentially involved in the regulation of broadly common inflammation associated pathways in skeletal muscle.

Interestingly, TWEAK^[61] has a role in stimulating the proliferation of normal neonatal rat cardiomyocytes, increasing cell numbers accompanied by expression of cell proliferation markers Cyclin D2 and Ki67, and other cell cycle factors. In contrast, adult rat cardiomyocytes cannot be stimulated by TWEAK because of the developmental downregulation of its receptor Fn14 in adult cells, coincident with the loss of proliferation capacity in mammalian cardiomyocytes several weeks after birth. Fn14 is present in neonatal cardiomyocytes, interacting with TWEAK to activate downstream signalling *via* ERK and PI3K signalling pathways, as well as *via* inhibiting glycogen synthase kinase-3 β .

FAPs are quiescent progenitor cells resident in normal muscle that can facilitate myofibre regeneration after muscle damage by providing factors which stimulate proliferating myogenic progenitor cells. In dystrophic muscle disease, FAPs typically proliferate and give rise to their differentiated progeny, fibroblasts and adipocytes which replace muscle tissue. However, Saccone *et al.*^[62] (2014) found in

early-stage disease dystrophic (mdx) mouse muscles that HDAC inhibitors can activate and commit FAPs themselves towards regeneration of muscle tissue, by derepressing a "latent" myogenic program. The inhibition of HDAC induces two core components of the myogenic transcriptional machinery, MyoD and BAF60C, which upregulate expression of miR-1-2, miR-133, and miR-206. The structural subunits of the BAF chromatin remodelling complex (BAF60a, BAF60b and BAF60c) bind to Brg1 (the core complex ATPase) and provide functional specificity^[63]. BAF60c is a specific member of the complex during myogenesis, and is essential for the myogenesis process. Interestingly, BAF60a and BAF60b are targets for downregulation by miR-133 and miR-1/206, suggesting that such negative regulation increases the availability of (non-target) BAF60c to join the muscle remodelling complex. Furthermore, a recent study of inflammatory myopathies^[64] including dermatomyositis, polymyositis, and inclusion body myositis found increased expression of the inflammatory cytokine TNF α was associated with decreased expression of miR-1, miR-133a, and miR-133b in all subtypes, plus the decreased expression of miR-206 in dermatomyositis. TNF α inhibited the expression of myogenic miRNAs in cells in an NF- κ B-dependent manner, while the overexpression of miR-1, miR-206, or miR-133a/b could relieve the TNF α blockage of myogenic cell differentiation. Overall, the dysregulation of myomiR expression in muscle degenerative diseases was demonstrated to be intrinsic to the disease progression.

In diseased cardiac tissues, pre-inflammatory reactions involve upregulation of *CNN* genes, and *in vitro* the downregulation of *CNN2* blocks multiple proinflammatory and profibrotic pathways in mouse activated primary cardiac fibroblasts (PCFBs)^[65]. Immune cell chemotaxis towards *CCN2*-depleted PCFBs is also reduced strongly. *CCN2* is a direct regulation target of miR-133b, and silencing of *CCN2* expression by siRNA strongly decreases the expression of stretch-induced chemokines, matrix metalloproteinases, extracellular matrix and a cell-to-cell contact protein, indicating that *CCN2* is involved in control of multiple signal pathways for muscle regeneration. Exogenous factors also influence muscle recovery after injury. PRP plasma is an enriched source of autologous platelet α -granule-derived growth factors and cytokines which can stimulate tissue healing^[66]. When PRP is applied to injured rat soleus muscle, the recruitment, proliferation and differentiation of cells for muscle recovery is stimulated. Molecular analysis showed that 5 d after PRP treatment the expression of proinflammatory cytokines IL-1 β , and TGF- β 1 was increased strongly, which in turn induced expression of myogenic factors MyoD1, Myf5 and Pax7, and muscle IGF-1Eb isoform, and muscle recovery was strongly accelerated. Concomitantly miR-133a and miR-1 were downregulated (miR-133a markedly), while SRF was

upregulated, phosphorylated α B-crystallin was increased, as were apoptotic factors (NF- κ B-p65 and caspase 3) which together indicate enhanced cell survival. Overall, PRP contributes to repair of injured skeletal muscle by *via* the control of secondary pathways (regulated by myomiRs and heat shock proteins) that modulate both inflammatory and myogenic pathways, with each contributing to the regulated tissue regeneration.

ROLES OF THE MYOMIRS IN OTHER TISSUES

Limb regeneration in lower vertebrates

Whilst miR-133 plays a central role is the repair of damaged muscle and nerves in mammals, in lower vertebrates such as amphibians and teleost fish which retain the capacity for regeneration of entire limbs after damage or loss, here the downregulation of miR-133 plays a central role in organizing the reactivation of cells for the repair of complex tissues. Yin and Poss^[67] (2008) found that miR-133 controls complex biological processes involving formation of the regeneration blastema, a proliferative mesenchymal cell mass which is the progenitor for regeneration of the lost structures, ultimately developing into organized organs, including connective tissues, muscle, blood vessels and nerve tissues^[67]. When zebrafish fins are excised, the depletion of miR-133 is controlled by increased Fgf signalling (Tables 1 and 2). In normal developed fins, high levels of miR-133 are maintained, accompanied by a cessation of Fgf signalling, indicating that high miR-133 levels normally suppresses tissue proliferation factors and signalling pathways, maintaining developed tissue homeostasis^[68]. Increased miR-133b also influences spinal cord regeneration in adult zebrafish, reducing the level of RhoA protein, an inhibitor of axonal growth, and stimulating spinal cord regeneration of axons from neurons in particular brain structures^[69].

MAMMALS

Corneal repair

In mouse cornea after laser ablation injury^[70], miR-133b is the most strongly reduced miR (amongst others) during wound recovery, allowing increased expression of its targets which include CTGF growth factor, SMA, and COL1A1. Transforming growth factor β 1-treated rabbit corneal fibroblasts also produced significant decrease in miR-133b, associated with significantly increased expression of CTGF, SMA, and COL1A1, and helped to minimise scar development during corneal recovery. See Table 2 for other tissues.

NERVE CELL DIFFERENTIATION

The miR-133b has roles in early stem cell differentiation leading to nerve development. ADSC stem

cells can be induced to differentiate into neuron-like cells by IGF- I signalling, which increases miR-133b expression *via* the downregulation of beta-III-tubulin, Pitx3 and IGF-IR by translational repression of their proteins^[71]. Neural differentiation from ADSC involves a feedback control mechanism in which IGF-I upregulates miR-133b, while miR-133b in turn downregulates the signal receptor activity, IGF-IR. Xin *et al.*^[47,72] found multipotent MSC cells regulate the growth of neurites *via* direct exosomal transfer of miR-133b to neural cells. Middle cerebral artery occluded rat brains elevate miR-133b in MSC exosomes, which stimulates neural regeneration. Exosomes transfer to adjacent astrocytes and neurons, reducing the expression of selected miR-133b targets, including CTGF and RhoA^[72]. The first identified molecular role for miR-133b was in neural tissue^[73] where it regulated the maturation of mammalian midbrain dopaminergic neurons (DNs), along with the paired-like homeodomain transcription factor Pitx3, which itself then regulated the transcription of miR-133b in a feedback control loop.

ADIPOCYTE DIFFERENTIATION

Several studies found that miR-133 a/b isomers play key roles in the differentiation of brown fat tissues from precursor cells after cold exposure^[74-76]. Strong reduction of the transcription regulator MEF2 caused reduction of miR-133a, allowing an increase in the adipocyte progenitor specific Prdm16 (a miR-133 target), which promotes the differentiation of both myogenic precursor cells and white fat precursor cells into brown adipocytes. Adult mouse skeletal muscle satellite cells can differentiate into brown adipose *via* miRNA-133 targeting of Prdm16, leading researchers to suggest that the presence of these miRs may indicate energy dissipating cell lineages (muscle, nerve), compared to energy storing cells, such as white adipose tissue^[76]. Mice with knockdown of *miR-133a1/a2* genes respond to cold exposure more strongly than wild-type animals and have increased insulin sensitivity and glucose tolerance associated with activation of the brown fat and thermogenic gene programs in subcutaneous white adipose tissue.

CHONDROCYTE DIFFERENTIATION

MiR-1 is highly expressed in the hypertrophic zone of growth plate cartilage, some 8-fold higher than in the proliferation zone^[77]. MiR-1 strongly promotes chondrocyte proliferation and differentiation, including induction of the expression of chondrocyte markers Indian hedgehog and Col X, and acts by targetting HDAC4. Additionally HDAC4 negatively regulates chondrocyte hypertrophy by inhibiting Runx2, a critical transcription factor for chondrocyte hypertrophy. In contrast, miR-1 is repressed strongly during hypertrophic

Table 1 Roles and targets of the myomiRs, miR-1, -206, -133a, -133b

Factor(s)	Regulation	Regulator	Tissue/cell	Ref.
Fish and lower vertebrates: Development and regeneration				
Ttk protein kinase (mps1)	Upregulated mps1: a target of miR-133	Downregulation of miR-133 by Fgf	Regeneration of Zebrafish caudal fin (appendage)	[68]
RhoA	Downregulation of RhoA mRNA	Upregulation of miR-133b expression	Regenerating adult zebrafish spinal cord, axon outgrowth	[69]
RhoA	Downregulation of RhoA protein	Upregulation of miR-1 and miR-133 expression	Zebrafish muscle gene expression and regulation of sarcomeric actin organization	[166]
Cell cycle factors mps1, cdc37 and PA2G4, and cell junction components cx43 and cldn5	Upregulated mps1, cdc37, PA2G4, cx43, cldn5	Downregulated miR-133(a1) stimulates cardiac cell regeneration	Regenerating zebrafish cardiac muscle	[167]
miR-133b	MiR-133b found in developing somites, little in CNS tissues		Whole zebrafish embryos - normal development	[168]
SRF activates muscle specific genes and miRs; HDAC4 represses muscle gene expression	MiR-1 targets HDAC4, promoting myogenesis	In contrast, miR-133a represses SRF, enhancing myoblast proliferation	<i>X. laevis</i> embryos: skeletal muscle proliferation and differentiation in cultured myoblasts <i>in vitro</i> and in embryos <i>in vivo</i>	[7]
nAChR subunits UNC-29, UCR-63; MEF2	Subunits UNC-29, UCR-63, and MEF2 downregulated	miR-1 upregulated	<i>C. elegans</i> muscle at the neuromuscular junction	[34]
Mammalian pluripotent cells				
Muscle-specific microRNAs: miR-1 and miR-133a	MiR-1 and miR-133a have opposing functions during differentiation of progenitor cardiac muscles	Muscle-specific microRNAs, miR-1 and miR-133(a) upregulated	Promotion of mesoderm formation from mouse ES cells	[13]
Notch signalling, promotes neural differentiation and inhibits muscle differentiation; opposes miR-1 effects	Dll-1 translationally repressed	miR-1 upregulation, promotes cardiomyocyte differentiation	Mouse and human ES cell differentiation into muscle	[13]
SRF ^{-/-} EBs reflecting the loss of hematopoietic lineages in the absence of SRF	Early endoderm markers, Afp and Hnf4a: strongly down regulated	Increased miR-1 and miR-133a relieve the block on mesodermal differentiation	Mouse endoderm	[13]
Blood cell -specific genes, such as Cd53, CxCl4, and Thbs1, dramatically down regulated	Cd53, CxCl4, and Thbs1 expression was reinitiated by reintroduction of miR-1 or miR-133			
mES(miR-1)- and mES(miR-133a)-EBs compared to in control EBs	Nodal stimulated expression of endoderm markers Afp and Hnf4a in control EBs. Dramatically lower levels in mES(miR-1)- and mES(miR-133a)- EBs	miR-1 or miR-133 can each function as potent repressors of endoderm gene expression	mES cells, that lack either miR-1 or miR-133(a) during differentiation into EBs	[13]
IGF-1	IGF-1 signalling and miR-133 co-regulate myoblast differentiation <i>via</i> a feedback loop	IGF-1 upregulates miR-133; miR-133 downregulates IGF-1R	Myogenic differentiation of C2C12 myoblasts; Mouse during development from embryonic to mature skeletal muscle	[24]
IGF-1R				
IGF-1	IGF-1 signalling and miR-1 coregulate differentiation of myoblasts <i>via</i> a feedback loop	IGF-1 signalling downregulates miR-1 by repression of FoxO3a; miR-1 down-regulates IGF-1	Differentiating C2C12 myoblasts	[25]
Reversine [2-(4-morpholinoanilino)-N6-cyclohexyladenine]	Decrease in active histone modifications; including trimethylation of histone H3K4/H3K36, phosphorylation of H3S10; Stimulates expression of polycomb genes Phc1 and Ezh2	miR-133a expression strongly inhibited by reversine; reduced acetylation of H3K14 at miR-133a promoter	Reversine dedifferentiates murine C2C12 myoblasts back into multipotent progenitor cells, <i>via</i> extensive epigenetic modification of histones resulting in chromatin remodelling, and altered gene expression	[20-23]
FZD7 and FRS2	miR-1 promotes cardiac differentiation; miR-1 targets FZD7 and FRS2	Activation of WNT and signalling cause MCPs differentiation into cardiomyocytes	Mouse and human ES cells	[169]
miR-206/133b cluster	PAX7 gene expression unchanged; miR-206/133b cluster is not required for development, and survival of skeletal muscle cells	miR-206/133b cistron knock-out mice cells	Muscle satellite cell differentiation <i>in vitro</i>	[170]
Differentiating skeletal muscle				
DNA polymerase alpha	Repression of Idl-3 protein expression Repression of p180 subunit of DNA polymerase alpha	miR-206 up-regulated	Mouse skeletal muscle differentiation	[42]
MEF2 transcription factor	MEF2 activates of miR-1-2 and 133a-1 transcription; binds muscle-specific enhancer	Bicistronic primary transcript of miR-1-2 and 133a-1	Development of mammalian skeletal muscle	[9]

MRFs, Myf5, MyoD, Myogenin and MRF4	Myf5 essential for miR-1 and miR-206 expression during skeletal muscle myogenesis	Forced expression of MRFs in neural tube induces miR-1 and miR-206 expression	Chicken and mouse embryonic muscle	[171]
PTB and neuronal homolog nPTB, exon splicing factors	Downregulation of PTB protein by miR-133 (and miR-206)	Concurrent upregulation of miR-133 and induction of splicing of several PTB-repressed exons	During myoblast differentiation, microRNAs control a developmental exon splicing program	[172]
BDNF	BDNF downregulated	miR-206 upregulated	Differentiation of C2C12 myoblasts into myotubes	[48]
Fstl1 and Utrn Utrophin A (muscle)	Fstl1 and Utrn downregulated Utrophin A down-regulated by both miRs	miR-206 upregulated Upregulated miR-133b, miR-206	Skeletal muscle differentiation C2C12 mouse myoblasts, mouse soleus muscle	[40] [173]
CNN3 gene	Negative correlation between miR-1 expression and CNN3 mRNA expression	Normal skeletal muscle	Tongcheng (Chinese) and Landrace (Danish) pigs	[174]
FGFR1 and PP2AC, members of ERK1/2 signalling pathway	miR-133 (a and b) activities increase during myogenesis	miR-133 directly downregulates expression of FGFR1 and PP2AC	Mouse C2C12 myoblast cells	[31]
ERK1/2 signalling pathway activity	ERK1/2 signalling activity suppresses miR-133 expression	Downregulation of expression of miR-133	A reciprocal mechanism for regulating myogenesis	
BAF chromatin remodelling complex (BAF60a, BAF60b and BAF60c)	Positive inclusion of BAF60c in the BAF chromatin remodeling complex	Expression of miR-133 and miR-1/206	Progression of developing somites in chick embryos	[63]
BAF chromatin remodelling complex	Negative regulation of BAF60a and BAF60b; exclusion from BAF chromatin remodelling complex	Expression of miR-133	Progression of developing somites in chick embryos	[63]
BAF chromatin remodelling complex	Exogenous upregulation of BAF60a and BAF60b		Delay in developing somites in chick embryos	[63]
Mitochondrial UCP2 and UCP3	MyoD activates miR-133a expression which in turn directly downregulates UCP2 mRNA	Feedback network involving MyoD-miR-133a-UCP2	Mouse skeletal and cardiac muscles; UCP2 imposes developmental repression	[56]
Mitochondrial UCP2 and UCP3	Exogenous overexpression of myogenin and MyoD transcription factors	Strong increase in UCP3 promoter, expression, weak effect at the UCP2 promoter	Mouse C2C12 myoblasts	[57]
Proliferating myogenic skeletal muscle cells				
MiR-206/133b cluster	MiR-206/133b cluster is not required for survival and regeneration of skeletal muscle	Muscle regeneration proceeds in Mdx mice <i>in vivo</i>	miR-206/133b cistron knock-out mice	[170]
Enhanced translation of specific mitochondrial genome-encoded transcripts	miR-1 enters muscle mitochondria and binds mtRNA targets along with Ago factor	Increased expression of mtRNA targets	Proliferating myogenic skeletal muscle cells after muscle injury	[53]
mTOR (serine/threonine kinase)	MyoD stability regulated by mTOR	Regulates miR-1 expression <i>via</i> MyoD availability	Regenerating mouse skeletal muscle and differentiating myoblast cells	[32]
AMPK-CRTC2-CREB and Raptor-mTORC-4EBP1 pathways	mTORC regulates timing of satellite cell proliferation during myogenesis	Knockdown of mTORC reduces miR-1 expression	Myogenic satellite SCs proliferating and differentiating into myogenic precursors following rat skeletal muscle injury	[58]
HDAC4 regulates Pax7-dependent muscle regeneration	Pax7 stimulates SCs differentiation toward the muscle lineage, and limits adipogenic differentiation	HDAC4 upregulated in SCs differentiating into muscle cells	Myogenic satellite SCs	[175]
pcRNA encoded by the H strand of the rat mitochondrial genome	Introduction of mt pcRNAs into injured muscle restoring mitochondrial mRNA levels; Intramuscular ATP levels were elevated after pcRNA treatment of injured muscle	Enhanced organellar translation and respiration; similarly reactive oxygen species were reduced; Resulted in accelerated rate of wound resolution	Injured rat skeletal muscle is associated with general downregulation of mitochondrial function; reduced ATP, and increased ROS	[176]
Cardiac muscle precursor cells				
GATA binding protein 4, Hand2, T-box5, myocardin, and microRNAs miR-1 and miR-133	Reprogrammed human fibroblasts show sarcomere-like structures and calcium transients; Some cells have spontaneous contractility	Forced over-expression of GATA binding protein 4, Hand2, T-box5, myocardin, and microRNAs miR-1 and miR-133	Human embryonic and adult fibroblasts activated to express cardiac markers	[15]
SRF, MyoD and Mef2 transcription factors	miR-1-1 and miR-1-2 During cardiogenesis miR-1 genes titrate critical cardiac regulatory proteins, control ratio of differentiation to proliferation	miR-1 genes upregulated; Elevated miR-1 targets downregulation of Hand2	Cardiac muscle precursor cells	[30]
Histone deacetylase inhibitor, trichostatin A forces differentiation, yet reduced miR-1 and miR-133a	miR-1 and miR-133a reduce cardiac specific Nkx2.5 protein and Cdk9	miR-1 and miR-133a increase during spontaneous differentiation of cardiac myoblasts	Mouse cardiac stem cells (ES cells)	[10]

Specific inhibition of HDAC4 modulates CSCs to facilitate myocardial repair	Positively proliferative myocytes increased in MI hearts receiving HDAC4 downregulated CSCs	CSCs with downregulated HDAC4 expression improved ventricular function, attenuated ventricular remodeling, promoted regeneration and neovascularization in MI hearts	Mouse CSCs transplanted into MI mouse hearts	[177]
Snai1	Overexpression of miR-133a (miR-133), Gata4, Mef2c, and Tbx5 (GMT) or GMT plus Mesp1 and MyocD improved cardiac cell reprogramming from mouse or human fibroblasts	miR-133a directly represses Snai1 expression, which silences fibroblast signatures; a key molecular process during cardiac reprogramming	Mouse/human fibroblasts more efficiently reprogrammed into cardiomyocyte-like cells	[16]
β1AR signal transduction cascade	Adenylate cyclase VI and the catalytic subunit of the cAMP-dependent PKA are components of β1AR transduction cascade	miR-133 directly targets β1AR, Adenylate cyclase VI and PKA	TetON-miR-133 inducible transgenic mice, subjected to transaortic constriction, maintained cardiac performance with attenuated apoptosis and reduced fibrosis <i>via</i> elevated miR-133 expression	[17]
ROS, MDA, SOD and GPx	miR-133 produced a reduction of ROS and MDA levels, and an increase in SOD activity and GPx levels	Overexpression of miR-133, a recognized anti-apoptotic miRNA	<i>In vitro</i> rat cardiomyocytes	[18]
Caspase-9	miR-133 directly suppresses caspase-9 expression resulting in downregulation of downstream apoptotic pathways	Overexpression of miR-133	<i>In vitro</i> rat cardiomyocytes	[18]
Spred1	miR-1 directly targets Spred1	miR-1 is upregulated in hCMPCs during angiogenic differentiation	hCMPCs	[178]
miRNA-1 and miRNA-133a	miRNA-1 and miRNA-133a have antagonistic roles in the regulation of cardiac differentiation	Forced overexpression of miR-1 alone enhanced cardiac differentiation, in contrast overexpression of miR-133a reduced cardiac differentiation, compared to control cells	Pluripotent P19.CL6 stem cells Overexpression of both miRNAs promoted mesodermal commitment and decreased expression of neural differentiation markers	[179]
Cardiac muscle Induction of GATA6, Irx4/5, and Hand2	Cardiac myocytes show defective heart development, altered cardiac morphogenesis, channel activity, and cell cycling	miR-1-2 ^{-/-} gene knockout	Cardiac myocytes with knockout of both miR-1-2 genes	[180]
mt-COX1 mRNA	3'-UTR of mt-COX1 mRNA bound by miR-181c and Ago1 factor	Overexpression of miR-181c significantly decreased mt-COX1 protein, but not mt-COX1 mRNA level	Overexpression of miR-181c increased mitochondrial respiration and reactive oxygen species in neonatal rat ventricular myocytes	[54]
mt-COX1 mRNA	<i>In vivo</i> elevation of miR-181c in rat heart, reduces levels of mt-COX1 protein	Results in reduced capacity for strenuous exercise and evidence of heart failure	Rat cardiac muscle	[55]
Carvedilol, a β-adrenergic blocker	Induces upregulation of miR-133	Cytoprotective effects against cardiomyocyte apoptosis	Rat cardiac tissue, <i>in vivo</i>	[18]
GLUT4, and SRF	Both miRs downregulate SRF and KLF15	Both miR-133a and miR-133b target KLF15	Mouse cardiac myocytes	[181]
GLUT4 expression	Both basal and insulin-stimulated glucose uptake are increased	KLF15	Mouse muscle cell lines	[182]
MEF2 transcription factor	MEF2 directly activates transcription of miR-1-2 and 133a-1 binding muscle-specific enhancer between the genes	Bicistronic primary transcript of miR-1-2 and 133a-1	Development of mammalian cardiac muscle	[9]
Myocardium tissue	Enriched in miR-1, miR-133b, miR-133a		Heart structures of rat, Beagle dog and cynomolgus monkey	[183]
Gelsolin	One common miR-133a isomiR targets gelsolin gene more efficiently than standard isomer; New second rat miR-1 gene	Many isomiRs were detected by deep sequencing at higher frequency than the canonical sequence in miRBase	miRNA/isomiR expression profiles in the left ventricular wall of rat heart	[184]
CTGF	CTGF downregulated by both miRs	Exogenous upregulation of miR-133b (and miR-30c)	Cultured cardiomyocytes and ventricular fibroblasts	[185]
MT1-MMP	miR-133a upregulated	miR-133a targets MT1-MMP	Human left ventricular fibroblasts	[186]

Injured and regenerating cardiac muscle				
SERCA2a	Akt/FoxO3A-dependent pathway Activated SERCA2a reduces phosphorylation of FoxO3a, allowing entry to nucleus and activation of miR-1 expression	Downregulation of miR-1 expression in failing heart muscle	Failing mouse heart muscle	[187]
IGF-1	IGF-1 signalling and miR-1 co-regulate differentiation of myoblasts <i>via</i> a feedback loop	IGF-1 signalling down-regulates miR-1 by repression of FoxO3a; miR-1 down-regulates IGF-1	Mouse heart muscle during cardiac failure states	[25]
Bim and Bmf	Only miR-133a expression enhanced under <i>in vitro</i> oxidative stress	miR-133a targets proapoptotic genes Bim and Bmf	Rat adult CPCs miR-1 favors differentiation of CPCs, whereas	[188]
Bim and Bmf	CPCs overexpressing miR-133a improved cardiac function by reducing Bim and Bmf	CPCs overexpressing miR-133a improved cardiac function, increasing vascularization and cardiomyocyte proliferation, reduced fibrosis and hypertrophy	CPCs overexpressing miR-133a in rat myocardial infarction model	[188]
MT1-MMP activity increased in both. Ischemia and reperfusion regions	Interstitial miR-133a decreased with ischemia <i>in vitro</i> and <i>in vivo</i> ; reperfusion returned to steady-state	Phosphorylated Smad2 increased within the ischemia-reperfusion region	Ischemia-reperfusion Yorkshire pigs (90 min ischemia/120 min reperfusion)	[186]
Cardiovascular disease				
CNN2	Strong upregulation of CNN2 expression Elevated miR-133	miR-133b downregulated; miR-133b directly targets CNN2	Pre-inflammatory events in diseased cardiac tissues	[65]
Circulating platelet derived microparticles			Patients with stable and unstable coronary artery disease	[189]
Acute MI causes upregulation of circulating serum miRs	miR-1, -133a, -133b, and -499-5p were about 15- to 140-fold elevated over control		Acute STEMI patients and experimental mouse MI model	[190]
Circulating miRNAs in serum of cardiovascular disease patients	Released miR-1 and miR-133a are localized in exosomes, and are released by Ca(2+) stimulation	Levels of miR-1, miR-133a, reduced in infarcted mouse myocardium	miR release indicates myocardial damage	[191]
LVM after valve replacement in aortic stenosis	microRNA-133a is a significant positive predictor of LVM normalisation	miR-133 is a key element of the reverse remodelling process	Patients following valve replacement	[192]
Circulating levels of miR-133a	Elevated miR-133a (11-fold)		Troponin-positive acute coronary syndrome patients	[193]
Circulating levels of miR-133a	Elevated miR-133a	Improved potential regression of Left Ventricular Hypertrophy after valve replacement	Patients with aortic stenosis surgery	[194]
Apelin treatment reduces elevated circulating miRs	Elevated miR-133a, miR-208 and miR-1 reduced	High-fat diet elevated miRs and increased left ventricular diastolic and systolic diameters, and wall thickness	Obesity-associated cardiac dysfunction in mouse model	[195]
NAC treatment	Expressed miR-1, miR-499, miR-133a, and miR-133b were strongly depressed in the diabetic cardiomyocytes	NAC restored expression of miR-499, miR-1, miR-133a, and miR-133b significantly in the myocardium	Diabetic rat hearts	[196]
Myocardial junctin elevated	miR-1 targets junctin	NAC reduces junction levels	Development of diabetic cardiomyopathy in rat hearts	[196]
CAD associated ischemic heart failure	miR-133 expression decreased with increased severity of heart failure		Patients with CAD	[197]
Runx2	miR-133a targets Runx2		Transition of VSMCs to osteoblast-like cells	[198]
Increased alkaline phosphatase activity, osteocalcin secretion and Runx2 expression	miR-133a was decreased during osteogenic differentiation		Transition of VSMCs to osteoblast-like cells	[198]
Circulating miR-133a and 208a levels	Cardiac muscle-enriched microRNAs (miR-133a, miR-208a) elevated		Patients with coronary artery disease	[199]
Hypertrophic cardiac muscle				
Cx43 increased	miR-1 targets Cx43	Downregulation of miR-1 mediates induction of pathologic cardiac hypertrophy	Hypertrophic rat cardiomyocytes <i>in vitro</i> and <i>in vivo</i>	[200]
Cx43 downregulated	miR-1 targets Cx43	Cx43 protein downregulated in miR-1 Tg mice compared to WT mice	Cardiac-specific miR-1 transgenic (Tg) mouse model	[201]

Twf1 upregulated	miR-1 targets Twf1	Strong downregulation of miR-1 in pathologic hypertrophic cardiac cells compared to normal, induces Twf1 expression	<i>In vivo</i> in hypertrophic mouse left ventricle; and <i>in vitro</i> in phenylephrine-induced hypertrophic cardiomyocytes	[202]
RhoA, Cdc42, Nelf-A/WHSC2	Increased levels of RhoA, Cdc42, Nelf-A/WHSC2	Reduction miR-133a	Hypertrophic cardiac muscle	[6]
Calcineurin, agonist of cardiac hypertrophy	Increased Calcineurin activity; Cyclosporin A inhibits calcineurin	Reduced miR-133a; Prevents miR-133 down-regulation	Hypertrophic cardiac muscle; Cardiac hypertrophy reduced	[203]
NFATc4	NFAFc4 targetted by miR-133a	miR-133a	Cardiomyocyte hypertrophic repression	[204]
Interdependent Calcineurin-NFAT and MEK1-ERK1/2 signalling pathways in cardiomyocytes	MEK1-ERK1/2 signalling augments NFAT and NFAF gene expression; Activated calcineurin activates NFAT, inducing cardiac hypertrophy	MEK1 is part of mitogen-activated protein kinase (MAPK) cascade; MEK1 activates ERK directly	Hypertrophic growth response of mouse cardiomyocytes	[205]
Innervating skeletal muscle				
Innervated skeletal muscle	MyoD, Myf5, Mrt4, nAChR α Each is strongly repressed	Myogenin expression	Mouse skeletal muscle	[50,51]
Denervated muscle (unstimulated)		Myogenin expression up-regulated MyoD, Myf5, Mrt4, nAChR α All strongly stimulated	Mouse skeletal muscle	[51]
Electrically stimulated - Denervated muscle	Myogenin, MyoD, Myf5, Mrt4, partly stimulated; nAChR α inhibited		Mouse skeletal muscle	[51]
HDAC4	miR-1 promotes myogenesis by targetting HDAC4	miR-133 enhances myoblast proliferation by targetting SRF	Skeletal muscle proliferation and differentiation in myoblast cultures	[7]
Neural activity effect on muscle (HDAC4 - MEF2 Axis)	Loss of neural input leads to concomitant nuclear accumulation of HDAC4	HDAC4 inhibits activation of muscle transcription factor MEF2; results in progressive muscle dysfunction	MEF-2 activity strongly inhibited in denervated mouse skeletal muscle and in ALS muscle	[49]
Innervation and formation of airway smooth muscle	Sonic hedgehog (Shh) / miR-206/ BDNF	Shh signalling blocks miR-206 expression, which in turn increases BDNF protein	Shh coordinates innervation and formation of airway smooth muscle	[206]
nAChR subunits (UNC-29 and UNC-63); retrograde signalling	Subunits UNC-29, UCR-63 and MEF2 downregulated	miR-1 upregulated	<i>C. elegans</i> muscle at the neuromuscular junction	[34]
MEF2	Hnrpu, Lsamp, MGC108776, MEF2, Npy, and Ppfibp2 downregulated	miR-206 upregulated	Rat skeletal muscle/re-innervating muscle	[43]
HDAC4	HDAC4 (miR-206 target, prospective miR-133b target) downregulated	miR-206/-133b upregulated (and miR-1/-133a downregulated)	Mouse fast twitch skeletal muscle/re-innervating muscle	[12]
Regenerating injured muscle				
Hnrpu and Npy downregulated	miR-1 upregulated	miR-1, -133a, downregulated 1 mo after denervation, then increased 2 × at 4 mo after re-innervation	Rat skeletal soleus muscle after sciatic nerve injury and subsequent re-innervation	[43]
Ptprd downregulated	miR-133a upregulated			
Hnrpu, Lsamp, MGC108776, MEF2, Npy, and Ppfibp2 downregulated	3 × increase in miR-206 1 mo later, after reinnervation; elevated at least 4 mo	Predominant type II fiber at 4 mo, after nerve re-innervation	Rat skeletal soleus muscle after sciatic nerve injury and subsequent re-innervation	[43]
PP2A B56a	PP2A B56a downregulated	133a upregulated	Canine heart failure model: myocytes	[207]
CaMKII-dependent hyperphosphorylation of RyR2	VF myocytes had increased reactive oxygen species and increased RyR oxidation	miR-1 upregulated	Canine post-myocardial infarction model	[208]
Collagen upregulated	TGF- β 1 and TGF β RII: upregulated	miR-133a or miR-590: downregulated	Canine model of acute nicotine exposure. Atrial fibrosis <i>in vivo</i> ; cultured canine atrial fibroblasts <i>in vitro</i>	[209]
miR-208 upregulated	miR-1 and miR-133a downregulated		Human MI compared to healthy adult hearts	[210]
Myogenic proteins, MyoD1, myogenin and Pax7	Induced expression of MyoD1, myogenin and Pax7 several days after miR injection	Exogenous injection of miR-1, -133 and -206 promotes myotube differentiation	Regenerating injured mouse skeletal muscle	[211]
Cyclin D1/ Sp1	Cyclin D1/ Sp1 downregulated	miR-1/133 upregulated	Regenerating rat skeletal muscle	[212]
PRP, source of pro-inflammatory cytokines	Stong upregulation of the mRNA of pro-inflammatory cytokines IL-1 β and TGF- β 1; stimulation of both inflammatory and myogenic pathways; elevated heat shock proteins and increased phosphorylation of α B-cristallin	Stimulated tissue recovery <i>via</i> increased myogenic regulators MyoD1, Myf5, Pax7, and IGF-1Eb (muscle isoform) together with SRF; acts <i>via</i> increased expression of miR-133a with reduced levels of apoptotic factors (NF- κ B-p65 and caspase 3)	Regenerating flexor sublimis muscle of rats, 5 d after injury and treated with PRP	[66]

Muscle degeneration				
Pro-inflammatory cytokine TWEAK	TWEAK upregulated	miR-1-1, miR-1-2, miR-133a, miR-133b and miR-206 downregulated	Degenerating/ wasting mouse skeletal muscle	[59]
HMOX1 mediated by codependent inhibition of c/EBP δ binding to myoD promoter	HMOX1 inhibits differentiation of myoblasts and modulates miRNA processing HMOX1 effects partially reversed by enforced expression of miR-133b and miR-206	Downregulation of miR-1, miR-133a, miR-133b, and miR-206. Downregulation of MyoD, myogenin and myosin, and disturbed formation of myotubes. Upregulation of SDF-1 and miR-146a	Degenerating/ wasting mouse skeletal muscle	[60]
Dystrophic muscular disease				
Circulating serum microRNAs	miR-1, miR-133a, and miR-206 highly abundant in Mdx serum	miR-1, miR-133a, and miR-206 downregulated or modestly upregulated in muscle	Muscle tissue from patients with Duchenne muscular dystrophy (Mdx)	[213]
Laminin α 2 chain deficiency	miR-1, miR-133a, and miR-206 are deregulated in laminin α 2 chain-deficient muscle	Laminin α 2 chain-deficient mouse	Congenital muscular dystrophy type 1A tissue	[214]
Dystrophic process advances from prominent inflammation with necrosis and regeneration to prominent fibrosis	Deficiency in calpain leads initially to accelerated myofiber formation followed by depletion of satellite cells	Pax7-positive SCs highest in the fibrotic patient group; correlated with down-regulation of miR-1, miR-133a, and miR-206	Muscle from Limb-girdle muscular dystrophy 2 type 1 patients	[215]
Transgenic overexpression of miR-133a1 (in dystrophin point mutation Mdx mice)	Extensive overexpression in skeletal muscle, lesser increase in heart	Normal skeletal muscle and heart development	Mdx mice (model for human muscular dystrophy), extensor digitorum longus muscle	[216]
miR-206 located in nuclear in both normal and DM1 tissues by <i>in situ</i> hybridization	Only miR-206 showed an over-expression in majority of DM1 patients	No change in expression of profiled miRs, miR-1, miR-133 (miR-133a/-133b), miR-181 (miR-181a/-181b/-181c)	Skeletal muscle (vastus lateralis) of from patients with myotonic dystrophy type 1 (DM1)	[217]
FAPs facilitate myofiber regeneration	HDAC inhibitors can activate FAPs towards muscle regeneration	Inhibition of HDAC induces MyoD and BAF60C expression, which causes up-regulation of miR-1-2, miR-133, and miR-206 expression	Early stage disease dystrophic mouse muscles, regeneration of myofibres	[62]
TDP-43	TDP-43 interacts with miR-1/-206 isomers, but not miR-133 isomers	Depleted miR-1/-206 allow targets IGF-1 and HDAC4 to accumulate in ALS muscle	Mouse ALS model injured motor neurons and muscle	[33]
Inflammation response in muscle				
Inflammatory myopathies	Increased expression of TNF α	Associated with decreased expression of miR-1, miR-133a, and miR-133b	Inflammatory myopathies including dermatomyositis, polymyositis, and inclusion body myositis	[64]
hBSMCs sensitized with IL-13	Increased muscle RhoA	Reduction of muscle miR-133a	Sensitized human bronchial smooth muscle cells (hBSMCs)	[218]

IGF: Insulin-like growth factor; IGF-1R: IGF-1 receptor; MRFs: Muscle regulatory factors; PTB: Polypyrimidine tract-binding protein; Fstl1: Follistatin-like 1; Utrn: Utrophin; mTOR: Mechanistic target of rapamycin; pcRNA: Polycistronic RNAs; SRF: Serum response factor; Snail1: Snail family zinc finger-1; β 1AR: β -adrenergic receptor; PKA: Protein kinase A; MDA: Malondialdehyde; Spred1: Sprouty-related EVH1 domain containing protein 1; mt-COX1: Mitochondrial cytochrome c oxidase subunit 1; SERCA2a: Sarcoplasmic reticulum calcium ATPase 2a; LVM: Left ventricular mass; NAC: N-acetylcysteine; CAD: Coronary artery disease; StAR: Steroidogenic acute regulatory protein; mES: Mouse embryonic stem; EBs: Endodermal bodies; SCs: Stem cells; CSCs: Cardiac stem cells; MI: Myocardial infarcted; hCMPCs: Human-derived cardiomyocyte progenitor cells; VSMCs: Vascular smooth muscle cells; CPCs: Cardiac progenitor cells; STEMI: ST-segment elevation MI.

(late-stage) differentiation of chondrocytes in growth cartilage^[78]. This differentiation could be reversed by transfection and overexpression of miR-1 which repressed the expression of aggrecan, the major cartilaginous proteoglycan gene in human chondrocytic HCS-2/8 cells and normal chicken chondrocytes. Thus miR-1 is a major effector in early growth and cell proliferation, and its repression at late differentiation stages is important for maintaining cartilage integrity.

MYOMIRS AND IMMUNOLOGICAL RESPONSES

Several reports of myomiR involvement in specialized immunological processes have recently emerged.

Stumpova *et al.*^[79] (2014) found that several miRNAs, including miR-133b, were strongly expressed during *in vitro* maturation of human tolerogenic dendritic cells induced by exogenous IL-10 and TGF- β in comparison to miRs expressed in IL-4-induced and IFN- γ activated dendritic cells. MiR-133b and -206 have been previously reported to be expressed during differentiation of immunocompetent mouse Th17 cells, with miR-133b/-206 cistron transcription occurring along with expression at the nearby *Il17a/f* gene locus^[80]. This feature of T cell differentiation towards an IL-17-producing phenotype is shared between $\alpha\beta$ and $\gamma\delta$ T cells, where the specific co-regulation of miR-133b and miR-206 with the *Il17a/f* locus extends to human Th17 cells, suggesting presence of miR-133b/-206 in T cells may be biomarkers for Th17-type immuno-reactions.

Table 2 Roles and targets of the myomiRs, miR-1, -206, -133a, -133b in other precursor cells and tissues

Factor(s)	Regulation	Regulator	Tissue/cell	Ref.
Nerve tissues				
Pitx3	Pitx3 downregulated	miR-133b	Mammalian midbrain DNs	[73]
Exosome-mediated transfer of miR-133b from MSC to brain astrocytes	miR-133b transfer from multipotent mesenchymal stromal cells to neural cells	miR-133b upregulated	Mouse MSCs to neural cells	[47]
Ctgf and RhoA	Ctgf and RhoA downregulated	miR-133b upregulated	Multipotent MSCs/Rat brain parenchymal cells	[72]
miR-133b null mice: Striatum dopamine levels unchanged, Pitx3 expression unaffected; motor coordination unaltered	miR-133b has no significant role on mDA neuron development and maintenance <i>in vivo</i>	Normal numbers of mDA neurons during development and aging of miR-133b null mice	Mouse mDA neuron development in Δ -miR-133b mutant mice	[45]
Acute or chronic morphine administration, or morphine withdrawal	miR-133b levels not affected		Rat VTA/ nucleus accumbens shell	[219]
GPM6A, a neuronal glycoprotein	microRNA-133b upregulation	Reduction in gmp6a at mRNA and protein level. Cell filopodium density was reduced	Hippocampus and prefrontal cortex of neonatal male rats stressed when in utero	[220]
Tac1 gene (neurotransmitter substance P)	Tac1 downregulated	miR-206 upregulated	MSCs-derived neural cells	[221]
Ketamine (antidepressive) administration	BDNF, a direct target gene of miR-206, was upregulated	miR-206 was downregulated by ketamine	Rat hippocampus tissue	[222]
Adipogenic tissues				
IGF-1 and IGF-1R	IGF-1 signalling and miR-133b co-regulate ADSC differentiation <i>via</i> a feedback loop	miR-133b downregulation of Pitx3; IGF-1 upregulates miR-133b; miR-133b downregulates IGF-1R	Adipose tissue-derived stem cell differentiation into neuron-like cells	[71]
Pdrn16	miR-133a directly targets Pdrn16.	Downregulation of miR-133 results in differentiation of pre-adipocyte precursors into BAT	Mouse adipocyte differentiation to BAT	[74]
Pdrn16	miR-133 directly targets Pdrn16	Downregulation of miR-133 resulted in differentiation of pre-adipocyte precursors into BAT	Mouse primary brown adipocyte (and myogenic) progenitor cells - differentiate into BAT or SAT	[75]
Pdrn16	miR-133 targets Pdrn16 controlling brown adipose determination in skeletal muscle satellite cells	miR-133 downregulates Pdrn16	Adult mouse skeletal muscle stem cells (satellite cells) differentiate into BAT	[76]
HDAC4 downregulation directs SCs towards adipocyte differentiation	Brown adipose master regulator Pdrn16 is upregulated, while its inhibitor miR-133 is also downregulated	HDAC4 downregulated in SCs differentiating into adipocyte progenitor cells	Myogenic satellite SCs	[175]
GLUT4 expression	Both basal and insulin-stimulated glucose uptake are increased	KLF15	Mouse 3T3-L1 preadipocytes differentiating into adipocytes	[182]
Intrinsic insulin resistance	Elevated miR-133b	Undefined role	Adipose tissue of women with PCOS	[223]
Upregulation of LIM homeobox 8 and Zic family member 1 and downregulation of Homeobox C8 and Homeobox C9	Undefined relation of upregulated miR-206, miR-133b	Undefined relation with parallel upregulation of brite/beige markers, TBX1 and TMEM26	Human BAT from the supraclavicular region	[224]
Obesity development	Downregulation of miR-133b, miR-1	Undefined role	Adipose tissue from obese male C57BL/6 mice	[225]
LXR α regulation of lipogenic genes	miR-1/miR-206 represses LXR α expression at both mRNA and protein levels	miR-1/miR-206-induces a decrease in lipogenic gene levels and lipid droplet accumulation	Mouse hepatocytes	[226]
Osteogenic tissues				
Development of bone on organic or inorganic substrates		miR-133 differentially expressed in osteoblasts grown on different substrates	Osteoblast	[227]
Runx2	miR-133 directly down-regulates Runx2	miR-133 up-regulated	Osteogenic differentiation from C2C12 mesenchymal cells	[228]
HDAC4	HDAC4 downregulates Runx2	miR-1 targets HDAC4, increasing Runx2 activity	Chondrocyte proliferation in cartilage growth plate	[77]
Aggrecan	miR-1 promotes late-stage differentiation of growing cartilage cells	miR-1 targets Aggrecan gene expression	Chicken chondrocytes and human HCS-2/8 cells	[78]
Alveolar cells				
VAMP2/ lung surfactant secretion	miR-206 targets VAMP-2	miR-206 overexpression decreased lung surfactant secretion	Lung alveolar type II cells	[229]

Hormonal regulation				
L-thyroxine	miR-206/miR-133b downregulated	L-thyroxine treatment	L-thyroxine treated hypothyroidic skeletal muscle from thyroidectomized patients	[230]
	miR-206/miR-133b upregulated	-	Hypothyroidic human skeletal muscle	
Thyroid hormone/TEAD1	Thyroid hormone inhibits the slow muscle phenotype by upregulation of miR-133a1 which downregulates TEAD1	miR-133a1 is enriched in fast-twitch muscle and regulates slow-to-fast muscle fiber type conversion	Mouse muscle	[231]
Thyroid hormone/miR-133a1 TEAD1	myosin heavy chain I expression downregulated	TH indirectly downregulates myosin heavy chain I <i>via</i> miR-133a/TEAD1	Mouse muscle	[232]
L-thyroxine	pre-miR-206 and pre-miR-133b downregulated 50-500x increase expression of miR-1/-133a and miR-206/-133b	L-thyroxine -	L-thyroxine treated hypothyroidic mouse liver; Hypothyroidic mouse liver	[232]
Reduced insulin-mediated glucose uptake in cardiomyocytes	Downregulation KLF15, which downregulates GLUT4	Forced overexpression of miR-133a and miR-133b	Rat cardiac myocytes	[181]
Cardiac myocyte glucose metabolism	Upregulation KLF15, which upregulates GLUT4	Silencing endogenous miR-133	Rat cardiac myocytes	[181]
Metabolic control of glucose uptake by GLUT4 transporter	Downregulates KLF15, which results in downregulation of GLUT4 levels	Chronic heart failure has depressed miR-133a and -133b levels	Rat cardiac myocytes during chronic heart failure and cardiac hypertrophy	[181]
Atrial natriuretic factor expression upregulation	Enhanced at LVH and dramatically increased at CHF stage	Both miR-133a and miR-133b downregulated at CHF stage	LVH and CHF in salt-sensitive Dahl rats	[181]
Estrogen	Estrogen replacement strongly decreased IGF-1 protein level in muscles at 1 wk		Ovariectomized rat skeletal muscle	[233]
Multiple targets	miR-133a upregulated in BTBR mice		Pancreatic islets, adipose tissue, and liver from diabetes-resistant (B6) and diabetes-susceptible (BTBR) mice	[234]
Augmentation of adipocyte differentiation by norepinephrine does not alter myomiR levels	miRNAs miR-1, miR-133a and miR-206 specifically expressed both in brown pre- and mature adipocytes	miRNAs miR-1, miR-133a and miR-206 were absent from white adipocytes	Mouse brown adipocytes	[235]
Foxl2	miR-133b targets Foxl2; miR-133b inhibits Foxl2 binding to StAR and CYP19A1 promoter sequences	Foxl2 regulates StAR and CYP19A1 transcriptionally	Estradiol production in ovarian granulosa cells	[236]
Exosome release and cell to cell transfer				
Exosome-mediated transfer of miR-133b from MSCs to brain astrocytes	miR-133b transfer from multipotent mesenchymal stromal cells to neural cells	miR-133b upregulated	Mouse multipotent MSCs to neural cells	[47]
Cell to cell transfer of exosome-enriched extracellular particles	miR-133b promotes neural plasticity and recovery of function after stroke induced damage	miR-133b upregulated	Rat multipotent MSCs <i>via</i> transfer of exosome-enriched extracellular particles	[72]
Transplanted stem cells				
MSCs expressing miR-1	Upregulated miR-1	Increased rate of recovery, enhanced survival of transplanted MSCs and cardiomyogenic differentiation	Experimental ligation of the mouse left coronary artery to model myocardial infarction	[237]
Knockdown of Hes-1, member of Notch pathway	Upregulated miR-1 promotes the differentiation of MSCs into cardiac lineage	Role in survival of transplanted MSCs and cardiomyogenic differentiation	Mouse MSCs	[238]
Notch signalling and cardiomyocyte markers, Nkx2.5, GATA-4, cTnT, and Cx43	MSCs expressing exogenous miR-1		Mouse MSCs	[238]
Tissue inflammation				
Selective release of miRs during inflammation into serum	miR-133 selectively released		Review	[239]
Inflammation and cancer	MicroRNA, free radical, cytokine and p53 pathways		Review	[240]
Immunological switch which shapes tissue responses	TWEAK/Fn14 pathway		Review	[241]
Tumor biology	HMOX1		Review	[242]
GM-CSF	Direct suppression of GM-CSF expression by miR-133	Elevated expression of miR-133a/-133b during oxidative stress	Mouse alveolar epithelial cells during oxidative stress	[82]

PI3K/ Akt and IGF-1 pathways	Activation of PI3K/ Akt and IGF-1 pathway activities	Downregulation of miR-133a (and other miRs) by AOM/DSS induced chronic inflammation	Mouse model: AOM/DSS-induced colitis-associated gastro-intestinal cancer	[83]
CTGF, SMA, and COL1A1	Increased expression of CTGF, SMA and COL1A1, which are miR-133b targets	Strong downregulation of miR-133b (and other miRs)	TGF- β treated rabbit corneal fibroblasts; Recovering mouse cornea after laser ablation,	[70]
IL-10 and TGF- β	Exogenous IL-10 and TGF- β induces miR-133b expression	Upregulation of miR-133b	Human tolerogenic dendritic cells during maturation	[79]
IL-17-producing T-cells	Upregulation of IL17a/f gene expression	miR-133b/-206 cistron transcription occurs along with nearby IL17a/f gene expression	Immunocompetent mouse Th17 cells	[80]
NLRP3 inflammasome which processes IL-1 β by caspase-1 cleavage	miR-133a-1 suppresses activation of inflammasomes <i>via</i> suppression of expression of mitochondrial UCP2	miR-133a-1 overexpression in cells increases caspase-1 p10 and IL-1 β p17 cleavage,	Differentiated mouse THP1 cells	[81]
Concanavalin A-induced fulminant hepatitis		miR-133a is the most strongly differentially upregulated miR	Mouse liver following ConA injection	[243]
Infection/immune response to influenza virus (H1N2)	miR-206 expression		Experimental influenza infection in pig lung	[244]
HIF-1 α , and its regulator Four-and-a-half LIM (Lin-11, Isl-1 and Mec-3) domain 1 (Fhl-1)	Downregulation of miR-206 and upregulated HIF-1 α and Fhl-1 in hypoxic lung tissue and PSMCs	miR-206 targets HIF-1 α directly. Hypoxia-induced down-regulation of miR-206 promotes PH in PSMCs	Hypoxia-induced PH in hypoxic rat model in cultured hypoxic PSMCs	[245]
miR-206/NR4A2/NFKB1; Indirectly: inflammatory cytokines (IL6, IL1B, CCL5)	NFKB1 stimulates inflammatory cytokines (IL6, IL1B, CCL5)	Liposaccharides induce miR-206 expression which targets NR4A2 downregulation, which in turn allows upregulation of NFKB1 activity	Astrocyte-associated inflammation during recovery from chronic central nervous system injury	[246]
Cellular factors influencing myomir expression/activity				
miR-1/miR-133a				
Skeletal muscle				
Positive regulator	Negative regulator	Regulated target miR	Tissue/cell	Ref.
Myogenin, MyoD		Upregulates miR-1-1 and miR-133a-2 Upregulates miR-1-2 and miR-133a-1	Primary human myoblasts; C2C12 cells	[11]
SRF, MyoD and MEF2		Upregulates miR-1-2	Muscle somites	[30]
MEF2		Upregulates miR-1 and miR-133a	Skeletal muscle	[9]
KSRP (part of Drosha and Dicer complexes)	miR-206 binds 3'-UTR of KSRP and inhibits its expression	KSRP upregulates miR-1 expression	Skeletal muscle	[35,37]
RNA-binding protein LIN28		LIN28 upregulates miR-1 expression; LIN28 promotes pre-miR-1 uridylation by ZCCHC11 (TUT4)	Cardiac muscle of patients with muscular dystrophy	[36]
	MBNL1	MBNL1 downregulates miR-1 expression; MBNL1 binds to UGC motif in the loop of pre-miR-1 and competes for the binding of LIN28; MBNL1 blocks DICER processing of pre-miR-1	Cardiac muscle of patients with muscular dystrophy	[36]
CX43 and CACNA1C calcium channel	CX43 and CACNA1C both increased in both DM1-/DM2-affected hearts, contributing to the cardiac dysfunctions	CX43 and CACNA1C are direct targets of miR-1 repression	Cardiac muscle of patients with muscular dystrophy; CACNA1C and CX43 encode the main calcium- and gap-junction channels in heart	[36]
Utrophin A	miR-206 and KSRP are negative regulators of utrophin A	Overexpression of miR-206 promotes the upregulation of utrophin A, <i>via</i> the downregulation of KSRP	Normal and dystrophic muscle cells; miR-206 can switch between (1) direct repression of utrophin A expression, and (2) activation of its expression by decreasing KSRP, allowing close regulation	[37]
	Myostatin	Downregulates miR-1, miR-133a, miR-133b, miR-206	Mouse (35 d) pectoralis skeletal muscle	[29]
	SRF	Downregulates miRs-133a	Skeletal muscle	[1,3]
Prmt5 and Prmt4		Upregulates myomiR expression during differentiation	Mouse skeletal muscle	[247]
Smooth muscle				
Sp-1 transcription factor	pERK1/2	Upregulates miR-133(a)	VSMCs	[248]

Brg1		Upregulates miR-133 (ChIP complex with SRF)	Smooth muscle	[249]
Cardiac muscle				
GATA4, Nkx2.5, Myocardin, SRF		Upregulates miR-1 and miR-133a	Differentiating cardiac muscle	[5]
SRF plus Myocardin		Upregulates miR-1-1 and miR-1-2	Cardiomyocytes	[30]
	Calcineurin	Downregulates miR-133a	Hypertrophic cardiac muscle	[203]
miR-206/ miR-133b				
Skeletal muscle				
Mrf5		Upregulates miR-1, miR-206	Skeletal muscle	[171]
Myogenin, MyoD		Upregulates miR-206	Primary human myoblasts; C2C12 cells	[11]
MyoD		Upregulates linc MD1 (encodes miR-133b)	Differentiating myoblasts	[11, 38]
		Binds to (E-box) enhancer of miR-206, miR-133b	skeletal muscle (mouse)	[12,40]
		Upregulates miR-206/miR-133b	Differentiated human foetal skeletal muscle cells	[250]
	FGF2 allows upregulation of Sp1/Cyclin D1 Myostatin	Downregulates p38-mediated miR-1/133 expression	Regenerating rat skeletal muscle	[212]
		Downregulates miR-133a, mir- 133b, miR-1, and miR-206	Mouse (35 d) pectoralis skeletal muscle	[29]
	TWEAK downregulates myoD and MEF2c	Downregulates miR-1-1 and miR-133	Degenerating/wasting skeletal muscle	[59]
	HMOX1 downregulates MyoD and myogenin	Downregulates all myomiRs	Inflamed skeletal muscle	[60]
L-Thyroxine treatment		Downregulation of pri-miR-206 and pri-miR-133b	Human skeletal muscle	[230]
		No effect on miR-1/miR-133a pairs		
Smooth muscle				
p-ERK	Activated extracellular signal- regulated kinase p-ERK inversely correlated with VSMC growth	Downregulates miR-133 expression	VSMCs	[248]
Other tissues				
Myogenin		Binds miR-206 enhancer (ChIP)	Fibroblast cell line:	[40]
IGF-I signalling		Upregulates miR-133b	Mouse Adipose derived stem cells	[71]
L-Thyroxine deficiency	Upregulated Col5a3	Strong upregulation of miR-133a and -133b	Hypothyroid mouse liver	[232]
	Downregulated Slc17a8, Gp2, Phlda1, Klk1d3, Klk1 and Dmbt1 Upregulated Vldlr and Akr1c19, and downregulated Upp2, Gdp2, Mup1, Nrp1, and Serpini2	Strong upregulation of miRs -1, -206		
L-Thyroxine treatment	Pre-miR-206 and Pre-miR-133b down-regulated	Upregulation of Gdp2 and Mup1	Hypothyroid mouse liver <i>in vivo</i> , and in vitro mouse hepatocyte AML12 cells	[232]
PA2G4, mps1, cdc37, cx43, cldn5; cx43 is a miR-133 target	Upregulation of cell cycle factors mps1, cdc37, and PA2G4, and cell junction components cx43 and cldn5	Suppression of miR-133a1 stimulates cardiac cell proliferation	Regeneration of damaged Zebrafish cardiac muscle, associated with reduced miR-133a1	[167]
Fgf	Upregulated Fgf	Downregulates miR-133	Zebrafish regenerating fin blastema	[67]
SHP (nuclear receptor)	Downregulation of miR-206 in nuclear receptor SHP(-/-) mice		SHP(-/-) mice strain, mouse liver	[251]
AP1 transcription factor complex	AP1 induced miR-206 promoter transactivity and expression; this is repressed by YY1	ChIP analysis shows physical association of AP1 (c-Jun) and YY1 with miR-206 promoter	SHP(-/-) nuclear receptor mice strain, mouse liver	[251]
NR3B3	YY1 promoter transactivated by ERRgamma; this inhibited by SHP (NROB2)	Nuclear receptor ERRgamma (NR3B3) binding site on the YY1 promoter	Mouse liver	[251]
Novel cascade "dual inhibitory" mechanism governing miR-206 gene transcription by SHP	(1) SHP inhibition of ERRgamma leads to decreased YY1 expression	(2) Derepression of YY1 on AP1 activity, leads to activation of miR-206	Mouse liver	[251]
Il17a/f locus	miR-133b and miR-206 expression	Coregulated with IL-17 production	$\alpha\beta$ and $\gamma\delta$ T cells	[80]

IGF: Insulin-like growth factor; MRFs: Muscle regulatory factors; PTB: Polypyrimidine tract-binding protein; Fstl1: Follistatin-like 1; Utrn: Utrophin; mTOR: Mechanistic target of rapamycin; pcRNA: Polycistronic RNAs; SRF: Serum response factor; Snail1: Snail family zinc finger-1; β 1AR: β -adrenergic receptor; PKA: Protein kinase A; MDA: Malondialdehyde; Spred1: Sprouty-related EVH1 domain containing protein 1; mt-COX1: Mitochondrial cytochrome C oxidase subunit 1; SERCA2a: Sarcoplasmic reticulum calcium ATPase 2a; LVM: Left ventricular mass; NAC: N-acetylcysteine; CAD: Coronary artery disease; StAR: Steroidogenic acute regulatory protein; VTA: Ventral tegmental area; BAT: Brown adipose tissue; SCs: Stem cells; PCOS: Polycystic ovary syndrome; LVH: Left cardiac ventricular hypertrophy; CHF: Chronic heart failure; DN: Dopaminergic neurons; mDA: Midbrain dopaminergic; AOM: Azoxymethane; DSS: Dextran sulfate sodium; PH: Pulmonary hypertension; PSMCs: Pulmonary artery smooth muscle cells.

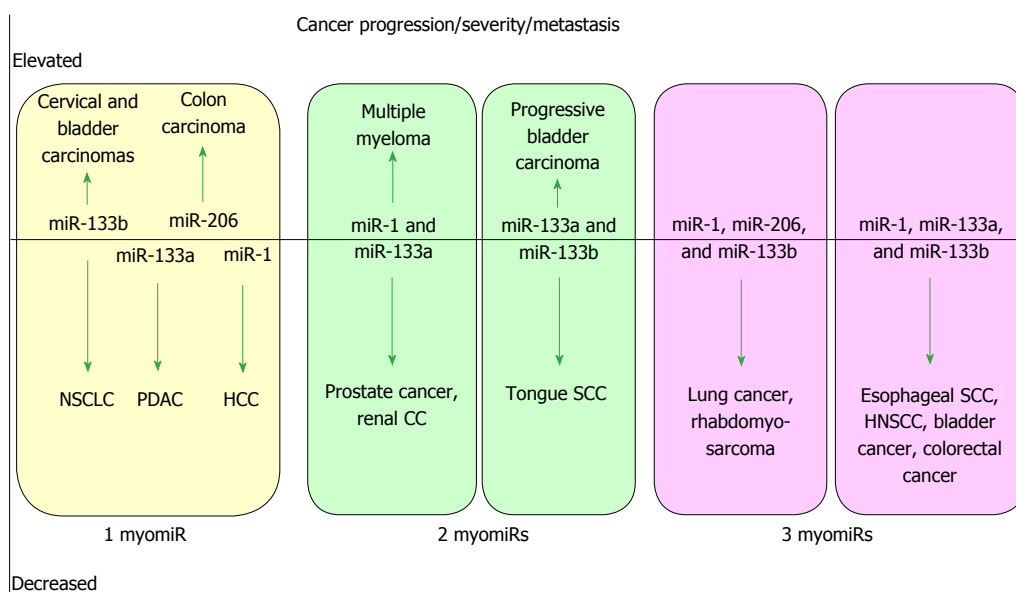


Figure 3 Roles of the myomiRs during cancer progression. Altered expression of myomiRs is associated with cancer progression and metastasis. Note that the changed level of myomiR typically contributes to cancer progression. Reduced levels of a single myomiR are associated with PDAC^[103], NSCLC^[105] and HCC^[98], whilst an elevated single miR is associated with cervical carcinoma^[121] and bladder cancer (TCC)^[122]. Increased expression of miR-1/-133a is associated with t(14;16) translocation multiple myeloma^[152], whilst reduced expression is associated with prostate cancer^[102] and RCC^[138], respectively. Reduced expression of other myomiR combinations is also associated with TSCC^[111], ESCC^[132], HNSCC^[114,143], GIST^[132], bladder cancer (TCC)^[93], lung cancer^[85,99], colorectal cancer^[86,87] and rhabdomyosarcoma^[88].

Bandyopadhyay *et al.*^[81] (2014) examined the effect of overexpression or suppression of miR-133a-1 in differentiated mouse THP1 cells which express the NLRP3 inflammasome, finding that miR-133a-1 overexpression suppresses expression of mitochondrial UCP2, resulting in increased caspase-1 p10 activity which subsequently causes IL-1 β p17 cleavage. siRNA silencing of UCP2 expression enhanced H₂O₂ stimulated inflammasome activity, and conversely, overexpression of UCP2 decreased the inflammasome activation, suggesting that the mechanism by which miR-133a-1 suppresses inflammasome activation involves the direct targeting of UCP2.

Further, the pulmonary cytokine GM-CSF is normally produced in lung alveolar epithelial cells (AECs) during a defense response. GM-CSF expression is suppressed by oxidative stress *via* altered mRNA turnover^[82], in which miR-133a and -133b are upregulated in primary AECs. *In vitro* cell experiments confirmed that miR-133a and miR-133b bind the 3'-UTR of GM-CSF and suppress its expression, and that their inhibition can reverse oxygen-induced suppression of GM-CSF expression, suggesting that miR-133 isomers are important regulators of GM-CSF expression in AECs during oxidative stress. Studies of mouse colorectal epithelial cells involving the azoxymethane/dextran sulfate-induced mouse model of colitis-associated cancer found that the induced chronic colorectal epithelial cell inflammation downregulates miR-133a and other miRs associated with the modulation of PI3K/Akt and IGF-1 associated pathways, particularly during the earlier inflammatory stages^[83].

Overall, whilst myomiRs miR-1-2, miR-133 and miR-206 have central roles muscle regeneration and (neuromuscular) repair, and in the regulation of aspects of inflammation and immunological responses during regeneration of injured and diseased muscles, miR-133a and miR-133b also appear to be involved in other regulatory processes affecting, repair, inflammation and immunological responses a number of other non-muscle cells/tissues, suggesting that these may involve common signalling pathways in a range of tissues in which myomiR play a regulatory role on the levels of signalling pathway components.

MYOMIRS AND CANCER

This review also examines the numerous cancers with prominent dysregulation of one or more of the canonical myomiRs, and the molecular events influenced by them. We focus on the deregulated myomiRs and their key target genes (up- or down-regulated) which have demonstrable effects *in vitro* on cell migration, proliferation or apoptosis, or those which are *in vivo* risk factors for cancer progression or patient survival. Although cancers characteristically have changed expression of large numbers of different protein genes and numerous different miRs which may contribute to the cancer pathology, these other deregulated genes and factors are not discussed except in relation with the myomiRs or their known targets. Table 3 lists the deregulated myomiRs reported in many different cancers. In addition, Tables 4 and 5 list targets and pathways influenced by

Table 3 The deregulated expression of the canonical myomiRs in different cancers

MiR-1	MiR-206	MiR-133a	MiR-133b	Cancer type	Ref.
		+	+	Progressive bladder cancer (TCC)	[122]
-		-		Bladder cancer (TCC)	[93,156]
-		-	-	Bladder cancer (TCC)	[92,144]
-	-	-	-	Bladder cancer	[127] ¹
-	-	-	-	Bladder cancer	[128] ¹
-		-	-	Muscle-invasive bladder cancer	[92]
	-			Proliferating breast cancer	[108]
	-			ER α -positive breast cancer	[109]
-			-	Breast cancer	[95]
			+	Progressive cervical carcinoma	[87,121]
-	-			Chordoma	[90]
	+			Colon cancer	[116]
-				Colon cancer	[101]
		-		CRC	[104,136]
			-	CRC	[86,87]
			+/-	Liver metastasis compared to primary CRC	[118]
-			-	Colon cancer	[95]
		-	-	Progressive GIST	[132]
		-		HNSCC	[143]
			-	HNSCC	[114]
	-			EEC	[110]
		-		ESCC	[132]
-		-	-	ESCC	[94]
	-			Laryngeal SCC cells	[113]
-		-		MSSCC	[137]
		-		TSCC	[111,112]
-				HCC	[98]
+			+	Liver cancer	[95]
-				Lung cancers: (NSCLC, adenocarcinomas, lung SCC, large cell carcinoma, and bronchoalveolar cell carcinoma)	[100]
	-			High metastasis lung tumors	[107]
		-		Lung SCC tissue; lung-SCC cell lines	[115]
			-	NSCLC	[85,105]
-	-			Lung adenocarcinomas; NSCLC cells	[99]
		-	-	Lung carcinomas	[85,115,131]
-			-	Lung cancer	[95]
-			-	Lymphoma	[95]
		+		AML	[120,151]
		+		Multiple myeloma	[152]
			+	Progressive prostate cancer	[123]
-	(-)	-		Prostate cancer	[102]
-			-	Prostate cancer	[95]
-			-	Recurrent prostate cancer compared to non-recurrent cancer	[96]
		-	-	Hormone-insensitive prostate cancer cells	[101]
-		-	-	Osteosarcoma	[91]
-			-	Ovarian cancer	[95]
		-		PDAC	[103]
		-		RCC	[138]
-	-			Rhabdomyosarcoma	[88,89]
-			-	Testicular cancer	[95]

¹DS: Deep sequencing; TCC: Transitional cell carcinoma; CRC: Colorectal cancer; GIST: Gastrointestinal stromal tumor; HNSCC: Head and neck squamous cell carcinoma; EEC: ER α -positive endometrioid adenocarcinoma; ESCC: Esophageal squamous cell carcinoma; MSSCC: Maxillary sinus squamous cell carcinoma; TSCC: Tongue squamous cell carcinoma; HCC: Hepatocellular carcinoma; NSCLC: Non-small-cell lung cancer; AML: Acute myeloid leukaemia; PDAC: Pancreatic ductal adenocarcinoma; RCC: Renal cell carcinoma.

downregulated and upregulated myomiRs respectively, and Table 6 lists validated myomiR targets related to enhanced cancer progression.

An excellent review by Nohata *et al.*^[84] (2012) reported changes in the expression of the miR-1/-133a and miR-206/-133b cistron clusters in numerous cancers, typically finding reduced expression of different combinations of myomiRs, such as in lung cancer^[85],

colorectal cancer^[86,87], rhabdomyosarcoma^[88,89], chordoma^[90], osteosarcoma^[91], muscle-invasive bladder cancer^[92], bladder cancer (transitional cell carcinoma) (TCC)^[93], ESCC^[94], breast cancer^[95] and prostate cancer^[95-97]. In different cancers (Figure 3) deregulation typically involves downregulation of one or more myomiR isomers, indicating they normally function as tumor suppressors in the tissues, limiting

Table 4 Targets and pathways influenced by the downregulated myomiRs in different cancers

Downregulated miR-1				
miR-1 downregulation influences multiple cancer-related pathway processes, and promotes cell proliferation and motility	Epigenetic promoter hypermethylation reduces miR-1/-133a expression in (a subset of) human prostate tumors	Reduced miR-1, miR-133a (and miR-206)	Human prostate tumors	[102]
Actin filament network-associated genes: FN1, LASP1, XPO6, CLCN3 and G6PD; Cell cycle and DNA damage control genes: BRCA1, CHK1, MCM7; Histone acetylation: HDAC4; Oncogenes: NOTCH3 and PTMA	miR-1 downregulation associated with upregulation of multiple cancer-related pathway processes	Reduced miR-1; Exogenous introduction of miR-1 or miR-206 caused similar inhibition of various cancer-related pathway genes	Human prostate cell lines, LNCaP, 22Rv1, PC-3 and RWPE-1	[102]
HSPB1	HSPB1 restores oncogenic pathways in prostate cancer cells	Downregulates miR-1 expression	Progressive prostate cancer PCa cells	[252]
XPO6 and TWF1 (PTK9)	Inverse expression between miR-1, XPO6 and TWF1 proteins in prostate cancer cell lines	Downregulated miR-1 expression	Prostate cancer cell cultures	[253]
CCND2, CXCR4, and SDF-1 α	Inverse expression between miR-1 and CXCR4 and SDF-1 α protein levels in thyroid carcinomas	Strongly downregulated miR-1 expression in thyroid adenomas and carcinomas	Thyroid adenomas and carcinomas	[254]
MET	MET upregulated	Reduced miR-1	Colon cancer	[101]
MET, Pim-1 (Ser/Thr kinase), FoxP1 and HDAC4	miR-1 downregulated, miR-1 targets MET, Pim-1, and may regulate FoxP1 and HDAC4	Reduced miR-1, -133b	Colon cancer	[87]
Fibronectin1	Fibronectin1 upregulated	MET, Pim-1, FoxP1 and HDAC4 are often upregulated in lung cancer	NSCLC tissue and A549 cell line	[100]
Met, Twf1 and Ets1 and Bag4	Met, Twf1 and Ets1 and Bag4 activities upregulated	miR-1 downregulated	Laryngeal SCC Hep2 cells	[255]
Mediator complex subunit 1 (Med1) and 31 (Med31)	Med1 and Med31 activation result in increased Met activity	miR-1 downregulated	Mouse cutaneous squamous cell carcinomas	[256]
NOTCH3 upregulates Asef expression, activating the Asef promoter, enhancing cell migration	NOTCH3 upregulated	Reduced miR-1; miR-1 targets Med 1 and Med 31	Osteosarcoma	[257]
Overexpression of PIK3CA correlates with low miR-1 expression in NSCLC tissues	71% of NSCLC samples had high PIK3CA expression	Reduced miR-1; miR-1 targets NOTCH3	Colorectal tumor cells	[258]
SLUG expression downregulated by miR-1	Transcriptional repressor of E-cadherin, or an inducer of epithelial-to-mesenchymal transition	69% of NSCLC samples had low miR-1 expression	Predictors of lymph node metastasis in NSCLC tissues	[259]
SLUG expression high in chordoma tissue	miR-1 inhibited cell proliferation both time- and dose-dependently in chordoma	Overexpression of miR-1 induces morphological change from a mesenchymal to an epithelial character	NSCLC A549 cell line	[260]
MET expression high in chordoma tissue	miR-1 downregulated 97% of chordoma samples	Transfection of MiR-1 inhibited Slug expression	mR-1 transfected chordoma cells	[261]
SRSF9/SRp30c	Exogenous upregulation of miR-1 expression	Slug overexpressed in advanced chordoma tissues and chordoma cells	Decreasing miR-1 expression levels correlated with severity of clinical prognosis	[262]
ANXA2 is essential for glioblastoma growth and invasion	ANXA2 is highly abundant protein in glioblastoma-derived extracellular vesicles	Novel apoptosis pathway involving SRSF9/SRp30c mediates tumor suppression	Bladder cancer (TCC) cells	[263]
EDN1	miR-1 downregulated in gastric cancer	miR-1 directly targets ANXA2; Reduced miR-1 in glioblastoma	Human Glioblastoma cells; miR-1 orchestrates glioblastoma extracellular vesicle function	[264]
EDN1	Elevated expression of EDN1 and reduced miR-1 level	miR-1 causes ET-1 silencing in gastric cancer cell lines	Gastric cancer tissue compared with adjacent normal tissue	[265]
Overexpressed EDN1	Enhanced <i>in vitro</i> cell proliferation and cell migration. Upregulation of several cell cycle/proliferation- and migration-specific genes	miR-1 directly targets EDN1	Human liver cancer tissues	[266, 267]
AKT inhibitor diminished the unfolded protein response and eliminated EDN1-induced cell migration	EDN1 effects act <i>via</i> activation of the AKT pathway	Upregulated UPR pathway mediators, spliced XBP1, ATF6, IRE1, and PERK at both RNA and protein levels	293T cells	[267]
		Results to enhance the UPR and subsequently activate the expression of downstream genes	293T cells	[267]

Edn1	Induced steatosis, fibrosis, glycogen accumulation, bile duct dilation, hyperplasia, and HCC	Liver-specific edn1 expression	Transgenic Zebrafish liver	[267]
API5	API5 expression upregulated thus inhibiting apoptosis	miR-1 expression downregulated	Human liver cancer tissues	[268]
Phosphorylation of ERK and AKT; LASP1	Apoptosis activated, API5 reduced Overexpression of miR-1 inhibits phosphorylation of ERK and AKT and reverses EMT process <i>via</i> inhibition of MAPK and PI3K/ AKT pathways	Overexpression of miR-1 MAPK and PI3K/ AKT pathways	HepG2 liver cancer cells Transgenic miR-1 expressing CRC cell lines	[269]
LASP1 expression upregulated	Upregulated LASP1 stimulates EMT resulting in cell proliferation and migration	miR-1 downregulated	Colorectal tumor tissue	[269]
PIK3CA	Increased expression of PIK3CA	Downregulated miR-1 expression in lung cancer	NSCLC tissue with poor patient prognosis	[259]
PIK3CA indirectly regulating pAKT and survivin proteins	Overexpressed miR-1 downregulated PIK3CA causing reduced pAKT and survivin proteins	Exogenously overexpressed miR-1 targets PIK3CA directly.	NSCLC A549 cell line	[270]
Signalling pathways such as TGF- β , ErbB3, WNT and VEGFA, and cell motility or adhesion	Ectopic expression of miR-1 and miR-145 downregulates VEGFA and AXL, respectively	Highly downregulated expression of miR-1, miR-133, miR-143 and miR-145 in gall bladder cancer	Gall bladder tumor samples and GBC NOZ cell line	[271]
lncRNA UCA1	Lnc RNA UCA1 upregulated in bladder cancer (TCC); Inverse relationship between miR-1 and lnc UCA1	Downregulated miR-1 expression in bladder cancer (TCC); miR-1 targets lnc RNA UCA1 for downregulation	Human bladder cancer (TCC) tissue	[156]
Downregulated miR-133a				
Moesin	Moesin upregulated	Reduced miR-133a	HNSCC	[143]
ARPC5	ARPC5 upregulated	Reduced miR-133a	HNSCC	[272]
ARPC5 and GSTP1	ARPC5 and GSTP1 upregulated	Reduced miR-133a (and miR-206)	Lung carcinoma	[115]
IGF-1R, TGFBR1, and EGFR are downregulated	Restoration of ectopic-expression of miR-133a in NSCLC suppresses metastatic capacity	miR-133a inhibits cell invasiveness and cell growth <i>via</i> suppression of IGF-1R, TGFBR1 and EGFR	NSCLCs	[131]
	Low expression of miR-133a is characteristic of pancreas tissue	Reduced miR-133a	PDAC	[103]
CDC42	CDC42 upregulated causing downstream activation of PAKs	miR-133 downregulated	Gastric cancer tissues	[273]
GSTP1	Upregulated GSTP1	Downregulation of miR-133a in cancer	Bladder cancer (TCC) cell lines	[144]
	Enforced downregulation of GSTP1 inhibits cell proliferation and growth;	Enforced upregulation of miR-133a and miR-133b induces cell apoptosis		
GSTP1 in cancer specimens	GSTP1 upregulated	Reduced miR-133a	Bladder cancer (TCC) tissue	[144]
Actin-binding protein, FSCN1	Upregulated FSCN1; Enforced downregulation of FSCN1 inhibits cell proliferation, migration and invasion	Downregulation of miR-133a; Forced UP exp of miR-133a inhibits cell proliferation, migration and invasion	Bladder cancer (TCC) tissue	[274]
EGFR/ AKT signalling pathway	Upregulated EGFR; Activated pAkt-1	Downregulated miR-133a; Enforced expression of miR-133a inhibits EGRF translation; causes inhibition of Akt protein phosphorylation and its nuclear translocation	Human MCF-7 and MDA-MB-231 breast cancer cell lines	[275]
Bcl-xL and Mcl-1 expression	Upregulated Bcl-xL and Mcl-1	Downregulated miR-133a correlated with tumor progression and poor patient prognosis;	Primary human osteosarcoma tissues; Osteosarcoma cell lines	[276]
E3 ubiquitin protein ligase	Downregulation of p21 and p53 proteins	Downregulated miR-133a	Primary CRC tissues	[277]
Enhanced sensitivity to doxorubicin and oxaliplatin	Enhancing apoptosis and inhibited cell proliferation	Ectopic upregulation of miR-133a	CRC cell lines	[277]
LASP1 upregulated	miR-133a expression downregulated	miR-133a targets LASP1	CRC tissues and cell lines	[278]
FTL protein upregulated	miR-133a expression downregulated	miR-133a targets downregulation of FTL protein	Patient breast cancer tissue	[279]

Increased sensitivity to chemotherapeutic drugs doxorubicin and cisplatin	Exogenous upregulation of miR-133a expression	Downregulation of FTL protein	Human MCF-7 breast cancer cells	[279]
Poor survival during breast cancer; upregulated FSCN1	Loss of miR-133a expression	FSCN1 is a direct target gene of miR-133a	Breast cancer tissue	[280]
FSCN1 downregulated	Restoration of miR-133a expression	Inhibited breast cancer cell growth and invasion	Breast cancer cell line	[280]
lncRNA Malat1/Srf/miR-133 regulatory loop	Malat1 transcript has a functional miR-133 target site, miR-133 acts as a competing endogenous RNA, regulating Malat1 levels	<i>In vitro</i> depletion of Malat1 in C2C12 cells reduces Srf activity, Srf is an enhancer of miR-133 expression; feed-back regulation loop involving miR-133	Mouse myoblast C2C12 cells	[164]
lncRNA MALAT1	MALAT1 is overexpressed in 46% of ESCC tissues, primarily in high-stage tumors, high expression correlates with lymph node metastasis	<i>In vitro</i> depletion of MALAT1 suppresses tumor cell proliferation, cell migration and invasion; G2/M phase arrest was induced and the ratio of apoptotic cells increased	Human ESCC	[162]
WIF1/lncRNA MALAT1	WIF1 (strong tumor suppressor) is systematically downregulated in glioblastoma	WIF1 down regulation correlates with strong upregulation of MALAT1. <i>In vitro</i> depletion of MALAT1 suppresses tumor cell proliferation	Glioblastoma	[163]
Downregulated miR-133b				
Fascin-1 mRNA	FSCN1 upregulated	Reduced miR-133b	High-grade GIST tissue	[281]
BCL-2 family (MCL-1 and BCL2L2)	MCL-1 and BCL2L2 upregulated	Reduced miR-133b	Lung cancer	[85]
FAIM antiapoptotic protein and GSTP1	miR-133b directly targets FAIM and GSTP1	Downregulated miR-133b	miR-133b expression significantly downregulated in 75% of prostate cancer tumor specimens	[282]
Gli1	Gli1 upregulated	Gli1 inversely correlated with downregulated expression of miR-133b	Gastric cancer	[283]
Bcl-w and Akt1	Bcl-w and Akt1 proteins overexpressed significantly	miR-133b significantly downregulated	Bladder cancer tissues	[284]
		miR-133b downregulated in tumors compared to surrounding tissue	Gastric and esophageal adenocarcinomas	[285]
			Endometrial sarcoma, leiomyosarcoma, and mixed epithelial-mesenchymal tumors	[286]
Downregulated miR-206				
Notch3/ miR-206	Downregulated Notch3, blocking of the anti-apoptotic activity of Notch3	Forced expression of miR-206 strongly induced apoptotic cell death <i>via</i> ; also inhibited cell migration and focus formation	HeLa cells	[287]
Met	Upregulated Met	miR-206 downregulated	Human rhabdomyosarcoma	[288]
HGFR	Upregulated HGFR	miR-206 downregulated	Human breast cancer cells	[289]
KLF4	Upregulated KLF4	miR-206 downregulated	RK3E breast epithelium cells	[108]
KLF4; RAS-ERK signalling	Upregulated KLF4 promotes RAS-ERK signalling Endogenous KLF4 binds the promoter regions stimulates expression of miR-206	miR-206 downregulated	TNBC cells	[290]
RASA1 and SPRED1		miR-206 inhibits translation of the RAS pathway suppressors RASA1 and SPRED1	Suppression of RASA1 or SPRED1 increased levels of GTP-bound, wild-type RAS and activated ERK 1/2	
VEGF	VEGF upregulated in Laryngeal SCC tissues	MiR-206 strongly downregulated in LSCC tissues	Laryngeal SCC cancer tissue and cells	[113]
VEGF	VEGF upregulated in ccRCC tissues	MiR-206 strongly downregulated in ccRCC tissues	ccRCC tissues assayed by Deep Sequencing	[291]
Cdc42, MMP-2 and MMP-9	Upregulated Cdc42, MMP-2 and MMP-9	miR-206 downregulated	Human breast cancer tissues	[292]
ER α	miR-206 directly targets ER α 3'-untranslated region	MiR-206 inhibited by ER α agonists, indicating a mutually (double) inhibitory feedback loop; miR-206 downregulated	Estrogen stimulated breast cancer cell lines	[293]
	Upregulated ER α		MCF-7 breast cancer cells	[109]
ER α	Upregulated ER α	miR-206 downregulated	EEC tissue	[294]
				[110]

K-Ras	K-Ras is direct target of miR-206; MiR-206 expression significantly downregulated and k-Ras upregulated on OSCC tissues	Low miR-206 potentiates metastases, and shorter overall survival	OSCC tissue samples and cell lines	[295]
MiR-206	Enforced upregulated of miR-206 attenuated cell proliferation, increased apoptosis and inhibited cell migration and invasion	MiR-206 strongly downregulated in lung cancer tissues	Lung cancer - tissues and cell lines	[107]
EGFR/MAPK signalling switches MCF-7 breast cancer cells from ER α -positive, Luminal-A phenotype to ER α -negative, basal-like phenotype	EGFR signalling represses estrogenic responses in MCF-7 cells by enhancing miR-206 activity Elevated miR-206 reduces cell proliferation, enhances apoptosis, and reduces numerous estrogen-responsive genes	miR-206 downregulates steroid receptor co-activators SRC-1 and SRC-3 and GATA-3 transcription factor, directly	MCF-7 breast cancer cells	[296]
Greater lymph node metastasis, venous invasion, and at a more advanced stage	miR-206 expression strongly downregulated	Correlates with tumor progression	Human gastric cancer tissue	[297]
CCND2	miR-206 expression strongly downregulated	Correlates with upregulation of CCND2 and cancer progression	Human breast cancer	[298]
MET	miR-206 expression strongly downregulated	Upregulation of MET	Human gastric cancer	[299]
Prognostic signature of metastatic colorectal cancer	miR-206 expression strongly downregulated	Upregulation of MET	Papillary thyroid carcinoma	[300]
Notch3, Hes1, Bcl-2 and MMP-9;	Exogenous upregulation of miR-206 expression;	Prognostic signature of metastases: miRs 21, 135a, 335, 206 and let-7a	Metastatic CRC	[301]
p57, Bax and caspase-3	miR-206 is a potent tumor suppressor	Notch3, Hes1, Bcl-2 and MMP-9 downregulated at both mRNA and protein level;	Human HHC Hep2 cells.	[302]
STC2, HDAC4, KLF4, IGF1R, FRS2, SFRP1, BCL2, BDNF and K-ras	Exogenous upregulation of miR-206 expression; miR-206 is a potent tumor suppressor	p57 and Bax upregulated, and cleaved caspase-3 protein upregulated	Reduced apoptosis, and cell migration in HepG2 cells overexpressing miR-206	[303]
Cyclin C, CCND1 and CDK4	Cyclin C, CCND1 and CDK4 upregulated in melanoma tissue; Exogenous upregulation of miR-206 expression reduced growth and migration/invasion of several melanoma cell lines; G1 arrest in melanoma cells	STC2, HDAC4, KLF4, IGF1R, FRS2, SFRP1, BCL2, BDNF, and K-ras downregulated strongly in SCG-7901 cells overexpressing miR-206	Gastric carcinoma SCG-7901 cells	[304]
Coronin, actin-binding protein	Silencing of coronin expression reduced tumor cell migration and altered the cellular actin skeleton and cell morphology, but did not effect cell proliferation	hsa-miR-206 downregulated in melanoma tissue	Reduced apoptosis, and cell migration in SCG-7901 cells overexpressing miR-206	[305]
RNA binding protein DEAD-END (DND1), DNA cytosine deaminase (AICDA), and APOBEC3	DND1 blocks miRNA interaction with 3'-UTR of specific mRNAs, restores protein expression; APOBEC3G binds DND1 counteracts repression and restores miRNA activity	Overexpression of miR-206 in melanoma cells strongly downregulated cyclin C, CCND1 and CDK4	Human melanoma cancer tissue, and cell lines	[306]
Advanced clinical stage, T classification, metastasis and poor histological differentiation		Downregulated miR-206 allowed upregulation of coronin, a direct target; Upregulated miR-206 reduced TNBC cell migration and cell proliferation	TNBC cell lines	[307]
Ellagic acid inhibits E2-induced mammary tumorigenesis	Reverses the downregulation of miR-206	APOBEC3G blocks DND1 to restore miR-206 inhibition of CX43 translation	Mouse cells	[308]
Actin-like 6A (BAF53a), a subunit of the SWI/SNF chromatin remodeling complex	Elevated BAF53a	Significant association with decreased miR-206 expression	Paired human osteosarcoma and normal adjacent tissues	[309]
Actin-like 6A (BAF53a)	BAF53a transcript is significantly higher in primary rhabdomyosarcomas than in normal muscle	Downregulation of miR-206	ACI model rat mammary tissue	[309]
		Restoration of miR-206 expression downregulated BAF53a, which inhibits proliferation and anchorage independent growth; BAF53a and is a direct target of miR-206	Primary rhabdomyosarcoma tumors	[309]

Wnt and transcription factors Tbx3 and Lef1	Exogenous upregulation of miR-206 expression	Inhibition of Wnt, Tbx3 and Lef1 activities	Estrogen receptor alpha (ER- α)-positive human breast cancer; developing mammary buds	[310]
ANXA2 and KRAS	Stimulation of KRAS activity then induces NFKB1 expression;	Downregulated miR-206 in PDAC	PDAC tissues and cell lines	[311]
Induces NFKB1	Increased KRAS results in stimulation of cytokines CXCR2, CXCL1, CCL2, as well as CSF2 (GM-CSF) and VEGFC	Increased cell cycle progression, cell proliferation, migration and invasion		
Downregulated miR-1 and miR-133a				
PNP	PNP upregulated	Reduced miR-1, -133a	Prostate cancer	[97]
TAGLN2	TAGLN2 upregulated	Reduced miR-1, -133a	RCC	[136]
TAGLN2 and PNP	TAGLN2 and PNP upregulated	Reduced miR-1, -133a	MSSCC	[137]
PTMA and PNP	PTMA and PNP upregulated	Reduced miR-1, -133a	Bladder cancer (TCC)	[312]
LASP1	LASP1 upregulated	Reduced miR-1, 133a, (and miR-218)	Bladder cancer (TCC)	[313]
	Forced expression of each miR decreased LASP1 in cell lines			
DNA methylation regulates miR-1-1 and miR-133a-2 cistron expression	Inverse correlation with TAGLN2 levels	CpG islands upstream of miR-1-133a hypermethylated	Colorectal carcinoma tissue and liver cancer tissue	[314]
Downregulated miR-1 and miR-133b				
	miR-1 and miR-133b have sufficient power to distinguish recurrent specimens from non-recurrent prostate cancer	miR-1 and miR-133b are significantly downregulated in recurrent prostate cancer tissue specimens	Recurrent prostate cancer tissue	[96]
Downregulated miR-1 and miR-206				
NRF2 upregulated	Downregulated miR-1 and miR-206 expression	Upregulated expression of NRF2 induces increased expression HDAC4	Primary lung adenocarcinoma; DU145 human prostate cancer cell line	[99]
Loss of NRF2	Decreased expression histone deacetylase (HDAC4)	Results in increased expression of miR-1 and miR-206; which inhibits PPP expression; Reduced PPP acts as a regulatory feedback loop stimulates HDAC4 expression	A549 human NSCLC cell line	[99]
c-Met	c-Met upregulated	miR-1 and -206 downregulated	Human rhabdomyosarcoma	[89]
ARPC5 and GSTP1	ARPC5 and GSTP1 upregulated	Reduced miR-133a (and miR-206)	Lung SCC cell lines	[115]
Downregulated miR-133a and miR-133b				
PKM2	PKM2 upregulated	Downregulated miR-133a, -133b	TSCC	[111]
FSCN1	FSCN1 upregulated	Downregulated miR-133a, -133b, (miR-145)	ESCC	[132]
		miR-133a, miR-133b downregulated	ESCC	[94]
KRT7	KRT7 upregulated	Downregulated (miR-133a and miR-133b)	Bladder cancer (TCC) and <i>in vitro</i> in BC KK47 cells	[315]
Downregulated miR-1, miR-206 and miR-133				
myomiRs	Patient to patient variation in the up or down regulation of miR expression in both tumor and matched normal tissues	In tumors strong down regulation of highly expressed miR-1/133a; (downregulation of weakly expressed miR-206/-133b)	Bladder cancer assayed by deep sequencing	[316]
Candidate tumor suppressor miRNAs in RCC	Each of miR-206, miR-1, miR-133b strongly downregulated	Restored expression strongly inhibited cancer cell proliferation,	RCC	[317]
Shorter overall survival and disease-free survival	Correlated with increased downregulated of miR-133b and/or miR-206	Both miR-133b and miR-206 significantly downregulated	Osteosarcoma tissues	[318]
Cell invasion and metastasis	miR-1, miR-133a, miR-133b downregulated	miR-133a, miR-133b involved in invasion and metastasis	ESCC	[94]

UPR: Unfolded protein response; PAKs: P21-activated kinases; EDN1: Endothelin 1; AKT1: V-akt murine thymoma viral oncogene homolog 1; HCC: Hepatocellular carcinoma; API5: Apoptosis inhibitor 5; LASP1: LIM and SH3 domain protein 1; XPO6: Exportin-6; CLCN3: Chloride channel, voltage-sensitive 3; G6PD: Glucose-6-phosphate dehydrogenase; BRCA1: Breast cancer 1, early onset; MCM7: Minichromosome maintenance complex component 7; PTMA: Prothymosin alpha; TWF1: Twinfilin actin-binding protein 1; CXCR4: Chemokine (C-X-C motif) receptor 4; HDAC4: Histone deacetylase 4; ANXA2: Annexin A2; SRSF9/SRp30c: Splicing factor serine/arginine-rich 9; ARPC5: Actin-related protein 2/3 complex subunit 5; GSTP1: Glutathione S-transferase pi 1; FSCN1: Fascin homolog 1; FTL: Ferritin light chain; BCL2L2: B-cell CLL/lymphoma 2-like 2; EGFR: Epidermal growth factor receptor; PNP: Purine nucleoside phosphorylase; PKM2: Pyruvate kinase, muscle type M2; KRT7: Keratin 7.

Table 5 Targets and pathways influenced by the upregulated myomiRs in different cancers

Factor(s)	Regulation	Regulator	Tissue/cell	Ref.
Upregulated miR-133b				
Activated p-ERK, pAKT1 cause <i>in vitro</i> proliferation of cervical cancer cell lines, and promote <i>in vivo</i> tumorigenesis and metastasis	Downregulation of MST2, CDC42, RHOA	Upregulated miR-133b	Human cervical carcinoma tissue compared to surrounding normal cervical tissue	[121]
Decreased patient survival		Upregulated miR-133b	Progression bladder cancer	[122]
Androgen receptor	miR-133b directly represses CDC2L5, PTPRK, RB1CC1, CPNE3	miR-133b directly upregulated by AR	Hormone-sensitive human prostate cancer (LNCaP) cells stimulated by androgen	[123]
Activated neuroendocrine neoplasia proliferation	Mutation in von Hippel-Lindau tumor suppressor, E3 ubiquitin protein ligase gene (VHL)	Upregulated miR-133b expression in VHL- deficient pheochromocytoma	Human pheochromocytoma (PCCs) and paraganglioma (PGLs) neuroendocrine neoplasias	[319]
Upregulated miR-206				
Cell reprogramming factor KLF4	KLF4 downregulated in colon cancer tissue, associated with increased miR-206	miR-206 strongly upregulated in colon cancer tissues	Human colon cancer tissue	[116]
Upregulated miR-1 and miR-133				
Decreased survival of R172 IDH2-mutated subset of CN-AML patients, increases resistance to chemotherapy	Distinctive gene and microRNA expression profiles accurately predicted R172 IDH2 mutations	Upregulated expression of miR-1 and miR-133	<i>De novo</i> CN-AML patient bone marrow and blood samples	[151]
EVI1 increases aggressive cancer growth	EVI1 expression upregulated in established patient samples ChIP assays show EVI1 binds to miR-1-2 gene promoter directly	Upregulated expression of miR-1-2 and miR-133-a-1	EVI1 expressing AML subset of patients	[120]
CCND2	miR-1 and miR-133a were specifically overexpressed in the cases with t(14;16) translocation, correlates with down-regulated CCND2 expression	Upregulated miR-1 and miR-133-a	Multiple myeloma	[152]
Secreted myomiRs				
miRs selectively released into serum (within exosome microparticles)	miR-1, miR-133a, and miR-133b selectively released		Human breast cancer	[320]
Circulating microRNA	Tumor-derived exosomes		Human non-small-cell lung cancer	[321]

AKT1: V-akt murine thymoma viral oncogene homolog 1; CN-AML: Cytogenetically normal acute myeloid leukemia.

the abundance of factors involved in aspects of cell proliferation^[84].

Some cancers however show that elevation of myomiR levels promotes cancer progression (Figure 3), indicating that in such cell environments the deregulated myomiR is oncogenic and their targets here are normal tumor-suppressor cell factors. These are both regulatory roles that myomiRs normally exert, either by influencing particular cell signalling pathways that maintain a differentiated non-proliferating state in mature cell development stages, or by influencing other signalling pathways involved in cell proliferation and tissue genesis stages. Notably, the reports of deregulated myomiRs in cancers often reveal significant relation between the myomiR concentration and tumor severity, with greatest deregulation of expression statistically associated with metastasis, or with tumors of high metastatic potential, or with decreasing prospects of patient survival^[88,98-102]. Yet, double knockout mice lacking both *miR-133a1/2* gene alleles^[14] do not display an elevated incidence of tumors in any organs or tissues *in vivo*, similarly animals with knockout of *miR-206* expression^[46], or knockdown of *miR-133b* or double knockdown of *-133b/-206*^[44] are not reported to be associated with tumors, indicating

that the singular lack of myomiR(s) alone is not (usually) sufficient to initiate tumorigenesis. Hence the oncogenic role of the deregulated myomiRs is a significant potentiating one, dysregulating cell signalling pathways in concert with the multiplicity of other molecular and cell environmental changes occurring in the developing tumor.

It is also notable that expression of cistronic myomiR isomers is not co-ordinated in different carcinomas, emphasizing individual deregulation of control of expression of each *myomiR* gene. For example, at least one miR of either cistronic pair is expressed in 16/20 cancers having altered miR-133 expression, showing also that each miRs has independent expression in non-muscle cells and tissues. Since the myomiRs influence numerous developmental pathways in normal muscle and other tissues, the differential expression of the myomiR isomers may potentially influence the pathology of cancers differently.

Cancer and downregulated myomiRs

Reduced expression of myomiRs was associated with numerous cancers, such as with PDAC^[103] colorectal cancer^[86,104], NSCLC^[105], HCC^[98], prostate

Table 6 Validated myomiR targets related to cancer progression

Up regulated cell factor	Down regulated cell factor	Cancer type	Ref.
Downregulated myomiRs			
Downregulated miR-1			
Mediator complex subunit 1 (Med1) and 31 (Med31) upregulated	miR-1 downregulated	Osteosarcoma	[217]
Slug expression upregulated; enhanced cell migratory and invasive activities	miR-1 downregulated	Chordoma	[128]
Slug expression upregulated; stimulation of EMT process	miR-1 reduced increasingly with cancer progression	Prostate adenocarcinoma	[260]
Downregulated miR-133a			
ARPC5 upregulated;	Downregulated miR-133a (> miR-206)	Lung SCC	[115]
		HNSCC	[272]
CAV1 upregulated;	miR-133a downregulated	HNSCC	[322]
Moesin upregulated;	miR-133a downregulated	HNSCC	[143]
FSCN1 upregulated;	miR-133a/ miR-133b downregulated	ESCC	[132]
		Bladder cancer (TCC)	[274]
GSTP1 upregulated;	miR-133a downregulated	HNSCC	[323]
		Bladder cancer (TCC)	[144]
		Lung SCC	[115]
LASP1 upregulated;	miR-133a downregulated in 83% of colorectal tumors	Colorectal cancer	[136]
CAV1 downregulated with miR-133a levels, and is lowest in metastatic cancers;	Contrastingly, higher levels of miR-133a correlate with poor prognosis and increased metastasis		
FSCN1 upregulated in non-metastatic tumors			
LASP1 upregulated;	miR-133a downregulated	Bladder cancer (TCC)	[313]
PKM2 upregulated;	miR-133a downregulated	TSCC	[111]
Moesin upregulated;	miR-133a downregulated	HNSCC	[143]
EGFR upregulated;	miR-133 downregulated	Hormone-sensitive prostate cancer cell lines	[324]
Human TERT telomerase catalytic subunit upregulated;	miR-133a downregulated		[325]
TCF7 transcription factor upregulated;	miR-133a downregulated		[325]
FSCN1 and MMP14 upregulated;	miR-133a downregulated	ESCC	[326]
Reduced miR-133a expression correlated significantly with advanced clinical stages, poor histological differentiation and lymph node metastasis	Marked downregulation of miR-133a in primary EOC tumors and OVCAR-3 cell line	Epithelial ovarian cancer (EOC), and in OVCAR-3 cell line	[327]
Downregulated miR-133b			
FSCN1 upregulated;	miR-133a/-133b downregulated	ESCC	[132]
FSCN1 mRNA upregulated;	miR-133b downregulated	Progressive GIST	[281]
BCL2L2 upregulated;	miR-133b downregulated	Lung cancer	[85]
MCL1 upregulated;	miR-133b downregulated	Lung cancer	[85]
MET upregulated;	miR-133b downregulated	Colorectal cancer	[87]
MET protein upregulated;	miR-133b downregulated	high grade osteosarcoma tumor samples and cell lines	[328]
EGFR upregulated;	miR-133b downregulated	NSCLC	[105]
Multiple cell factors elevated;	miR-133b downregulated	Prostate cancer	[282]
FGFR1 downregulated;	miR-133b downregulated	Gastric cancer	[329]
Gli1 protein downregulated by miR133b, Gli1 target genes, OPN and Zeb2, are indirectly regulated	miR-133b downregulated	Gastric cancer	[283]
TAp63 suppresses metastasis; downregulation target of miR-133b	miR-133b is a transcription target of TAp63, downregulated	Colon cancer cells	[330]
Chemokine (C-X-C motif) receptor 4 protein downregulated by miR133b; upregulated in advanced cancer	miR-133b downregulated	CRC	[331]
TBP-like 1 mRNA and protein are upregulated in CRC	miR-133b downregulated in CRC	CRC	[332]
Strong additional down regulation of miR-133b aids liver metastatic niche for CRC cells	miR-133b downregulated 3 × (significant) in liver metastasis compared to primary CRC	Metastatic cancer arising from primary hCRC	[333]
Interestingly, miR-133b is not downregulated significantly in lung metastasis compared to primary CRC			
SP1 targeted directly by miR-133, causing reduced expression of MMP-9 and Cyclin D1	miR-133a and -133b downregulated	Gastric cancer	[334]
miR-133b target MMP-9 is upregulated	miR-133b downregulated	RCC	[335]

Downregulated miR-206				
ER α		ER α downregulates miR-206	ER α -positive breast cancer;	[294]
		miR-206 downregulated	Double feedback loop	[109]
		miR-206 downregulated		[293,336]
ER α		miR-206 downregulated	EEC tissue	[110]
SRC-1, SRC-3 and GATA-3 proteins contribute to estrogenic signalling		miR-206 downregulated	ER α -positive breast cancer	[296]
			Signalling contributes to Luminal-A phenotype	
KLF4 over expressed in proliferating cells and cancers.		miR-206 levels are KLF4 dependent. KLF4 and miR-206 feedback pathway oppositely affect KLF4 protein translation	Breast cancer cells and normal cells	[108]
FGBP1		miR-206 gene double knockdown	miR-206 ^{-/-} mouse skeletal muscle.	[12]
VEGF upregulated		miR-206 downregulated	Laryngeal SCC cells	[113]
VEGF upregulated		miR-206 downregulated	CRC tumors compared to matched normal tissue; (¹ DS assay)	[337]
miR-206 correlates with negative ER status, negative PR status, and negative HER-2 status		Downregulated miR-206	Breast cancer tumor tissue	[338]
miR-206 was downregulated in clinical TNBC tumor samples, one of its targets, actin-binding protein coronin was upregulated		Downregulated miR-206 associates with increased metastasis potential in breast cancers	High metastatic capacity TNBC tumors	[305]
Downregulated miR-1 and miR-133a				
PNP upregulated		miR-1/miR-133a downregulated	MSSCC	[137]
			Prostate cancer	[97]
			Bladder cancer (TCC)	[312]
TAGLN2 upregulated;		miR-1/miR-133a downregulated	MSSCC;	[137]
			RCC	[138]
			HNSCC	[339]
			Bladder cancer (TCC)	[93]
			Bladder cancer (TCC)	[312]
PTMA upregulated		miR-1 and miR-133a downregulated		
Downregulated miR-1 and miR-206				
MET levels correlated inversely with miR-1/206 expression		miR-1/206 downregulated	Up-regulation of MET in rhabdomyosarcoma	[89,291]
HGFR upregulated		miR-1/206 downregulated	Breast cancer cells	[289]
G6PD; PGD; TKT; GPD2 upregulated		miR-1/206 downregulated	Primary lung adenocarcinoma	[99]
Upregulated myomiRs				
Upregulated miR-133b				
miR-133b	miR-133b strongly upregulated	MST1, CDC42, RHOA, and DUSP1 downregulated	Cervical carcinoma	[121]
miR-133b	miR-133b is directly upregulated by AR	miR-133b represses CDC2L5, PTPRK, RB1CC1, and CPNE3	PCa prostate cancer cell line	[123]
Upregulated miR-206				
miR-206	Strongly upregulated miR-206	KLF4 downregulated	Human colon cancer tissue	[116]
Upregulated miR-1 and miR-133a				
miR-1-2 and miR-133-a-1	Upregulated miR-1-2 and miR-133-a-1	EVI1 (transcriptional activator of miR-1 and miR-133b)	AML	[120,151]
miR-1 and miR-133-a	Upregulated miR-1 and miR-133-a	Downregulated CCND2	Multiple myeloma	[152]
Up-regulation of exogenous myomiR expression in cell lines				
Reduced cell proliferation	Estrogen receptor alpha	Overexpression of miR-206 has an inhibitory effect on cell proliferation	ER α -positive breast cancer cells over expressing mir-206	[289]
miR-133b	GSTP1 downregulated	Transgenic miR-133b overexpression	HeLa cervical cancer cells	[282]
miR-133b	FAIM downregulated	Transgenic miR-133b overexpression	HeLa cervical cancer cells	[282]
Apoptosis increased	TNF α -induced cell death is activated	Transgenic miR-133b overexpression	HeLa cervical cancer cells	[282]
Increased cell proliferation and migration	Downregulation of MST2	Transgenic miR-133b overexpression	CaSki cervical cancer cells	[121]
	Downregulation of CDC42			
	Downregulation of RHOA			
Increased cell proliferation and migration	Indirect upregulation of p-AKT1 activity	Transgenic miR-133b overexpression	CaSki cervical cancer cells	[121]
	Indirect upregulation of p-ERK activity			
RB1CC1 downregulated	Exogenous upregulation of miR-133b;	miR-133bm promotes cell apoptosis, but suppressed cell proliferation and cell-cycle progression in aggressive PC-3 cells	PC3 prostate cancer cell line	[106]
	miR-133b directly targets RB1CC1 in LNCaP cells	In contrast in low-aggression LNCaP cells, miR-133b stimulate cell proliferation and cell-cycle progression, but inhibit apoptosis	Hormone sensitive prostate cancer LNCaP cell line	

Cell proliferation decreased and apoptosis increased	Met, Twf1 and Ets1 and Bag4 activities downregulated	miR-1 expression is lower in mouse cSCCs compared to normal skin Transgenic miR-1 overexpression	Mouse cutaneous squamous cell carcinomas (cSCCs); A5 and B9 cSCC cell lines	[256]
Ets1 proto-oncogene	Repression of Ets1 expression inhibited HepG2 cell invasion and migration	Transgenic miR-1 overexpression	HCC HepG2 cells	[340]
lncRNA UCA1	Knockdown of lnc UCA1 expression phenocopied the effects of upregulation of hsa-miR-1	hsa-miR-1 decreased the expression of lnc UCA1 in bladder cancer cells in an Ago2-slicer-dependent manner	Human bladder cancer (TCC) cells	[156]
NOTCH3 signalling	miR-206 had a direct inhibition of NOTCH3 signalling and indirect interaction with other signalling pathways <i>via</i> CDH2 and MMP-9	miR-206 upregulation blocks the cell cycle, inhibits cancer cell proliferation and migration and activates cell apoptosis	SW480 (plus its metastatic strain) and SW620 colon cancer cell lines	[341]
FSCN1	miR-133b targets FSCN1 in GC cells; the direct knockdown of FSCN1 can also inhibit GC cell growth and invasion	Up regulation of miR-133b in GC cells inhibits cell proliferation, cell migration and invasion	miR-133b is significantly downregulated in GC tissues compared with adjacent normal tissues, as well as in GC cell lines	[342]
FSCN1	miR-133a targets FSCN1 in CRC cells; Overexpression of FSCN1 can reverse the inhibitory effect of miR-133a upregulation, reactivating CRC cell invasion	Up regulation of miR-133a expression and downregulation of FSCN1 protein expression both suppress colorectal cancer cell invasion	miR-133a is significantly downregulated in some colorectal cancer cell lines, as well as in colorectal cancer tissues compared with the normal adjacent tissues	[343]

¹DS: Deep sequencing. HNSCC: Head and neck squamous cell carcinoma; ESCC: Esophageal squamous cell carcinoma; ARPC5: Actin-related protein2/3 complex, subunit 5, 16 kDa; CAV1: Caveolin-1; FSCN1: Fascin homolog 1; GSTP1: Glutathione S-transferase pi 1; TSCC: Tongue squamous cell carcinoma; EGFR: Epidermal growth factor receptor; TAp63: Tumor protein p63; CRC: Colorectal cancer; KLF4: Kruppel-like factor 4; SRC-1 and SRC-3: Steroid receptor coactivator proteins; HGFR: Hepatocyte growth factor receptor; G6PD: Glucose-6-phosphate dehydrogenase; PGD: 6-Phosphogluconate dehydrogenase; TKT: Transketolase; GPD2: Glycerol-3-phosphate dehydrogenase; RHOA: Ras homolog family member A; AML: Acute myeloid leukaemia; EVI1: Ecotropic virus integration site 1 transcription factor; MST2: Mammalian sterile 20-like kinase 2; RB1CC1: RB1 inducible coiled-coil 1; UCA1: Urothelial cancer associated 1; GC: Gastric cancer.

cancer^[102,106], metastatic lung tumors^[107], proliferating breast cancer^[108], ER α -positive breast cancer^[109], EEC^[110], NSCLC^[85], TSCC^[111,112], laryngeal SCC^[113], and HNSCC^[114] (Table 3). Many of the reports identify reduction in a single myomiR, but in case studies of particular cancers often the different myomiRs have been identified as downregulated, for example in NSCLC tumor samples, miR-1^[100], -133a^[85,115], -133b^[85,115], -206^[107] or miR-1/-206^[99] have been reported, by implication dysregulation of all of the myomiRs. Similarly, for colorectal cancer, downregulated miR-1^[101], -133a^[104], or -133b^[86], or upregulated miR-206^[116], while in prostate cancer downregulated miR-1^[101,102] and miR-206^[102], miR-133a^[97,102] and miR-133b^[95,96] have been variously reported (Table 3). Either these studies have examined different subclasses of the particular cancer which have different myomiR profiles, or more likely the method of miR detection or the purpose of the study (*e.g.*, relating a particular miR to a particular deregulated target gene) may have influenced the findings reported, leading to some potentially conflicting and inconsistent reports. Additionally, the mature miR isomers are difficult to distinguish by molecular assay and some reports note cross-identification of miR-133b by Taqman mature miR-133a specific probe^[14], which is used in many studies. Others^[114,117] also make this point, that the different miR assay platforms generate different profiles which may obscure or confuse the identity of observed molecular changes, and at minimum generate apparently different miR profiles of the same tissues or diseases^[118,119].

It is essential to accurately determine the specific

expression of particular myomiR isomers and their alleles to understand the control processes of particular pathological states. The expressed alleles of the myomiRs can be assessed accurately by employing pri- or pre-miR RT-PCR assays. For example, only cistron miR-1-2/-133a1 is found expressed in AML, presumably due to specific activation of that cistron^[120] and amongst the myomiRs only pre-miR-133b is elevated in cervical cancer^[121], in bladder cancer^[122], and in progressive prostate cancer^[123]. We suggest that initial microarray profiling be confirmed by pri- or pre-miRNA assay of each miR isomer independently.

Significantly, a deep sequencing profile of miR expression in mouse heart^[124] found that miR-133b is expressed at about 1/6 of the level of miR-133a, reflecting that microarray-based studies may underestimate the relative levels of important miR isomers. Importantly, such deep sequencing analyses also question the canonical view that miR-133b is not expressed in cardiac tissue, reinforcing the need to employ sensitive and accurate analysis to extend our understanding of miR involvement in biological processes. Discrepancies in miR profiles detected between deep sequencing analysis of liver cancer^[344] and microarray/Taqman expression profiling^[95], and in comparison of level 3 expression data from the Cancer Genome Atlas (TCGA) with deep sequence data from ovarian cancer patients^[119] which found only 1 out of 12 survival-associated miRs identified by sequencing correlated by the TCGA data, emphasize the need for robust analytical and computational methods for in-depth profiling of tumors. Expression profiling of

prostate tumors from individual patients by deep sequencing revealed that the expression of numerous miRs changed according to tumor stage^[125]; however qRT-PCR of individual miRs at each tumor stage could not consistently confirm these alterations. A detailed survey of miRs using qRT-PCR accompanied by *in situ* hybridization to confirm the identity of the changed miR expression in matched prostate tumor tissue found less than 50% identity between major altered species when compared with deep sequencing analysis of pooled tumors by the same authors^[126]. Furthermore, a deep sequencing profile of expressed miRs in bladder cancer^[127] also showed that individual patient profiles varied greatly, and remarkably found also that the majority of deregulated miRs are upregulated in tumor tissue compared to matched normal tissue, with up to 3-5 × more upregulated than downregulated species in some patient tumor samples. Generally however, the myomiRs expressed in normal tissue are found downregulated in tumors when analysed by both qRT-PCR and by sequencing platforms. Significantly, another deep sequencing profile of expressed miRs in bladder cancer^[128] revealed a different myomiR expression profile to the above deep sequencing study^[127], yet confirmed the pattern of myomiR expression detected previously by the same group using microarray and qRT-PCR techniques^[93] (Table 3). Overall, these various platform comparisons suggest the need to re-evaluate the genome-wide miR expression profiles of different cancers by use of deep sequencing and then to confirm findings using independent molecular methods.

Cell factors deregulated in cancer by myomiRs

Teicher^[129] (2012) and Frith *et al.*^[130] (2013) noted in sarcoma tumors, which arise in diverse tissues of mesenchymal origin, that the upregulation of cMET (MET), HIF-1 α , IGFR-1 or EGFR, CDK4, MCL1 or mTOR is observed with some elevated frequency in (particular) sarcomas, related to increased cancer severity and enhanced tumor progression, with the significance of each altered pathway likely due to the differentiated tissue in which the sarcoma arises. Downregulated expression of myomiRs occurs in both some sarcomas and carcinomas, linked specifically to the upregulated expression of some of the above gene targets. The different deregulated myomiRs modulate the levels of numerous mRNA/gene targets, but often particular deregulated gene targets are seen in several different cancers. For example, MET is upregulated due to reduced miR-1 levels in colon cancer^[87,101], NSCLC^[100] and rhabdomyosarcoma^[89]. MET binds specifically with HGF, resulting in activation of pathways causing malignant progression *via* increased cell mobility, tissue invasion, and reduction of apoptosis. Reduced miR-1 targetting is only one of several cell alterations which can contribute to MET activation, yet the downregulation of miR-1 relates

significantly to increased cancer severity, indicating the likely importance of miR-1 as a pleiotropic suppressor of several pathways important for tumor development.

Oncogenic membrane receptors, such as IGFR-1 and EGFR are upregulated in association with reduced miR-133 expression in NSCLC^[131], and a similar EGFR upregulation occurs in hormone-sensitive prostate cancer and in breast cancer (Tables 4-6). Furthermore, a marked upregulation of cyclin C, cyclin D1 and CDK4 is seen in skin melanoma tissues associated with the reduced expression miR-206; whilst the BCL2 family of pro-survival molecules (Mcl-1 and BCL2L2) are both strongly upregulated due to a marked decrease in miR-133b levels (> 20-fold) in lung carcinoma tissue^[85]. In addition, a detailed investigation of the role of myomiRs in prostate cancer^[102] found that miR-1, miR-133a (and miR-206) were epigenetically suppressed through promotor modification in numerous tumor samples and in prostate cancer cell lines. *In vitro* studies with prostate cancer cells showed that the downregulation of miR-1 allowed overexpression of multiple target genes that regulate key pathways affecting cell proliferation and cell migration, such as the actin filament network (FN1, LASP1, XPO6, CLCN3, G6PD), cell cycle and DNA damage control (BRCA1, CHEK1, MCM7) and histone acetylation (HDAC4); and with further downregulation of miR-1 the activation of oncogenic genes such as NOTCH3 and PTMA. Cell studies also showed that miR-206 has similar effects on prostate cancer cell biology as miR-1, suggesting that the combined downregulation of the myomiRs contributes significantly to prostate cancer progression.

In addition, FSCN1 expression is elevated in progressive metastatic ESCC, in breast cancer and in high-grade GIST, in part due to the loss of miR-133 expression^[132]. FSCN1 is an actin-binding protein critical to cell adhesion, cell motility, and cell-cell interactions. In normal prenatal pig skeletal muscle, FSCN1 expression increases during major muscle developmental spurts, but postnatally it is expressed highly only in brain tissues during accelerated neural cell development^[133]. The CREB pathway is often activated in different metastatic human cancers, causing the upregulation of FSCN1 expression^[134]. In HNSCC patients, expression of RSK2 and FSCN1 proteins correlate closely. RSK2 protein expression potentiates filopodia formation and cell bundling, increasing cell invasiveness. Overexpression of FSCN1 can rescue the invasion phenotypes in RSK2 knockdown cells, linking RSK2-CREB signalling to the upregulation of FSCN1. In the highly metastatic PDAC^[135], relative FSCN1 expression correlates with expressed HIF-1 α levels, suggesting the hypoxic tumor microenvironment might induce FSCN1 expression, contributing to invasion and metastasis. Taken together, the downregulation of the myomiRs can contribute to the deregulated overexpression of oncogenic cell factors such as FSCN1, TAGLN2,

KLF4, MET (cMET), IGFR and others, each of which can potentiate dysregulation of other cell signalling pathways, enhancing oncogenesis and metastasis (Tables 4-6).

Interestingly, the oncogenic membrane receptors, IGF-1R, TGFBR1 and EGFR are upregulated in NSCLCs due to the downregulation of miR-133a, whilst NSCLC patients with highly expressed levels of miR-133a tend to survive longer^[131] presumably because these target oncogenic proteins are less strongly expressed. In contrast, although downregulation of miR-133a occurs generally in colorectal tumors, tumors with higher miR-133a levels are associated with increased metastasis, adverse clinical characteristics and poor prognosis^[136], suggesting that other cell factors contribute to these differences in outcome. Expression of other targets of miR-133a: LASP1, CAV1, and FSCN1 are also deregulated in a complex pattern. While LASP1 showed negative correlation with miR-133a levels, CAV1 instead had significant positive correlation, which increased in patients with distant metastases, while negative correlation of FSCN1 was only seen in non-metastatic patients. Again the relationship between the deregulated myomiR and its deregulated target(s) display a complexity which suggests the involvement of other factors in early developing and metastatic cancer stages.

The upregulation of yet other cell factors were seen with reduced myomiR expression, such as TAGLN2 with the downregulation of miR-1 and -133a in MSSCC^[137], in RCC^[138], and in CRC, liver cancer and in bladder cancer (TCC) (Table 6). Interestingly, TAGLN2 is a known tumor suppressor, yet its upregulated expression is associated with increased metastasis, and worse patient prognosis. This outcome may be related to findings in HCC where phosphorylation of TAGLN2 by PFTK1 (an oncogenic serine/threonine protein kinase) inactivates its actin-binding function, abrogating its suppression of cell motility^[139]. Furthermore, in mouse metaphase I oocytes after IGF-1 treatment, miRNA-133b was upregulated more than 30-fold and target TAGLN2 was downregulated, stimulating growth and maturation of the oocytes^[140]. These several reports indicate that both the miR-133 isomers may target TAGLN2.

OTHER PLEIOTROPIC PATHWAYS

The (semi-) homologous miRs-1 and -206 are downregulated in many cancers, acting as tumor suppressors (Table 3). The homologues share many of the same oncogenic targets, which may be released from negative regulation in tumors. Recently, Singh *et al.*^[99] (2013) demonstrated that downregulation of miR-1/-206 in lung adenocarcinomas (and NSCLC) have major effects on carbon flux and the activity of metabolic pathways associated with cell proliferation and growth. The inhibition of miR-1/-206 expression

results (indirectly) from elevated NRF2 which in turn increases the activity of redox-sensitive HDAC4, affecting myomiR expression. Importantly, key targets of miR-1-2 and -206 include enzymes of the pentose phosphate pathway (including G6PD, PGD and TKT) and the tricarboxylic acid cycle enzyme GPD2, which increase their activities and potentiate cancer cell proliferation and compromise patient survival. Pleiotropic factors, such as KLF4, HDAC4 and IGFR-1 are also elevated in gastric carcinoma and in breast cancer cells in part due to the loss of miR-206^[108], yet in contrast KLF4 is reduced in colon cancer tissue due to strong upregulation of -206^[116]. KLF4 is a zinc-finger transcription factor that binds to the TGF- β 1 promoter and is important during normal cellular differentiation and proliferation. KLF4 is highly expressed in normal cardiac fibroblasts where it promotes the formation of myofibroblasts^[141].

Some common molecular targets of the isoforms of miR-133 are elevated in different cancers (Tables 4 and 5). Moesin, a member of the ezrin-radixin-moesin protein family which links functionally between the plasma membrane and the actin-based cytoskeleton of cells and is found in invadopodia of metastatic cells^[142], is deregulated in HNSCC, breast cancer, CRC, and prostate cancer. In HNSCC cells both miR-133a/-133b target moesin specifically, and restoration of miR-133b levels in HNSCC cells, as well as in PC10 and H157 lung SCC cell lines, reduces moesin expression, inhibits cell proliferation, cell migration and invasion^[143]. The phenotype is also associated with elevation of other miR-133 targets such as ARPC5 and GSTP1^[115]. Both ARPC5 and GSTP1 levels are elevated in lung-SCC and elevated GSTP1 is seen in bladder cancer (TCC) associated with reduced levels of miR-133a^[144].

Several large cohort studies have examined the changes in expressed miR networks in a variety of cancers. Navon *et al.*^[95], Hart *et al.*^[125], Han *et al.*^[127], Itesako *et al.*^[128] and Volinia *et al.*^[145] noted that the downregulated expression of the *miR-133/-1/-206* genes is associated with a variety of solid tissue cancers, with numerous other miRs also having highly significant oncogenic roles in particular cancer types. In sum, the downregulation of the various myomiRs contributes significantly to the upregulation of oncogenic proteins linked to regulation of cell cycle progression in solid tumors, sarcomas and carcinomas.

UPREGULATED MYOMIRS IN CANCER

In yet other cancers in which the upregulation of a myomiR is associated with worsening metastasis or potentiation of cancer severity, the downregulation of different tumor-suppressor factors appears to be critical. In cervical cancer the upregulation of miR-133b is associated with downregulation of Mst2 protein kinase (STE20), Cdc42 and RhoA, which in turn lead to increased p-ERK and pAKT1 signalling activity,

resulting in tumorigenesis and metastatic cancer proliferation^[121]. RhoA, Cdc42 and other small Rho GTPases are components the Rho-kinase pathway which is a key controller of fundamental cellular processes such as cell motility, cell proliferation, cell division, cell differentiation, cell apoptosis, as well as morphological structure development, epithelial and skin morphogenesis, nerve system and limb development^[146-148]. The downregulation of these key factors restricts cell apoptosis, favouring tumorigenesis. Interestingly, elevated levels of RhoA, Cdc42, Nelf-A/WHSC2 are also observed in hypertrophic cardiac muscle, associated with reduced levels of miR-133a^[6], suggesting that both isomers of miR-133 may normally regulate these factors (in muscle). Similarly, the disruption of the Mst2 pathway is also associated with the disruption of cell apoptosis, of abrogating cell cycle regulation and with increased tumorigenesis in mouse intestinal epithelium^[149], specifically by derepressing the accumulation of Yes-associated protein^[150] which results in strong activation of β -catenin and Notch signalling. Elevation of Notch has also been seen in colorectal tumors associated with reduced miR-1 levels.

In AML the levels of expression of both miR-133a and miR-1 were elevated significantly^[120,151]; similar to multiple myeloma where upregulation of these myomiRs was associated with the downregulation of the cyclin CCND2^[152]. Wang *et al.*^[153] (2007) also found in patients with AML that downregulated CCND2 and CCND3 results in dephosphorylation of phospho-retinoblastoma protein and induction of G(1) cell-cycle arrest. These findings suggest that CCND2 may also be downregulated in AML in association with the upregulation of miR-133a and miR-1 levels, similar to multiple myeloma. During development or regeneration of normal muscle, the downregulation of CCND2 strongly enhances the myogenic terminal differentiation of muscle progenitor cells^[154]. Taken together, the upregulation of these myomiRs in particular cancers can contribute to the deregulated repression of cell factors such as MST2, RHOA, CDC42, CCND2 and others, which then also contribute to dysregulation of other cell signalling pathways, potentiating oncogenesis and metastasis. In contrast, a study of the miR network associated with altered mRNA profiles in AML^[155] found that miRs other than myomiRs had highly significant roles in key deregulated pathways. Overall, the several studies suggest that myomiRs also contribute to development of AML.

LONG NON-CODING RNA

Long non-coding RNAs (lncRNAs) are emerging as important new regulators of oncogenic pathways in cancers, and miRs are emerging as important regulators of lncRNA activities. Recently Wang *et al.*^[156] (2014) reported that deregulated expression of lncRNA UCA1, an important new oncogene in human

bladder cancer (TCC), can be downregulated by miR-1 *in vitro*. These two factors are inversely expressed in bladder cancer tissue *in vivo*. lncRNA UCA1 expression is induced by HIF-1 α which stimulates bladder cancer cell proliferation, migration, and invasion under hypoxic growth conditions^[157]. It is also induced by the transcription factor CCAAT/enhancer binding protein α ^[158], and in another route for potentiation of bladder cancer cell growth and reduction of cell apoptosis the transcription factor Ets-2 binds directly at the UCA1 promoter, stimulating UCA1 promoter activity^[159]. In bladder cancer cell lines the transgenic alteration of lncRNA UCA1 levels positively influences AKT expression and activity, and cell cycle progression could be reduced by inhibition of the PI3-K pathway, indicating that lncRNA UCA1 affected cell cycle progression through CREB^[160]. Taken together, lncRNA UCA1 regulates the cell cycle through CREB and *via* PI3K-AKT-dependent pathways in bladder cancer. Interestingly, several of the cellular factors which are induced by lncRNA UCA1 are also regulated at the expression level by myomiRs. miR-206 targets HIF-1 α directly, hence hypoxia-induced downregulation of miR-206 promotes pulmonary hypertension *via* elevated HIF-1 α in hypoxic rat model pulmonary artery smooth muscle cells (Table 6). Additionally, during regeneration of injured muscle, the AMPK-CRTC2-CREB and Raptor-mTORC-4EBP1 pathways are activated in satellite cells, which involve regulation by miR-1^[58]. The involvement of myomiRs in the regulation of these cellular factors in muscle cells suggests a potential for involvement of lncRNA UCA1 in the regulation of normal cellular processes involving the above protein factors.

Recently, the lncRNA MALAT1 which is upregulated during the differentiation of myoblasts into myotubes in normal muscle biogenesis^[161] was also reported to be upregulated in several non-muscle cancers associated with worsening patient outcomes^[162,163]. In skeletal muscle MALAT1 expression is downregulated by myostatin^[161], whilst the silencing of MALAT1 expression in the mouse myoblast C2C12 cells results in the reduction of SRF transcription factor at both RNA and protein levels as well as reduced myocyte differentiation^[164]. The MALAT1 transcript has a functional miR-133 target site, thus miR-133 acts as a competing endogenous RNA, regulating MALAT1 levels, which in turn modulates SRF activity. SRF also regulates the expression of miR-133a in C2C12 cells by its binding to the miR-133a enhancer^[22], indicating a complex regulatory loop involving SRF, miR-133 and MALAT1.

MYOMIR TARGETS IN MUSCLE AND TUMOURS

Considering the central role of the myomiRs in the cell biology of myogenesis and muscle cell differentiation,

in muscle metabolism, as well as in muscle remodelling and recovery from injury, it may be expected that they regulate the expression of numerous other target genes, in addition to the well documented pathway genes associated with key processes. In non-muscle-tissue cancers in which myomiRs play critical roles, the myomiRs influence expression of a variety of intermediary regulatory pathway genes, causing the potentiation of tumour development and metastasis. Literature searches (PubMed) show that essentially all myomiR targetted genes detected in different cancers have identified roles in several aspects of muscle cell biology. Thus, the myomiRs likely influence the expression of these gene targets in muscle in a normal regulatory manner, yet in non-muscle cancers the deregulated expression of myomiRs contributes to the dysregulated expression of these various gene targets, to the advancement of cancer development.

CONCLUSION

This review examines the diverse and complex regulatory functions of the cistronic myomiRs miR-133, -1 and -206 in numerous tissues. The myomiRs are intimately involved in the regulation of many processes of muscle development, muscle cell metabolism and homeostasis. Indeed some of the myomiRs are critical cell factors that commit stem cells onto the path of muscle cell differentiation and development, and their removal can elicit de-differentiation of committed muscle cells to an undifferentiated state. This centrality of cell regulatory functions suggests that, by necessity, these miRs would also be involved in redeveloping and repairing tissue after damage or injury, and that dysfunctional expression of myomiRs would play important roles during disease states. Furthermore, individual myomiRs have functional roles in the development of numerous non-muscle cells and tissues, beyond their original classification as muscle-specific factors, and hence the observation that myomiRs have roles in an increasing number of different cancer types should perhaps not be surprising. Whilst the myomiRs can display either tumor suppressor or tumor stimulator roles in different cells and tissues, independent cellular function assays confirm that the altered expression of the myomiRs typically correlates with the potentiation of cancer severity. This apparently contradictory ability of miRs to cause tumor suppression or tumor stimulation actions in different tissues relates to the multiple regulatory pathway genes targeted by each miR and the specific regulatory functions targeted in each tissue type. Interestingly, this ambiguity parallels the alternate roles of key signalling pathway regulatory genes in cancers, for example the increased expression of members of the FOX family of genes can cause either tumor suppression or tumor stimulation in different cancer types^[165]. Significantly, the number of validated

targets of each of the myomiRs has increased greatly in recent years, yet the extent to which each myomiR, miR-133, miR-1 or miR-206, contributes to specific tumorigenesis or tumor progression must await fuller clarification and integration with complex cellular regulatory pathway processes which are not yet fully defined.

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Inflammation, oxidative stress and renin angiotensin system in atherosclerosis

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Abstract

Atherosclerosis is a chronic inflammatory disease associated with cardiovascular dysfunction including myocardial infarction, unstable angina, sudden cardiac death, stroke and peripheral thromboses. It has been predicted that atherosclerosis will be the primary cause of death in the world by 2020. Atherogenesis is initiated by endothelial injury due to oxidative stress associated with cardiovascular risk factors including diabetes mellitus, hypertension, cigarette smoking, dyslipidemia, obesity, and metabolic syndrome. The impairment of the endothelium associated with cardiovascular risk factors creates an imbalance between vasodilating and vasoconstricting factors, in particular, an increase in angiotensin II (Ang II) and a decrease in nitric oxide. The renin-angiotensin system (RAS), and its primary mediator Ang II, also have a direct influence on the progression of the atherosclerotic process *via* effects on endothelial function, inflammation, fibrinolytic balance, and plaque stability. Anti-inflammatory agents [statins, secretory phospholipase A2 inhibitor, lipoprotein-associated phospholipase A2 inhibitor, 5-lipoxygenase activating protein, chemokine motif ligand-2, C-C chemokine motif receptor 2 pathway inhibitors, methotrexate, IL-1 pathway inhibitor and RAS inhibitors (angiotensin-converting enzyme inhibitors)], Ang II receptor blockers and renin inhibitors may slow inflammatory processes and disease progression. Several studies in human using anti-inflammatory agents and RAS inhibitors revealed vascular benefits and reduced progression of coronary atherosclerosis in patients with stable angina pectoris; decreased vascular inflammatory markers, improved common carotid intima-media thickness and plaque volume in patients with diagnosed atherosclerosis. Recent preclinical studies have demonstrated therapeutic efficacy of vitamin D analogs paricalcitol in ApoE-deficient atherosclerotic mice.

Key words: Atherosclerosis; Renin-angiotensin system; Inflammation; Oxidants/antioxidants imbalance; Anti-inflammatory drugs; Renin-angiotensin system blockers

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Core tip: There are several reviews in the literature contributed to the pathophysiology, therapeutic options and clinical trials for atherosclerosis. However this is a first review to report the latest cellular and molecular mechanisms of the pathways of atherosclerosis including inflammation, renin-angiotensin system, oxidants/antioxidants imbalance and the efficacy of several therapeutic strategies in improving cardiovascular outcomes, and recent clinical trials reducing the progression of the pathogenesis of atherosclerosis.

Husain K, Hernandez W, Ansari RA, Ferder L. Inflammation, oxidative stress and renin angiotensin system in atherosclerosis. *World J Biol Chem* 2015; 6(3): 209-217 Available from: URL: <http://www.wjgnet.com/1949-8454/full/v6/i3/209.htm> DOI: <http://dx.doi.org/10.4331/wjbc.v6.i3.209>

INTRODUCTION

Atherosclerosis, a continuing chronic disease with inflammation manifesting in the vascular system of blood vessels is the primary origin of cardiovascular diseases (CVDs) in the developed countries of the globe. Its burden is higher in developing countries of Asia, Africa and South America (> 5000 per 100000) than in the developed countries of North America, Europe and Australia (< 3000 per 100000). The mortality due to this disease is anticipated to be at the first place in the globe by 2020^[1]. It is represented by the development of vascular lesions or plaques in the blood vessels following inflammatory/oxidant response to endothelial damage^[2,3]. The plaque mainly composed of blood cells, foam cells, lipids and proteins with calcium accumulation^[3-5]. Finally it results to vascular expansion, vascular blockage, and inhibition of vascular blood flow leading to burst of the vascular wall^[6,7]. In cardiovascular disease, blockage and rupture of atherosclerotic coronary arteries cause myocardial infarction, whereas blockage of carotid arteries cause stroke^[2,6,8]. The impairment of the physiological functions of the endothelium is seen during initial phases of the atherosclerotic lesions due to oxidant damage. Endothelial damage is linked with heart and blood vessels risk factors such as diabetes, high blood pressure, nicotine, lipid disorders, obesity, and disorders of the metabolism^[2,6,9,10]. The renin-angiotensin system (RAS) also plays an important role in the advancement of atherosclerosis by influencing on physiology of the endothelium, inflammatory reactions, thrombosis, and oxidant injury^[9,10]. Ang II causes oxidant damage in vascular system by inducing oxidant species generation *via* activation of NADPH oxidase and these oxidant species oxidize cellular biomolecules including lipids, lipoproteins and DNA leading to endothelial impairment.

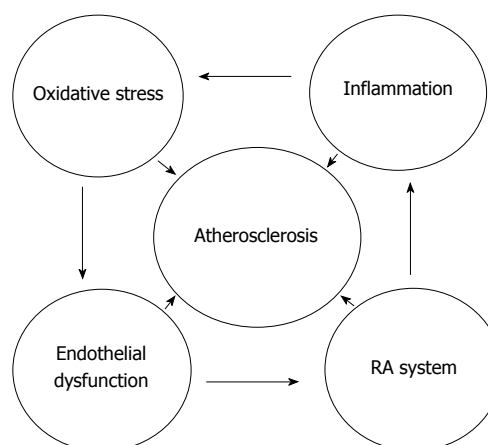


Figure 1 Relationships between inflammation, oxidative stress, renin-angiotensin system, endothelial dysfunction and atherosclerosis. RA: Renin-angiotensin.

The relationship between inflammation, oxidative stress, RAS system, endothelial dysfunction and atherosclerosis is depicted in Figure 1. This mini review presents precisely the mechanistic aspects of the events associated with atherosclerosis, implications of the inflammation, RAS and oxidative stress as well as the efficacy of several therapeutic strategies in improving cardiovascular system, physiology of the endothelium, and ameliorating the advancement of atherosclerotic events. Current clinical trials using anti-inflammatory, RAS blockers and antioxidants in attenuating the atherosclerotic lesions and preserving the pathophysiology of the endothelium is also reviewed.

INFLAMMATION AND ATHEROSCLEROSIS

Atherosclerosis is a concurrent inflammatory disease which first starts in the endothelium of the arterial wall^[3,4,11]. Impairment of the endothelium is the first physiological alteration in the pathophysiology of this disorder which is manifested by enhanced vascular constriction and depressed dilatation of the vascular endothelium as well as changes in the mediators of thrombosis. Endothelium-derived relaxing factor (EDRF) or nitric oxide (NO) plays an important role in preserving the endothelial vasodilatation and inhibiting the vasoconstriction triggered by angiotensin II and endothelin^[3]. Inflammatory processes are manifested by enhanced biosynthesis of mediators of inflammation and thrombosis. The mediators and reactions include interleukin-6, monocyte chemoattractant protein-1 (MCP-1), intercellular adhesion molecule-1 (ICAM-1), endothelial-selectin, adhesion/infiltration of monocytes, oxidation of low density lipoprotein (LDL) and production of foam cells^[12]. Foam cells are formed due to storage of excess cholesterol ester in the macrophages^[12]. The transport of cholesterol regulated by ATP-binding cassette transporter A1 (ABCA1) and transport of

Table 1 Cytokines and factors implicated in causing inflammation in atherosclerosis

Cytokines/factors	Abbreviations
Tumor necrosis factor- α	TNF- α , TNF- α
Interleukine-6	IL-6
Interleukine-1 β	IL-1 β , IL-1 β
Nuclear factor-kappa B	NF- κ B
Monocyte chemoattractant protein-1	MCP-1
C-reactive protein	CRP
Intracellular adhesion molecule-1	ICAM-1
Vascular cell adhesion molecule-1	VCAM-1
Monocyte colony-stimulating factor	M-CSF, MCSF
Transforming growth factor β 1	TGF β 1, TGF- β 1
Plasminogen activator inhibitor-1	PAI-1
Macrophage migration inhibitory factor	MIF
Cyclooxygenase-2	COX-2
Endothelin-1	ET-1
Angiotensin 2	Ang II, ANG II
Endothelial-selectin	E-selectin
Platelet-selectin	P-selectin
Angiotensinogen 2	ANGT 2
Leptin	LEP
Inducible nitric oxide synthase	iNOS
Matrix metalloproteinase	MMP
Low density lipoprotein	LDL
Serum amyloid A	SAA
Apolipoprotein E	ApoE
Tumor necrosis factor receptor -associated factor	TRAF
Reactive oxygen species	ROS
Angiotensin type 1 receptor	AT1R
Glutathione peroxidase 1	GPx1
Phospholipase A2	PLA2
Lipoprotein-associated phospholipase A2	Lp-PLA2
5-Lipoxygenase-activating protein (FLAP)	FLAP
5-Lipoxygenase	5-LO
Chemokine motif ligand 2	CCL2
Chemokine motif receptor 2 (CCR2)	CCR2
Toll like receptor 2	TLR2
Heat shock protein	HSP

oxidized LDL through CD36 regulate the excess of cholesterol ester in the macrophages^[12]. Apart from excess foam cells, growth of smooth muscle/endothelial cells^[3,13], collagens, matrix metalloproteinases (MMPs), fibronectin, and elastin are also responsible for plaque development^[2,3,11]. Evidences suggest that cytokines and tissue factors also regulate pathophysiology of the endothelium due to inflammatory reactions. The influence of different cytokines and factors modulating the pathophysiology of the vessel wall is depicted in Table 1. Among the biomarkers of inflammation C-reactive protein (CRP) is important which is generated by hepatic cells and is also modulated by IL-6, IL-1 and TNF- α ^[14]. Evidences suggest that raised blood CRP level is one of the inflammatory biomarkers and predictors of cardiovascular diseases^[15,16]. It is also implicated in the advancement of atherosclerotic lesions by regulating physiology of endothelium^[3,17,18]. It enhances the production of VCAM-1, ICAM-1, selectins, and MCP-1 in the endothelium through induction of powerful constrictor of the vessels ET-1 and IL-6^[3,17]. It ameliorates the synthesis of NO in the endothelium by depressing the transcription and translation of enzyme

NO synthase^[3,19]. It also plays a significant role in cooperating with the activities of other cytokines and factors. CRP induces the biochemical synthesis and physiological functions of PAI-1 in the endothelium^[19]. PAI-1 is known to be actively involved in thrombosis during atherosclerosis process and inhibits destruction of the fibrin clot by suppressing plasminogen activation^[20]. There is a good correlation between elevated blood PAI-1 concentration and death rate in patients with coronary heart diseases^[20]. Apolipoprotein E as well as low-density lipoprotein (LDL)-receptor knock out animals display speedy atherosclerotic lesions^[21,22]. These animals also have sizeable counts of macrophages/T cells in their plaques. Cross breeding of apolipoprotein-E knock out with T-cell knock out and mice with deficient macrophages (osteoporotic op/op) revealed the influence of immune cells in the progression of atherosclerosis^[23]. Inflammatory reactions are not only involved in progression of human vascular plaques generation but also have important role in the rupture of internal arterial plaques transforming chronic disorder into an acute thromboembolic disease. Several factors are implicated in the rupture of internal arterial plaques comprise of cytokines, cyclooxygenase-2, matrix metalloproteinases, and tissue factors^[1,4,23]. Experimental evidences support crucial role for inflammatory reactions as a connection between risk factors for atherosclerotic disorder and pathophysiologic complexity of the disease^[2]. Serum amyloid A (SAA) protein has also been implicated in the inflammatory reactions associated with atherosclerotic disorder and used as a biomarker for cardiac and vascular disorders as well as heart and vessels outcome^[24]. TNF- α is one of the inflammatory cytokines involved in commencement as well as development of atherosclerosis. It induces transcription factor nuclear factor- κ B (NF- κ B), a key factor in the pathways of inflammation. In the process of atherosclerosis NF- κ B induces the transcription of VCAM-1, ICAM-1, MCP-1, and E-selectin in smooth muscle/endothelial cells of the blood vessels^[25]. TNF- α depletes NO levels in the endothelium causing decrease of endothelial dilatation leading to dysfunction of the endothelium^[26,27]. TNF- α has been reported to cause apoptosis of the endothelial cells through dephosphorylation of protein kinase B (Akt) leading to endothelial damage^[28,29]. Resistin exerts inflammatory reactions/vasoactive effects in cultured cells of the endothelium^[8]. In atherosclerotic process resistin induces transcription of cellular factors such as VCAM-1 and MCP-1^[30]. Cells from endothelium exposed to resistin deplete the levels of TNF receptor-associated factor (TRAF-3) which is a well known inhibitor of the endothelial activation^[31]. It is suggested that augmented resistin concentration causes a significant dysfunction of the endothelium through activation of endothelial system. Furthermore, resistin exposure activates endothelial cells by increasing ET-1 release through induction of transcription of ET-1 indicating its role in the impairment of the endothelium^[32]. Leptin

up regulates ET-1 as well as NO synthase biosynthesis in the endothelial cells and augments generation of free radicals and oxidants^[33,34], causing oxidative stress^[35]. Leptin also increases the cellular growth as well as migration of cells of the endothelium^[36] and cells of smooth muscle^[37]. It induces the synthesis of MCP-1 in the cells of the aortic endothelium^[38]. It enhances the aggregation of the platelets and vascular thrombus formation through leptin receptor pathways^[33,34]. It directly augments concentrations of monocyte colony-stimulating factor (M-CSF)^[39], increases cholesterol levels in hyperglycemia^[40], and promotes new blood vessel formation^[41]. We have shown in our earlier studies elevated concentrations of factors involved in inflammatory pathway namely TNF- α , MCP-1, Cox-2, TGF- β 1, iNOS, and Mn-SOD in ApoE-deficient atherosclerotic mice^[42,43] proving the vascular inflammation as an integral process in the atherosclerotic pathophysiology.

OXIDATIVE STRESS AND ATHEROSCLEROSIS

Oxidative stress is referred as imbalance of the cellular oxidants and antioxidants in the body. In atherosclerosis process Ang II causes oxidants/antioxidants imbalance in the vascular system by inducing oxidant species generation *via* activation of NADPH oxidase^[44,45]. Earlier studies have shown that excess superoxide generation due to NADPH oxidase activation causes inflammation and further generation of inflammatory cytokines (TNF- α) through NF- κ B activation^[46-48]. The role of NF- κ B activation is also demonstrated in atherosclerosis^[47,49]. These reactive oxygen species (ROS) initiate vascular membrane lipid peroxidation leading to inflammation and production of TNF- α *via* NF- κ B induction^[46,47] and other factors namely VCAM-1, MCP-1, TGF- β 1, Matrix metalloproteinase 9 (MMP9), iNOS and Mn-SOD^[50-52]. The ROS up regulate atherosclerotic events namely cell infiltration, migration, adhesion and platelet activation. These ROS oxidize cellular biomolecules including lipids, proteins and nucleic acids causing endothelial impairments^[53]. However, cells are equipped with an intricate cellular defense system that includes antioxidant enzymes namely superoxide dismutase (SOD), catalase and glutathione peroxidase (GSHPx), tripeptide glutathione (GSH), antioxidant vitamins A, C, and E to scavenge oxidant species thereby attenuate oxidant injury^[54]. Most importantly, depletion of a major cellular antioxidant such as GSH has been reported to cause vascular dysfunction^[42,55-57]. The vascular system has also been shown to be equipped with antioxidant defense to combat oxidant damage^[42,55,56]. However depletion of cellular antioxidants increases oxidant species build up leading to pathological and physiological impairments^[42,43,55-57]. Several studies in ApoE knock out mouse model revealed down regulation of antioxidants in atherosclerosis^[42,43,58] which

suggests a relationship between declined antioxidants and enhanced pathological lesions. On the contrary, over expression of catalase which hydrolyses H₂O₂, suppressed tissue injury in ApoE knock out mouse model^[21] indicating the implications of H₂O₂ degrading enzyme in atherosclerosis. Furthermore, additional studies suggest defensive capacity of glutathione peroxidase (GPx1) in atherosclerosis^[58]. Down regulation of erythrocyte GPx1 level was linked with enhanced risk of heart and blood vessel injury and patients with atherosclerosis of carotid artery possess declined GPx1 levels^[58]. Knock down of GPx1 in animal enhanced lipoprotein oxidation and depleted NO levels causing impairment of endothelium^[58]. Diabetes-related atherosclerotic events are enhanced with deficit of GPx1 through increased inflammation and thrombosis in ApoE-deficient mouse model^[58]. Several studies reported a lower level of tissue antioxidants in patients with renal and cardiovascular diseases^[6,59-61].

RAS AND ATHEROSCLEROSIS

The renin-angiotensin system is thought to have an important implication in the pathophysiologic injury in atherosclerosis through induction of various cellular/molecular reactions^[10,61,62]. Previous studies showed that angiotensin II (Ang II) produced by vascular tissues increases generation of ROS and induces synthesis of several factors *via* induction of AT II type 1 receptor (AT1R), leading to stockpile of inflammatory mediators and proliferation/migration of vascular cells^[1,10,62]. These findings indicate that regional impact of AT1R activation in vascular wall plays a critical role in pathophysiology of concurrent inflammatory reactions through a direct action on local vascular cells. Several studies demonstrated an essential part of RAS in pathophysiology of heart and vascular system, including atherosclerotic disease^[1,6,10,62,63]. Angiotensinogen (ANGT), a precursor of ANG II, is synthesized in fat cells^[64]. Angiotensin II causes oxidative stress in cardiovascular systems by inducing ROS production *via* induction of NADPH oxidase^[44,49]. These oxidant species begin oxidation reaction of lipids in the membranes of the blood vessels leading to inflammation and generation of inflammatory cytokines (TNF- α) through NF- κ B activation^[1,2,11,23,63,65]. These ROS oxidize cellular biomolecules namely lipids, proteins and nucleic acids causing oxidation of membrane phospholipids resulting in the impairments of the heart and blood vessels^[1,2,11,23,57,63,65,66]. Vasoconstrictor Ang II instantly triggers the synthesis of ICAM-1, VCAM-1, MCP-1, and macrophage colony stimulating factor (M-CSF) in the walls of the blood vessels through induction of NF- κ B-regulated genetic products^[67]. Moreover Ang II stimulates production of oxidant species from NO leading to depletion of NO causing injury to blood vessels^[3]. Enhanced ANGT 2 levels is associated with new blood vessels formation^[68] and elevation of blood pressure^[44,56,57]. These two disorders are closely linked

with impairments of the endothelium. Additionally, CRP induces ATR1-receptor transcription and translation as well as enhanced AT1-receptor levels in blood vessel wall^[69]. Importantly, activation of ATR1-receptors promote Ang II-induced ROS generation, migration/proliferation/remodeling of the cells of the blood vessels^[70].

TREATMENT OF ATHEROSCLEROSIS

Use of anti-inflammatory agents

The influence of inflammatory reactions on the progression/maturation and rupture of thrombus in the vascular lumen of the blood vessels opens new therapeutic strategies for atherosclerotic disorder. One of the clinical trials (JUPITER) corroborates clinical advantage of the evaluation of the degree of inflammation in managing the therapeutic intervention to restrict the occurrence of the injury to the heart and vascular system. Academic and theoretical concepts of inflammatory reactions are being now utilized as a therapeutic tool in the clinics for the risk assessment as well as targeted therapeutics^[2]. Current therapeutics effectiveness in preventing atherosclerosis such as HMG-CoA reductase inhibitors (statins), acetylsalicylic acid (ASS), and RAS blockers deploy their influence through modulation of inflammation in the blood vessels^[71]. Attentions are also focused to various therapeutic agents decreasing different modulators of inflammation process. Among these drugs are derivatives of thiazolidinediones (glitazones), HMG-CoA reductase inhibitors (statins), acetyl salicylic acid, ACE inhibitors, and ATR blockers. Glitazones are activators of nuclear peroxisome proliferator-activated receptor (PPAR)- γ . Glitazones are known to down regulate various factors involved in the process of inflammation and their activities therefore they can ameliorate the advancement of atherosclerotic events. Glitazones are also known to deplete TNF- α level in fat cells and abrogate TNF- α -induced synthesis of VCAM-1 and ICAM-1 in the endothelium of the blood vessels^[8,17]. Rosiglitazone has been shown to reverse the progression of atherosclerotic activities of CRP in the endothelium of the blood vessels^[8]. Glitazones also depress leptin-induced migration of the cells of the endothelium^[33]. Glitazones inhibit resistin concentration in fat tissues^[72,73]. Alternatively, inhibitors of matrix metalloproteinase (MMP) activity as well as vaccines are presently under developmental processes and clinical trials^[23]. There are several anti-inflammatory agents have been used pre-clinically and clinically in the treatment of atherosclerotic cardiovascular diseases such as phospholipase A2 (PLA2) inhibitors^[74], phospholipase A2 of lipoproteins (Lp-PLA2) inhibitors^[75], 5-LO-activating protein (FLAP)^[76], arachidonate 5-lipoxygenase (5-LO)^[77], chemokine motif ligand 2 (CCL2), chemokine motif receptor 2 (CCR2) inhibitors^[78], methotrexate^[15,65], and

IL-1 pathway inhibitor^[79].

Use of antioxidants

Antioxidant therapy has been shown to ameliorate cardiovascular oxidative stress by scavenging excess ROS and up regulating the antioxidant defense system^[54,80]. The antioxidant N-acetylcysteine is reported to abrogate accelerated atherosclerotic events in ApoE knockout mouse models^[80]. Our recent study demonstrated that vitamin D analog paricalcitol ameliorated the oxidative vascular injury by suppressing ROS-generating enzyme NADPH oxidase activity and inflammatory mediators and by up regulating the antioxidant defense system in ApoE-deficient mice^[42,43]. There is a novel strategy against inflammation is being used by inhibiting the oxidation of lipoproteins to stop their entry into the cells of the blood vessels. The ARISE clinical trial investigated the influence of antioxidant succinobucol (AGI-1067) in patients who had myocardial infarction/ruptured atherosclerotic plaques had significant curative effects^[81]. In preclinical investigation using antioxidant enzyme GPx1 afforded significant protection in a mouse model of atherosclerosis^[58].

Use of RAS inhibitors

In preclinical and clinical investigations putative drugs used for high blood pressure have been reported to adequately suppress the events of atherosclerosis^[6,62,63]. The inhibitors or blockers/antagonists of RAS such as ACE inhibitors and ATR blockers act either by depleting the generation of Ang II or by blocking the binding of Ang II to its receptors. RAS inhibitors or blockers/antagonists have both ancillary and concurring actions that enhance the NO concentration, decrease oxidants/antioxidants imbalance, inhibit RAS-induced inflammation, as depicted by actions of these drugs on several biomarkers of inflammation^[6,10,17,23,62,63,82]. The above biochemical alterations achieve amelioration in the activities of the endothelium and pathophysiology of the vascular system in atherosclerosis. Additionally above biochemical alterations are also linked with advantage over lowering the blood pressure in patients with atherosclerosis and high blood pressure. These investigations have demonstrated that olmesartan medoxomil administration suppress the advancement of atherosclerotic process and significantly ameliorate the cardiovascular dysfunctions^[10,63]. ATR1 blockers have been shown to decrease the new blood vessel formation in ApoE-deficient mouse model of atherosclerosis, through toll like receptor 2 and 4 (TLR2/TLR4)-arbitrated events of inflammation and activation of MMP, consequently inhibiting the proliferation of the vascular lesions as well as rupture of the plaques^[83]. Our recent studies have shown that ACEI enalapril ameliorated the oxidative vascular injury by suppressing ROS-generating enzyme NADPH oxidase activity and inflammatory mediators and by up regulating the antioxidant defense

system in ApoE-deficient mice^[42,43].

Use of other agents

Preclinical as well as clinical studies have clearly demonstrated and provided the evidence that vitamin D and its analogues suppresses the various factors and biomarkers associated with disorders of vascular system and permeate cells of the immune system against the activities of the inflammation^[5,9,84-87]. The knock down of vitamin D receptor (VDR) and VDR-mediated signaling pathways in animals induces hypertension by elevating renin release from the kidneys and stimulates the process of atherosclerosis conceivably through regional induction of cellular RAS^[9]. The insufficiency or depletion of vitamin D causes hypertension and enhances the progression of atherosclerotic lesions in animals^[5]. Our recent study demonstrated that vitamin D analog paricalcitol ameliorated the oxidative vascular injury by suppressing ROS-generating enzyme NADPH oxidase activity and inflammatory mediators and by up regulating the antioxidant defense system in ApoE-deficient mice^[42,43]. In animal model when vaccines derived against oxidized LDL and heat shock protein (HSP) administered have demonstrated inhibition of inflammation and progression of atherosclerotic lesions^[88]. The prospect of clinical application of vaccines for prevention or treatment of atherosclerosis is presently under exploration^[23]. The antibodies raised against the oxidized lipoproteins not only reveal activity of the disorder but also confer the prevention and treatment of atherosclerosis^[7,63].

CONCLUSION

The investigations delineated in the present mini review furnish to our comprehending the role of inflammatory events, oxidants/antioxidants imbalance and RAS pathways in the pathophysiology of atherosclerosis as well as targeted therapeutic intervention. Preclinical as well as clinical investigations clearly demonstrate that inflammatory reactions operates all stages of atherosclerotic events, including commencement, advancement, and the complexity of the lesions. Inflammatory process is commonly associated with several risk factors for atherosclerotic plaque formation and modified pathophysiology of the blood vessels. Alteration of implicated risk factors has a clinical advantage through abrogating the inflammatory reactions and its consequences. Convincing verifications from clinical studies authenticates the application of the drugs inhibiting the inflammation process; RAS blockers and antioxidants as a therapeutic regimen that can prevent and treat the atherosclerotic lesions.

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Techniques to elucidate the conformation of prions

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Abstract

Proteinaceous infectious particles (prions) are unique pathogens as they are devoid of any coding nucleic acid. Whilst it is assumed that prion disease is transmitted by a misfolded isoform of the cellular prion protein, the structural insight of prions is still vague and research

for high resolution structural information of prions is still ongoing. In this review, techniques that may contribute to the clarification of the conformation of prions are presented and discussed.

Key words: Prion; Amyloid; Neurodegenerative disease; Protein structure; Fourier-transform infrared spectroscopy

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Core tip: Prions (proteinaceous infectious particles) are misfolded isoforms of cellular proteins that cause neurodegenerative diseases in mammals and humans. Several structural models are available for prions but a 3D-structure does still not exist. More structural information is demanded for the understanding of the conversion process and finally for the design of efficient therapeutic approaches. In this review, techniques that may contribute to the clarification of the conformation of prions are presented.

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INTRODUCTION

Prions are infectious proteins that cause fatal neurodegenerative diseases^[1-5]. Rearrangements of the structure of the cellular prion protein (PrP^C) are the key of infection. PrP^C is characterized by a high α -helical content and conversion in a β -sheet rich conformer (PrP^{SC}) transforms it into an infectious protein^[6,7]. According to the "prion hypothesis" the protein itself is the causative agent of disease even though several cofactors that may influence the conversion process are discussed in the literature^[2,8-12]. A 3D-structure of PrP^{SC} does still not exist, albeit several structural models for

PrP^{Sc} are described^[13]. Definite structural information about prions is indispensable for the understanding of the conversion process and eventually for the design of new therapeutic strategies.

In the present review, several techniques to assess the conformation of prions are presented and discussed with a main focus on the application of Fourier-transform infrared spectroscopy. The intention of this mini-review article is to give an overview of current techniques used in prion research to elucidate the structure of prions. For details readers are referred to the literature cited in the text.

FOURIER-TRANSFORM INFRARED SPECTROSCOPY

Fourier-transform infrared (FTIR) spectroscopy was one of the fundamental techniques that had demonstrated the high β -sheet content of PrP^{Sc} in contrast to PrP^C^[14-16]. This technique has several advantages compared to other analytical approaches. With FTIR spectroscopy protein samples can be analyzed (1) without labeling; (2) under native conditions; (3) with only minute amounts of material (in particular in case of micro-FTIR); and (4) in a dynamic process. Moreover results can be obtained within a very short time^[16,17]. FTIR spectroscopy elucidates primarily the secondary structure of proteins and originates essentially from C=O stretching vibrations of the amide groups of the protein backbone. For the structural characterization of PrP^{Sc} the amide I band is the most useful infrared absorption band^[18-20].

FTIR of proteins typically is performed in H₂O, D₂O or under dried conditions. For spectroscopic analyses the purity of sample material is of utmost importance^[16,21]. This is particularly challenging when sample material has to be extracted from infected tissue and when the protein of interest is present in only small amounts. Improved purification protocols for the extraction of prions from tissue have been published and allowed us to analyze tiny amounts of sample material by micro-FTIR^[16]. Micro-FTIR can be applied on dried protein samples using a FTIR spectrometer linked to an IR-microscope. This enables the performance of measurements on pure protein samples in the range of only a few nanograms.

Micro-FTIR has been shown to be an appropriate tool for the screening of different prion strains and *in vitro* generated prion protein (PrP)^[16,17]. Particularly in case of *in vitro* generated PrP only very small amounts of prions are expected to be extractable. This could impede an analysis by other structure-sensitive techniques [e.g., circular dichroism (CD) spectroscopy]. We recently recorded spectra from PrP^{Sc} molecules generated by protein misfolding cyclic amplification (PMCA) using micro-FTIR^[16]. PMCA mimics the conversion of PrP^C to PrP^{Sc} *in vitro*^[22,23]. Comparing spectra from native- and PMCA-derived PrP^{Sc} revealed structural differences

of molecules before and after PMCA. This technical progress provides the possibility to analyze the influence of cofactors on the conversion activity and putative correlating structural rearrangements in future experiments.

As mentioned above, micro-FTIR is also suitable to discriminate different prion strains^[14,24,25]. In prion research, strains are defined as prion-isolates that, after inoculation into distinct hosts, cause disease with consistent characteristics, such as incubation period, distinct patterns of PrP^{Sc} distribution and spongiosis in the brain (Figure 1). A concrete example of use would be strain typing for chronic wasting disease (CWD)^[17]. CWD is a prion disease occurring in cervids in North America and rapidly spreads among free ranging deer and elk^[26,27]. To test whether and to which extent there are different strains for CWD in the environment or whether new strains emerge in the future, micro-FTIR (with only 100 mg of infected tissue needed to obtain a sufficient amount of protein extract for FTIR analysis) can be applied. The use of small amounts of sample material allows the preparation of multiple samples within a short time. Spectra from CWD field samples collected in the past, present or future could be compared. As different strains can correlate with different zoonotic potentials a rapid structural scan by FTIR may be suitable for a proper risk assessment.

OTHER TECHNIQUES TO DETERMINE THE STRUCTURE OF PRIONS

Electron microscopy analyses directly reveal the morphological difference between PrP^C and PrP^{Sc} as the latter appears as amyloid fibrils^[28,29]. Moreover, it is possible to distinguish between some prion strains due to their distinct morphological shape. Electron crystallography had a limited scope to predict general conformational features because prions tend to aggregate^[30,31]. Atomic force microscopy (AFM) is used as an alternative technique that needs no staining of samples^[16,32]. AFM can be combined with infrared- and Raman-spectroscopy suitable for site-directed sample characterization. Tip enhanced infrared- or Raman-spectroscopy principally allow measurements on defined positions within a prion fibril^[33,34]. These techniques could be useful for the analyses of mixtures of prion fibrils ("quasi species") and would allow a precise biochemical characterization of prions^[17,35].

The impact of specific amino acids on the prion conversion process can be determined by mutational analyses when subsequently tested in animal-, cell culture- or *in vitro* conversion assays^[36-39]. By this means it could be demonstrated that specific amino acids accelerate or decelerate the conversion process and that the N-terminus is not essential for prion infectivity^[40]. Limited proteolysis with Proteinase K is used to discriminate PrP^C from PrP^{Sc} as the conversion in a β -sheet rich conformation (PrP^{Sc}) prevents the

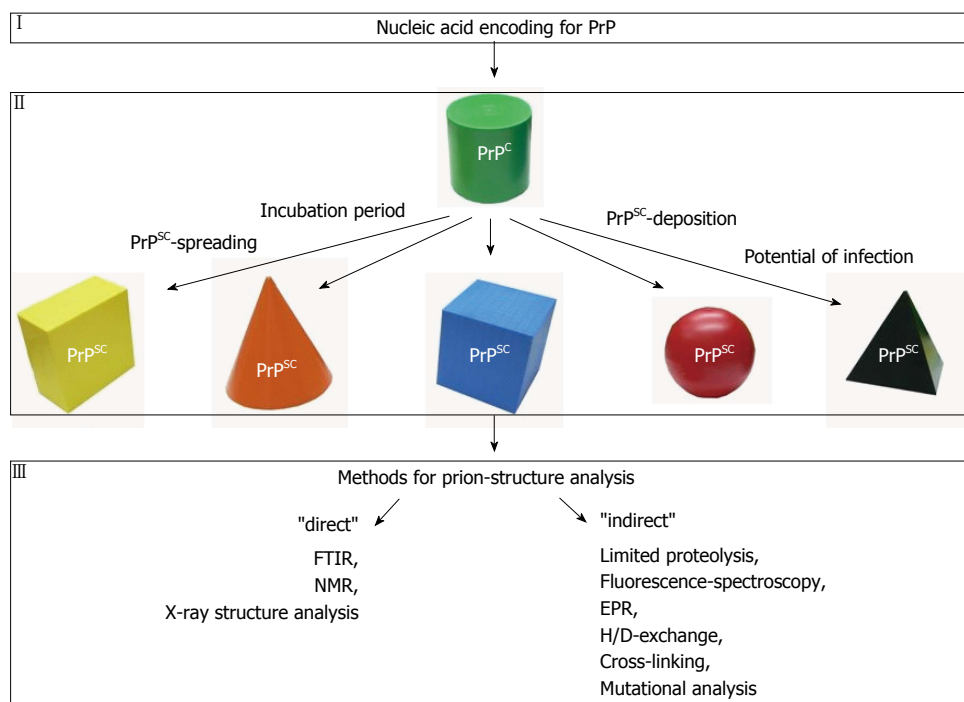


Figure 1 Different conformations of the prion protein. In mammals and humans both isoforms of prion protein (PrP) [the cellular isoform (PrP^C) and the misfolded isoform (PrP^{SC})] are encoded by the same gene (I). PrP^C is shown in green (II). Under specific conditions PrP^C can be transferred into a misfolded and then putatively infectious conformation (PrP^{SC}). Prion strains can adopt different conformations (indicated by different symbols and colors). When a misfolded isoform of the prion protein becomes infectious it is characterized by specific features as incubation period, PrP^{SC}-spreading, PrP^{SC}-deposition and a distinct potential of infection. For structural analyses of misfolded prion proteins several methods are available. While FTIR, NMR and X-ray structure analyses provide information about the secondary structure as well as 3D-structural information ("direct"), limited proteolysis, fluorescence spectroscopy, EPR, H/D-exchange, cross-linking or mutational analyses reveal more "indirect" structural information that is focused on specific residues within the protein. FTIR: Fourier-transform infrared.

C-terminus from degradation while the less structured N-terminus is cleaved off^[41-46]. Limited proteolysis at different pH values, with rising amounts of protease or in the presence of chaotropic salts can give further structural information and may help to discriminate prion strains with different conformations.

Recombinant PrP can (after site-directed mutagenesis) site-specifically be labelled with fluorophores or spin labels and then analyzed by fluorescence- or EPR spectroscopy, respectively. Such studies propose a parallel in-register β -sheet structure of PrP amyloids and enable the calculation of distances between specific residues within an amyloid fibril^[11,47-49]. It has to be mentioned that misfolded recombinant PrP without the addition of cofactors usually lacks infectivity and may therefore not exactly represent the *in vivo* structure of PrP^{SC}^[11].

The exchange of hydrogen for deuterium ions and subsequent analysis by mass spectrometry allows the detection of unstructured and highly structured regions within PrP^{SC}^[50-52]. The C-terminal part of prions shows low exchange rates indicating a highly structured conformation^[53].

While the above mentioned techniques reveal basic and to some extent indirect structural insights, high resolution structure information is needed to understand the conversion process of PrP. High resolution 3D-structural data from X-ray fiber diffraction and NMR are available for PrP^C but not for the complete PrP^{SC}

molecule. Because of the insolubility and the propensity to aggregate only parts of PrP^{SC} are structurally resolved until now^[54-57].

CONCLUSION

Misfolding of a cellular protein into an infectious isoform sharing the same primary structure is unique for prions. This phenomenon is currently discussed to be a general principle for other neurodegenerative diseases^[58-60]. Prions lack a coding nucleic acid thus diagnosis and the detection of misfolded isoforms cannot be done by genetics but *e.g.*, by structural characterization.

A lot of structural models for PrP^{SC} exist but as discussed recently by Requena and Wille "the results and/or their interpretation remain controversial"^[61]. For a better understanding of the conversion of PrP^C to PrP^{SC}, more structural data from biochemical and biophysical experiments are required for PrP^{SC} (Figure 1). Therapeutic strategies that aim at the prevention of this misfolding process would benefit from such a progress.

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Essential roles of four-carbon backbone chemicals in the control of metabolism

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Abstract

The increasing incidence of obesity worldwide and its related cardiometabolic complications is an urgent public health problem. While weight gain results from a negative balance between the energy expenditure and calorie intake, recent research has demonstrated that several small organic molecules containing a four-carbon

backbone can modulate this balance by favoring energy expenditure, and alleviating endoplasmic reticulum stress and oxidative stress. Such small molecules include the bacterially produced short chain fatty acid butyric acid, its chemically produced derivative 4-phenylbutyric acid, the main ketone body D- β -hydroxybutyrate - synthesized by the liver - and the recently discovered myokine β -aminoisobutyric acid. Conversely, another butyrate-related molecule, α -hydroxybutyrate, has been found to be an early predictor of insulin resistance and glucose intolerance. In this minireview, we summarize recent advances in the understanding of the mechanism of action of these molecules, and discuss their use as therapeutics to improve metabolic homeostasis or their detection as early biomarkers of incipient insulin resistance.

Key words: Butyric acid; D- β -hydroxybutyrate; Histone deacetylases; Histone deacetylases inhibitors; Insulin resistance

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Core tip: Recent research demonstrated that the four-carbon molecule butyrate, and butyrate-related molecules (4-phenylbutyric acid, D- β -hydroxybutyrate and β -aminoisobutyric acid), act as modulators of metabolism, favoring energy expenditure. Conversely, another butyrate-related molecule, α -hydroxybutyrate, is an early predictor of insulin resistance and glucose intolerance. In this minireview, we summarize the recent progress in the understanding of the mechanism of action of these molecules and discuss their possible therapeutic use to improve metabolic homeostasis and their usefulness as early biomarkers for insulin resistance.

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INTRODUCTION

Accordingly to World Health Organisation figures, the increased incidence of overweight and obesity worldwide constitutes a major risk factor for global deaths in both developing and industrialized countries^[1]. Obesity predisposes individuals to the development of several non-communicable diseases, including cardiovascular disease, dyslipidemia, insulin resistance and type 2 diabetes (T2D), and certain cancers^[2]. Furthermore, obesity engenders psychological consequences in affected individuals, especially in the young^[3], and recent research indicates that a predisposition to metabolic disease linked to obesity can be transmitted trans-generationally through epigenetic mechanisms^[4].

Obesity is the most straightforward consequence of an energy imbalance. Indeed, body weight results from the difference in energy intake, determined by food consumption, and total energy expenditure required to sustain vital functions, including basal metabolism, thermoregulation, and exercise. If the balance is negative, stored fat is mobilized to provide calories, resulting in weight loss. Reciprocally, excessive food intake results in fat accumulation, and ultimately obesity.

Lifestyle interventions, including changes in diet and increased physical activity, are the simplest way to control body weight. Yet, these interventions are often limited by social and economic constraints, such as restricted access to healthy food and environments conducive to physical activity, especially for people already obese or overweight^[5]. Consequently, extensive research has been conducted to discover therapeutic agents that might diminish or reverse the negative consequences of the western diet on body weight and associated health effects.

Interestingly, short chain fatty acids (SCFAs) and molecules related to the 4-carbon SCFA butyric acid (Figure 1) have been shown to exert beneficial effects on the control of body weight and metabolism. Here we summarize the current knowledge about these molecules, their biological source of production and physiological mode of action.

BUTYRIC ACID AND SHORT CHAIN FATTY ACIDS

SCFAs, also known as volatile fatty acids, are carboxylic acids containing up to 6 carbons in their aliphatic chain. Accordingly to the number of carbons, SCFAs include acetic (C2), propionic (C3), butyric (C4), valeric (C5) and caproic (C6) acids. SCFAs are mainly produced by the gut microbiota using dietary fibers as the major substrate. Therefore, the amount of SCFAs produced is

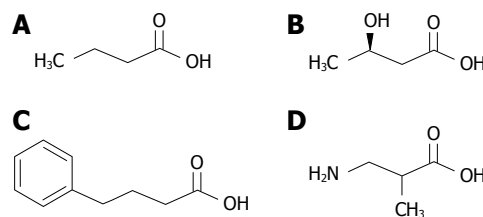


Figure 1 Molecular structures of (A) butyric acid, (B) D-β-hydroxybutyric acid, (C) 4-phenylbutyric acid and (D) β-aminoisobutyric acid.

affected by environmental, dietary and microbiological factors^[6], with a plant-based diet favoring the production of acetate and butyrate^[7].

The gut microbiota is acquired postnatally and its composition is affected by both host genetics and dietary habits. For example, in mice, a high fat and high sugar diet administered to different inbred strains induced a strain-specific propensity to obesity and strain-specific effect on the composition of their gut microbial communities^[8].

The dynamic composition of the gut microbiota is a reflection of the host's physiological status^[9]. A metabolic diseases like T2D, resulting from both genetic predisposition and environmental factors, is a good example of the interplay between the disease and the microbiota, as gut bacterial dysbiosis was observed in type 2 diabetic patients, with a decline in butyrate-producing bacteria^[9,10].

It has been suggested that diets that favor the production of SCFAs by the microbiota may have multiple positive effects on host metabolic regulation. For example, the intake of dietary fibers, which contains indigestible and fermentable carbohydrates, exerts a positive action on the central regulation of satiety and appetite, leading to reduced weight gain and adiposity^[11-13]. Some dietary fibers have been found to have a positive effect by decreasing weight gain and energy intake in mice when supplemented to a high fat diet^[13], preventing insulin resistance and the development of T2D^[14], and improving immune responses.

The interplay between SCFAs and the immune system

It has been shown that SCFA act on T cells to enhance the generation of Th1 and Th17 cell populations, boosting immunity to fight pathogens. At the same time SCFAs induce T cell production of IL-10 and favour the expansion of FOXP3-positive regulatory T cells, helping preventing inflammatory responses^[15]. The intestinal epithelium, which separates the exterior environment from the inner organism, is constantly exposed to extrinsic pathogens: viruses, bacteria and their products, that interact with the host's immune system^[16]. The gut microbiota interacts with the immune system and, depending on its composition, promotes or limits inflammatory responses in the intestine. In fact, a mixture of Clostridia strains orally administered in a mouse model of colitis, was shown to induce

T-regulatory (Treg) cells and promote the production of anti-inflammatory molecules including interleukin-10 (IL-10) and inducible T-cell co-stimulator (ICOS) in Treg cells^[17]. The interplay between microbiota and immune system is mediated by bacterially produced metabolites, especially butyrate and other SCFAs. Indeed, SCFAs increase the number of Treg cells through the potentiation of their extrathymic differentiation^[18]. Also, butyrate's histone deacetylase (HDAC) inhibitor properties induce histone H3 acetylation on the Foxp3 promoter in Treg cells resulting in Foxp3 transcriptional upregulation and alleviation of inflammation in the intestine^[18,19]. Another SCFA, propionate, exerts the same actions of butyrate on Treg cells, also through HDAC inhibition^[18].

Inflammation and the development of obesity:

The existence of a correlation between obesity and a sub-clinical inflammatory state is widely accepted^[20]. The chronic inflammation observed in adipose tissue in the obese is attributed to the infiltration of immune cells, mainly monocytes - which subsequently differentiate into resident macrophages - but also T cells. Such immune cell infiltration results in a sustained local production of pro-inflammatory cytokines, secreted by the infiltrating immune cells, but also by adipocytes^[21]. The establishment of a pro-inflammatory environment, in turn, renders the adipocyte insulin-resistant, pre-disposing the individual to development of T2D in the long term.

Biological actions of butyric acid: Butyric acid is one of the most well-studied SCFAs due to its presence in dairy products such as cheese and butter^[12], and its endogenous production in the human gut, mainly by butyric bacteria^[22]. Indeed, plasma butyrate results from its production in the gut, which is favored by foods high in fermentable fiber^[23], and impaired with high fat diets^[24].

When supplemented to mice diets, particularly in the context of a high fat diet, butyrate was shown to counteract the development of obesity^[25], insulin resistance and the emergence of T2D. The mode of action of butyrate was attributed to a rise in energy expenditure. In skeletal muscle, butyrate caused an increase in type I muscle fibers along with an augmentation of ATP consumption. This effect seems to be associated with butyrate being an HDAC inhibitor and the fact that it favored type I oxidative muscle fibers and mitochondrial biogenesis^[14]. Whether the beneficial actions of butyrate are due to a direct action on tissue metabolism will require further investigation, as a clinical study investigating the effects of perfusion of short-chain fatty acids - including butyrate - on glucose metabolism in healthy men failed to show beneficial changes of glucose metabolism^[26]. On the other hand, a decreased population of gut butyric bacteria has been demonstrated in type 2 diabetic patients^[9,10], indicating the prominent role of butyrate

in the maintenance of a healthy metabolism.

Butyrate has been shown to act as an histone deacetylase (HDAC) inhibitor^[27]. In fact, its presence leads to increased histone acetylation, giving butyrate a possible action on proliferation, differentiation and regulation of metabolism^[27,28].

Recent research has suggested a link between inhibition of class I / II HDAC by butyrate and other SCFAs^[29,30], and improved metabolic responses. In liver, class II a HDACs dephosphorylate and activate FOXO, favouring the expression of gluconeogenic FOXO target genes. Thus, HDACs inhibition may provide a potential therapeutic approach to alleviate insulin resistance by decreasing gluconeogenesis^[31].

Interestingly, butyric acid inhibits class I and class II HDACs in a competitive fashion^[32], and dietary administration of sodium butyrate has been shown to improve systemic insulin sensitivity and increase energy expenditure in mice *via* up regulation of mitochondrial function in skeletal muscle and brown fat, through PGC-1 α (peroxisome proliferator-activated receptor gamma coactivator 1-alpha) induction and elevation of AMPK (adenosine monophosphate-activated protein kinase) activity^[14]. A further mechanism accounting for the beneficial metabolic effects of butyrate is the induction of hepatocyte- and adipocyte- produced fibroblast growth factor 21 (FGF-21), a hormone promoting fatty acid oxidation in mice. Increased FGF-21 production depends on acetylated and active PPAR α (peroxisome proliferator-activated receptor alpha), and inhibition of the PPAR α -deacetylating enzyme HDAC3 contributes to maintaining PPAR α in its active state^[33].

4-PHENYLBUTYRIC ACID

4-phenylbutyric acid (4-PBA) is a chemically produced derivative of butyric acid; 4-PBA is obtained by the reaction of benzene with butyrolactone in the presence of aluminum chloride, followed by neutralization with a base (Burzynsky SR and Musial L, US patent US6372938 B1, 2002). 4-PBA was first used in therapies for urea cycle enzyme deficiencies, due to its ability to act as a chaperone for other enzymes^[34,35]. 4-PBA has also been shown to have wider therapeutic indications including alleviation of endoplasmic reticulum stress and associated pathological conditions such as inflammation, hypertension and diabetes^[36-40]. The chaperone activity of 4-PBA prevents protein conformational abnormalities and protein aggregation occurring in neurodegenerative diseases^[41,42] and in insulin resistance/T2D - as demonstrated by the capability of 4-PBA to restore glucose homeostasis in type 2 diabetic rodent models^[43]. Recent preclinical research in zebrafish and mouse models demonstrated that 4-PBA is a PDK (pyruvate dehydrogenase kinase) inhibitor^[44]. PDK, inhibits the pyruvate dehydrogenase complex (PDHC) catalytic activity *via* inhibitory phosphorylation and has been used to alleviate lactic

acidosis caused by PDHC deficiency by restoring the conversion of glycolytic pyruvate into acetylCoA. Another use of 4-PBA is as an HDAC inhibitor, mostly in cancer studies^[45-47]. Both 4-PBAs HDAC inhibitor activity and its chaperone-like activity might constitute upstream targets to regulate gene expression. Indeed, in metabolic diseases like insulin resistance, 4-PBA positively modulates energy expenditure by favoring the expression of key metabolic genes, including GLUT4^[48]. Furthermore, in 3T3-L1 pre-adipocytes, administration of 4-PBA was shown to inhibit adipogenesis through the inhibition of the unfolded protein response, and nutritional supplementation of 4-PBA to mice lowered fat pad weight and resulted in smaller adipocytes in this tissue^[49]. Considering its multiple modes of action, and in particular its activity as chemical chaperone, 4-PBA constitutes a very promising molecule to target metabolic states related to endoplasmic reticulum stress such as insulin resistance and fat over-accumulation in the adipose tissue^[50]. Taken together, these studies suggest that 4-PBA favors the glycolytic utilization of glucose, while inhibiting adipogenesis and, by extension, fatty acid oxidation.

HYDROXYBUTYRATE

Ketone bodies (KB) are the organism's main source of energy in periods of fasting or prolonged physical exercise, when glucose availability is scarce^[51]. KB are mainly derived from free fatty acids, although a small percentage of KB can originate from the metabolism of amino acids^[52].

Essentially produced by the liver, the two main KB are acetoacetate (AcAc) and D- β -hydroxybutyrate. KB are metabolically equivalent to fat yet are water-soluble and can be transported in blood to target tissues^[53]. Once in the target tissues, KB are converted back to acetyl-coA, through the sequential action of D- β -hydroxybutyrate dehydrogenase, converting D- β -hydroxybutyrate to acetoacetate; 3-ketoacyl-CoA transferase, which produces acetoacetylCoA from acetoacetate, which is finally converted to acetyl-coA by acetoacetyl-CoA thiolase. Importantly, 3-ketoacyl-CoA transferase is only present in organs using KB, and not expressed in the liver. Thus, the flow of ketone bodies is unidirectional, from the liver to peripheral organs. For a long time the liver was thought to be the only organ to produce KB, but it appears the brain can also synthesize them to a certain extent for its own energetic needs^[54].

α -hydroxybutyrate (α -HB), a D- β -hydroxybutyrate isomeric but metabolically unrelated molecule, is generated in the liver (1) in the cysteine formation pathway; (2) as a byproduct during the formation of α -ketobutyrate, - the final product of methionine and threonine catabolism^[55]; and also (3) under increased oxidative stress, a physiological state associated to insulin resistance. Recently, α -HB has been proposed as a

marker of oxidative stress, and increased α -HB circulating levels have been shown to be early biomarkers of insulin resistance in a non-diabetic population^[56]. Therefore, α -hydroxybutyrate in the blood might be a predictive sign of incipient metabolic disease^[57,58], and β pancreatic cells damage^[59], thus representing an ideal biomarker for early detection of predisposition to insulin resistance and T2D.

D- β -hydroxybutyrate, which is present in the serum in the micromolar concentration range under postprandial or short-term fasting conditions, can increase up to millimolar concentrations in case of fasting or intense exercise and becomes the major energy source for the brain and muscles^[60,61]. D- β -hydroxybutyrate is a central metabolic intermediate that is associated to decreased free fatty acids blood concentrations and at the same time prevents from the exhaustion of fat stocks during prolonged starvation^[58,61] and might also regulate the aging process, as administration of D- β -hydroxybutyrate to *Caenorhabditis elegans* has been shown to increase the worm's lifespan^[62].

Recent research has demonstrated that the biological action of the ketone body D- β -hydroxybutyrate extends beyond its role as metabolite. Indeed, D- β -hydroxybutyrate has been shown to act both as a signaling molecule by activating G-protein coupled receptors (GPCRs), and as a transcriptional regulator by acting as a HDAC inhibitor.

Related to its properties as signaling intermediate, D- β -hydroxybutyrate has been shown to be an agonist for two GPCRs, PUMA-G (also named HCAR2, hydroxycarboxylic acid receptor 2, or Gpr109)^[63] and the free fatty acids receptor 3 (FFA3, or Grp41)^[64].

A novel and exciting research field has finally recently emerged with the discovery that D- β -hydroxybutyrate can be a key regulator of gene expression by acting as an endogenous HDACs inhibitor^[60]. The HDACs inhibitory activity of D- β -hydroxybutyrate brings changes in histone acetylation and gene expression that seem to protect cells from oxidative stress^[65].

β -AMINOISOBUTYRIC ACID

β -aminoisobutyric acid (BAIBA) was first identified as a catabolite derived from the breakdown of pyrimidines^[66] and branched chain amino acids^[67]. More recent research has demonstrated that BAIBA can act to prevent weight gain in several ways. Through an action on lipid metabolism, BAIBA reduces plasma and liver triglycerides by increasing both liver fatty acid oxidation and ketogenesis, especially by inducing β -D-hydroxybutyrate synthesis^[68]. A further mode of action of BAIBA may involve the action of leptin. Leptin is an adipokine expressed by adipose tissue which contributes to the central hypothalamic regulation of food intake and energy expenditure, therefore favoring weight loss^[69]. Interestingly, *ob/ob* leptin deficient mice were not responsive to BAIBA,

Table 1 Summarizing table describing the biological and metabolic effects promoted by butyric acid and its analogues molecules

Molecule	Putative molecular target or mechanism involved	Overall biological response	Ref.
Butyric acid	Increased PGC1 α expression and AMPK activity	Increased type I muscle fibers, increased insulin sensitivity, reduced adiposity	[14]
	Inhibition of HDACs	Decreased glycaemia in streptozotocin-induced diabetes. Decreased pancreatic β -cells apoptosis	[28]
D- β -hydroxybutyrate	HDAC3 inhibition	Induction of FGF21, fatty acid oxidation and ketone bodies production	[33]
	Binds and activates GPCR PUMA-G/Gpr109	Inhibition of adipocyte lipolysis	[63]
	Binds and activates GPCR FFA3/Grp41	Regulates energy consumption through FFA3/Grp41 activation in the sympathetic nervous system	[64]
4-phenylbutyric acid	HDACs inhibition	Protection from oxidative stress	[65]
	HDAC5 inhibition	Increased GLUT4 gene expression and promotion of glucose metabolism	[48]
	Inhibition of oxidative stress and endoplasmic reticulum stress	Alleviation of diabetic nephropathy in streptozotocin-induced diabetes	[40]
β -aminoisobutyric acid	Activates PPAR α	Induction of hepatic β -oxidation and brown adipose tissue	[71]
	Restores normal plasma leptin levels	Increase of fatty acid oxidation and alleviation of diet-induced obesity in <i>ob/+</i> heterozygous mice	[69]
	Induces liver ketogenesis, increasing circulating D- β -hydroxybutyrate	Decreased body fat mass	[68]

HDACs: Histone deacetylases; GPCR: G-protein coupled receptor; FGF-21: Fibroblast growth factor 21; FFA3/Grp41: Free fatty acids receptor 3; PPAR α : Peroxisome proliferator-activated receptor alpha.

showing neither weight loss nor increased fatty oxidation upon BAIBA administration^[68]. BAIBA, like leptin, appears to favor lipid metabolism, and at the same time, can act indirectly by promoting leptin expression in white adipose tissue^[70].

Moreover, recent research has shown that BAIBA is produced in muscles and BAIBA plasmatic concentrations in rodents and humans are most likely representative of the amounts of BAIBA produced by skeletal muscle^[71]. BAIBA derived from skeletal muscle can be considered as a myokine (*e.g.*, a muscle-secreted molecule with hormonal-like properties on target organs) that mediates the metabolic crosstalk between skeletal muscle and other metabolically active tissues. BAIBA production is regulated by PGC-1 α , which is a general co-activator overexpressed in response to exercise^[71]. As a myokine, BAIBA is capable of inducing a transition in the adipose tissue from a white adipose tissue phenotype to a tissue endowed with brown adipose tissue characteristics, thereby favoring the expression of β -oxidation and thermogenic genes, including PPAR α and its target mitochondrial uncoupling protein 1 (UCP1), rather than lipogenic and lipid-storage genes^[71,72]. Although the direct mechanism of action of BAIBA upstream of PPAR α has not yet been elucidated, it has been proposed that BAIBA could be a possible therapeutic molecule as an anti-obesity agent, by favoring a white fat to brown fat phenotypic transition^[73].

CONCLUSION

Obesity and its metabolic consequences leading to increased morbidity and mortality can be currently considered as one of the major health burdens worldwide. Extensive preclinical and clinical research is thus necessary to identify novel agents that might

be therapeutically useful - along with nutritional and physical activity approaches - to counteract this pandemic.

The four-carbon backbone chemicals described in this review, through their multiple mechanisms of actions summarized in Table 1, may provide a new class of potential pharmacologically effective candidates, to be used alone or in combination to effectively reverse or alleviate obesity and the insulin resistant state.

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Current medical treatment of estrogen receptor-positive breast cancer

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Abstract

Approximately 80% of breast cancers (BC) are estrogen

receptor (ER)-positive and thus endocrine therapy (ET) should be considered complementary to surgery in the majority of patients. The advantages of oophorectomy, adrenalectomy and hypophysectomy in women with advanced BC have been demonstrated many years ago, and currently ET consist of (1) ovarian function suppression (OFS), usually obtained using gonadotropin-releasing hormone agonists (GnRHa); (2) selective estrogen receptor modulators or down-regulators (SERMs or SERDs); and (3) aromatase inhibitors (AIs), or a combination of two or more drugs. For patients aged less than 50 years and ER+ BC, there is no conclusive evidence that the combination of OFS and SERMs (*i.e.*, tamoxifen) or chemotherapy is superior to OFS alone. Tamoxifen users exhibit a reduced risk of BC, both invasive and *in situ*, especially during the first 5 years of therapy, and extending the treatment to 10 years further reduced the risk of recurrences. SERDs (*i.e.*, fulvestrant) are especially useful in the neoadjuvant treatment of advanced BC, alone or in combination with either cytotoxic agents or AIs. There are two types of AIs: type I are permanent steroidal inhibitors of aromatase, while type II are reversible nonsteroidal inhibitors. Several studies demonstrated the superiority of the third-generation AIs (*i.e.*, anastrozole and letrozole) compared with tamoxifen, and adjuvant therapy with AIs reduces the recurrence risk especially in patients with advanced BC. Unfortunately, some cancers are or became ET-resistant, and thus other drugs have been suggested in combination with SERMs or AIs, including cyclin-dependent kinase 4/6 inhibitors (palbociclib) and mammalian target of rapamycin (mTOR) inhibitors, such as everolimus. Further studies are required to confirm their real usefulness.

Key words: Breast cancer; Endocrine therapy; GnRH-agonists; Ovarian function suppression; Tamoxifen; Selective estrogen receptor modulator; Aromatase inhibitors

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Core tip: In women with breast cancer (BC), the two major endocrine therapies (ET) available are directed at blocking estrogen receptors (ERs) on cancer cells (selective estrogen receptor modulators), or against the key enzyme in the biosynthesis of estrogens (aromatase inhibitors). The new knowledge about molecular mechanisms regulating tumor progression, suggested the existence of a strict relationship between ERs, intracellular signaling pathways, and other growth factors receptor, which justify the escape of a portion of patients from the inhibitory effects of ET. To restore endocrine sensitivity of BC cells, molecular-target therapies are giving encouraging results, but further studies are required to confirm their real usefulness.

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INTRODUCTION

Breast cancer (BC) is the most common female cancer worldwide, and the second cause of cancer death in women^[1]. In the United States, the expected number of new BC cases in 2015 is approximately 231800, accounting for 29% of all cancers, with an annual change based on incidence of +0.3%^[2]. In the European Union, BC seems to have a more favorable trend, with a 10.2% fall from 2009^[3].

Estrogen receptors (ERs) are nuclear proteins regulating the expression of specific genes, and approximately 80% of BCs are estrogen receptor (ER)-positive, of which 65% are also progesterone receptor (PR)-positive^[4]. Thus, endocrine therapy (ET) should be considered complementary to surgery in the majority of patients, inducing tumor remission and providing consistent clinical benefit. ET has represented the standard adjuvant treatment for ER+ tumors since the 1970s, although the advantages of oophorectomy, adrenalectomy, and hypophysectomy in women with advanced BC had already been demonstrated many years before^[5]. ET for BC currently consist of (1) ovarian function suppression (OFS); (2) selective estrogen receptor modulators (SERMs); (3) selective estrogen receptor down-regulators (SERDs); and (4) aromatase inhibitors (AIs), or a combination of two or more drugs. The two major strategies are directed at blocking ERs of cancer cells (SERMs) or against the key enzyme in the biosynthesis of estrogens (AIs).

OVARIAN FUNCTION SUPPRESSION

For decades, in premenopausal women with operable BC in whom ovaries are the main source of estrogen and

progesterone, surgical oophorectomy has represented an effective adjuvant therapy. Ovary irradiation was rarely performed for this purpose. Recently, it has been shown that the timing of oophorectomy (*i.e.*, luteal phase of the menstrual cycle) does not affect disease-free survival (DFS) and overall survival (OS) of patients with BC^[6]. OFS can be also obtained using gonadotropin-releasing hormone agonists (GnRHa), which are generally considered drugs with reversible actions. Although in patients with BC undergoing simultaneously GnRHa administration and chemotherapy (CT) ovarian function is rarely preserved, this treatment has largely replaced surgery^[7,8].

A historic large overview of randomized trials testing the usefulness of ovarian ablation in patients with ER+ early BC, named EBCTCG (Early Breast Cancer Trialists' Collaborative Group) study, demonstrated that OFS significantly increases survival in women aged below 50 years and that these benefits are independent of axillary nodal status, compared to untreated patients^[9]. The advantages of adding a GnRHa to SERMs or CT in terms of reduced recurrence and deaths rate was confirmed in other studies^[10,11]. More recently, it has been shown that women who underwent adjuvant GnRHa monotherapy or standard CT regimen had similar DFS and OS, while a combination of GnRHa plus CT or SERMs significantly improved DFS and OS in comparison to CT alone^[12]. Further studies suggested that, in women with ER+ early BC, adjuvant treatment with OFS plus SERMs (tamoxifen) does not lead to any advantage, while the combination of OFS and AIs (exemestane) significantly reduces recurrences compared to the other regimen^[13,14].

In premenopausal women previously treated with SERMs (tamoxifen), extended therapy with GnRHa and an AI may lead to substantial adverse effects^[15]. In conclusion, for patients aged less than 50 years, there is no conclusive evidence that the combination of OFS and SERMs or CT is superior to OFS alone^[16].

SELECTIVE ER MODULATORS AND DOWN-REGULATORS

Tamoxifen (TAM) is the drug most extensively used when an ET is required, both in pre- and postmenopausal women, and is still considered the SERM of choice^[7]. TAM (Figure 1A) was initially studied as anti-fertility drug, demonstrating soon favorable effects in patients with ER+ BC, as well as on bone tissue and cardiovascular system^[17]. TAM acts as a partial nonsteroidal agonist in some tissues, such as liver, uterus and bone, but is a competitive receptor inhibitor in the breast and brain^[18]. In these tissues, TAM selectively blocks signaling at the level of ERs, also inhibiting the proliferation of ductal cells^[19]. TAM is hydroxylated by the cytochrome P450 enzyme system into 4-hydroxy tamoxifen (4HT), and further metabolized in the liver, thanks to a CYP2D6-mediated

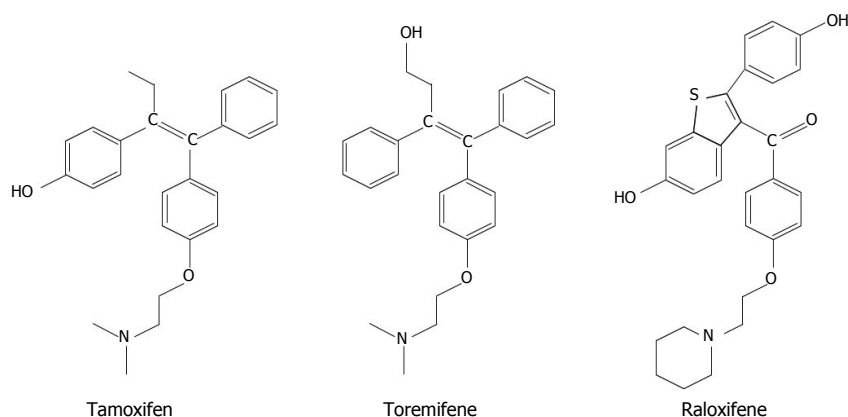


Figure 1 Chemical structures of the main selective estrogen receptor modulators.

oxidation, to endoxifen (4-hydroxy-N-desmethyl-tamoxifen)^[20]. Endoxifen and 4HT are the main active metabolites of TAM, exhibit similar ability in binding to ERs, and are more potent than TAM in suppressing estrogen-dependent cancer cells^[7]. The relationship between CYP2D6, endoxifen or 4HT serum levels and the response to TAM therapy has long been studied, but the hypothesized role of CYP2D6 genotype as prognostic factor affecting recurrence in patients with BC was not confirmed^[21,22]. However, CYP2D6 inhibitors, such as antipsychotic drugs, selective serotonin reuptake inhibitors (SSRIs), and some cardiac medications have the potential to affect response to SERM therapy^[23,24].

TAM significantly benefits only women with ER+ BC, and ASCO (American Society of Clinical Oncology) guideline recommendations consider a cut-off of 1% for immunohistochemical testing of ERs in BC cells^[25]. A number of studies and meta-analyses confirm its usefulness. According to the 2011 EBCTCG study, the reduction in mortality, expressed as reduction in annual odds of death, is approximately 20% after 5-years of TAM adjuvant therapy, and the 15-year recurrence rate ranges from 4.6% to 17.4%, according to age^[16,26]. The long-term mortality among TAM users was 23.9% compared with 33.1% of the control group, with limited age-related side effects, such as increased risk of thromboembolic disease (> 55 years) and endometrial cancer (> 45 years)^[26,27]. Extending the treatment to 10 years, the risk of recurrence and overall mortality are further slightly reduced, and thus the 2014 ASCO guidelines recommend the use of TAM for up to 10 years in pre- or perimenopausal women^[28,29].

With the aim of limiting the incidence of adverse effects of TAM, eventually improving its effectiveness, other SERMs have been proposed, such as toremifene and raloxifene, both approved by the US Food and Drugs Administration (FDA). *Toremifene* (Figure 1B) is similar to TAM in clinical activity, efficacy and tolerability, and is cross-resistant to TAM. It causes less vascular and endometrial adverse effects than TAM, but has less favorable actions on bone and lipids levels^[30]. Also in patients with metastatic BC, the results of TAM and toremifene therapy did

not differ significantly, and the latter represents a reliable alternative to TAM in menopausal women^[31]. *Raloxifene* (Figure 1C) is another nonsteroidal SERM causing less toxicity compared to TAM, especially used for the prevention of osteoporosis in postmenopausal women^[32]. However, as preventive therapy, raloxifene has been shown to be less effective than TAM, because it reduces the risk of cancer by 36% compared to 50% of the group treated with TAM^[33].

In conclusion, SERMs users exhibit a reduced risk of BC, both invasive and *in situ*, especially during the first 5 years of therapy, ranging from 53% to 79% according to age and menopausal status^[34].

Selective estrogen receptor down-regulators have different pharmacologic characteristics, biochemical structure, and molecular activity with respect of SERM, causing down-regulation and degradation of ERs, and inhibiting proliferation of estrogen-dependent BC cells^[35]. SERDs are pure ER antagonist, blocks ERs activity and accelerate their degradation, thus exhibiting exclusively anti-estrogen effects^[36]. Fulvestrant, the only SERD approved by FDA to treat patients with BC, has 100-fold the affinity of TAM for ER, in lack of adverse effect on endometrial ERs^[37,38]. It is useful especially in patients with advanced BC and as second-line therapy in TAM-resistant tumors^[36]. Fulvestrant sensitizes ER-negative BC to chemotherapy, showing a synergistic action with various cytotoxic agents, such as docetaxel^[39,40]. In the treatment of patients with metastatic ER+ BC, the combination of fulvestrant plus an AI (anastrozole) was superior to anastrozole alone, and fulvestrant as single-drug therapy represents a reasonable alternative to AIs^[41,42]. In the neoadjuvant setting, the use of fulvestrant is controversial, because of the slow response rate and possible non-response. In a phase II randomized study, tumor response rates were 20.6% and 22.9%, according to the dosage of the drug^[43].

AROMATASE INHIBITORS

The human CYP19 single-copy gene, located on chromosome 15q21.2, encodes the microsomal enzyme

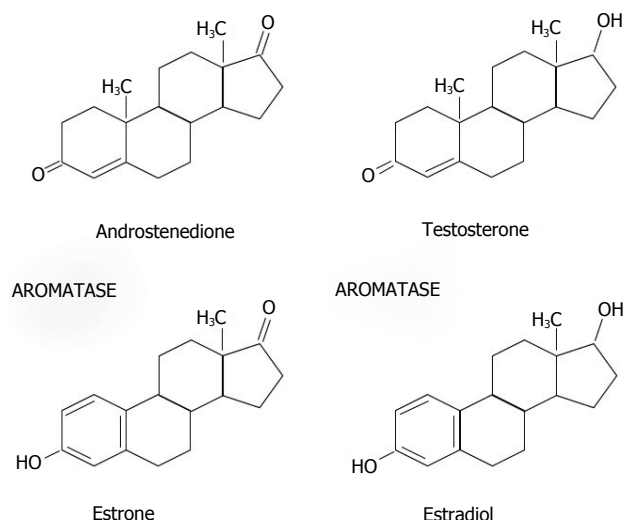


Figure 2 Actions of the aromatase on steroid hormones.

aromatase [cytochrome P450 19 (CYP19)], which catalyzes the conversion of androgens, such as testosterone and androstenedione, to estrogens, such as 17- β -estradiol and estrone^[44] (Figure 2).

Aromatase activity is expressed especially in the ovary (premenopausal women), testis, placenta, brain, bone and adipose tissue (postmenopausal women)^[45]. AIs block aromatase enzyme activity, safely reducing circulating estrogen levels only in postmenopausal patients. AIs are ineffective in premenopausal women with functionally active ovary in whom they increase gonadotropin secretion, and therefore estrogen production^[46]. The combination of AIs and OFS, using a GnRHa to block the pituitary, is usually an effective strategy. According to the conclusions of the STAGE study, 70.4% of postmenopausal patients with early BC treated with an AI (anastrozole) plus GnRHa (goserelin) obtained complete or partial response to therapy, compared with 50.5% of the control group treated with anastrozole plus TAM^[47]. Another study, did not found any difference in DFS between premenopausal women with ER+ early BC treated with TAM alone and those treated with anastrozole alone, who presented a slightly increased risk of death^[48]. A correlation between elevated body mass index and reduced effect of AIs has been reported in some studies^[49].

According to the chemical structure and mechanism of action, there are two types of AIs: type I are permanent steroidal inhibitors of aromatase, while type II are reversible nonsteroidal inhibitors (Table 1).

Aminoglutethimide, initially used as anti-epileptic drug, is the oldest nonsteroidal AI (Figure 3A). It inhibits also cytochrome P450scc, blocking the conversion of cholesterol to pregnenolone, and thus decreases the production of several steroids, including cortisol^[17]. The subsequent activation of the hypothalamic-pituitary-adrenal axis, causes hypersecretion of adrenocorticotrophic hormone (ACTH)

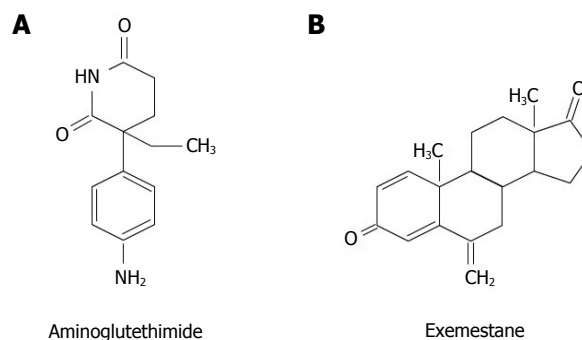


Figure 3 Chemical structures of aminoglutethimide and exemestane.

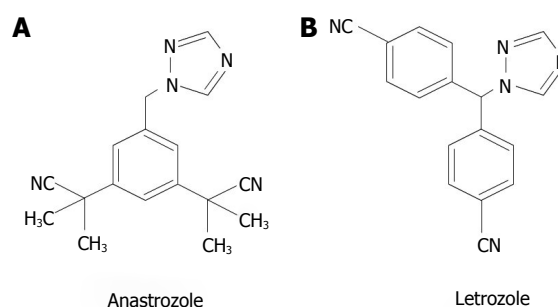


Figure 4 Chemical structures of the two main third-generation aromatase inhibitors.

leading to the need of increasing the dosage of the drug, and requires corticosteroids supplementation. Due to these side effects, aminoglutethimide has been replaced by better-tolerated and more effective AIs^[7].

Exemestane (Figure 3B) is a potent irreversible steroidal AI that acts as a false substrate for aromatase and suppresses selectively estrogens at any site^[50]. In some studies, exemestane plus OFS reduced the risk of recurrences in premenopausal women with ER+ early BC, while TAM plus OFS did not provided benefit^[51]. In a group of postmenopausal patients at slightly increased risk of cancer according to the Gail model who underwent exemestane therapy, the annual incidence of BC was 0.33% compared to 0.77% of control group^[52].

Anastrozole and letrozole (Figure 4) are two nonsteroidal third-generation AIs with similar potency and characteristics. Because their block of ERs is reversible, a continuous drug administration is required. An old comparison between anastrozole and letrozole as second-line therapy in advanced BC, reported an overall response rate of 12% vs 19%, respectively^[53]. In postmenopausal women with advanced BC, letrozole was more effective than fadrazole, a second-generation type II nonsteroidal AI with reversible action^[54]. The more recent ACOSOG (American College of Surgeons Oncology Group) study compared exemestane, anastrozole, and letrozole as neoadjuvant therapy of women with advanced BC, and reported that the clinical response rate was 62.9%, 69.1%, and 74.8%, respectively^[55]. Several meta-analysis underline the

Table 1 Main characteristics of type I and II aromatase inhibitors

Type	Chemical structure	Action	First generation	Second generation	Third generation
I	Steroidal	Permanent	Testolactone	Formestane	Examestane
II	Nonsteroidal	Reversible	Aminoglutethimide	Fadrozole	Anastrozole Letrozole

superiority of the third-generation AI (but not first- and second-generation AIs) compared with TAM, confirming that adjuvant therapy with AIs reduces the recurrence risk in patients with advanced BC, but does not affect mortality rate^[56,57].

ENDOCRINE THERAPY AND MOLECULAR-TARGET THERAPY

The new knowledge about molecular mechanisms regulating tumor progression, suggested the existence of a strict relationship between ERs, intracellular signaling pathways, and other growth factors receptor, which justify the escape of a portion of patients from the inhibitory effects of ET^[58]. To restore endocrine sensitivity of ER+ cancer cells, several other drugs have been studied, including cyclin-dependent kinase (CDK) 4/6 inhibitors (palbociclib), epigenetic modulators that inhibit histone deacetylase (HDAC), and other signal pathway inhibitors^[59]. Experimentally, combined HDAC inhibition and TAM reverse ET resistance, inducing apoptosis of TAM-resistant BC cells, and in women with advanced ER+ BC and disease progression or recurrence the progression-free survival (PFS) can be reduced adding palbociclib to letrozole^[60,61]. The phosphoinositide 3-kinase (PI3K)/protein kinase B (AKT) pathway and the serine/threonine kinase mammalian target of rapamycin (mTOR) are part of a signaling network regulating several cell processes, including growth, proliferation and survival of cancer cells^[62]. The addition of an mTOR inhibitor (everolimus) to exemestane in patients with ER+ BC and advanced disease refractory to both SERMs and AIs, can improve the progression-free survival (PFS) compared to everolimus alone^[52]. A recent meta-analysis showed that exemestane and everolimus in combination was more efficacious than fulvestrant alone in patients with relapsed BC previously treated with nonsteroidal AIs^[63]. Unfortunately, in women with ER+ BC, the addition of an anti-vascular endothelial growth factor (bevacizumab) to fulvestrant or letrozole did not change the resistance of the tumor to ET, and failed to improve PFS or OS^[64].

METASTATIC BREAST CANCER

As in part already reported above, anti-estrogen therapy plays a major role in the prolonged remission in women with metastatic and advanced ER+ BC. Several studies showed that the inhibition of the aromatase enzyme

system with third-generation AIs, such as exemestane, anastrozole and letrozole, is associated with better survival compared to SERMs^[56]. In terms of both PFS and OS, there are also advantages of treating patients with AIs compared to other ET, and letrozole seems to be the drug of choice^[65,66]. The adverse effects observed using letrozole or anastrozole are similar than that observed with TAM, and the time-to-progression was better^[67]. The combination anastrozole and fulvestrant in metastatic ER+ BC was superior to anastrozole alone^[41].

All endocrine therapies, in particular AIs, synergize their effects with several molecular target drugs, such as everolimus and palbociclib^[58,60]. It has also been shown that exemestane plus everolimus were more efficacious than everolimus alone and fulvestrant alone in women with advanced BC and progressive disease^[52,63]. The GINECO (Groupe d'Investigateurs Nationaux pour les Etudes des Cancers de l'Ovaire) study showed that the short-term clinical benefit rates obtained with TAM plus everolimus vs TAM alone were 61% and 42%, respectively, and that the time to progression increased from 4.5 to 8.6 mo^[68]. However, according to the conclusion of the BOLERO-2 (Breast Cancer Trials of OraL EverOLimus-2) study, the decision of prescribing AIs and mTOR inhibitors in combination should be individualized, taking into account the characteristics of single patient and considering risks and benefits of the treatment^[69]. Both CDK and HDAC inhibitors, such as palbociclib and etinostat, in combination with ET, can be useful in patients with advanced ER+ BC as third-line therapy^[70].

ADVERSE EFFECTS

Unfortunately, both TAM and AIs use is associated with several adverse events (AEs) and distressing symptoms, such as hot flushes, weight gain, sexual dysfunctions, decreased bone mineral density (BMD), osteoporosis and pathologic fractures, or musculoskeletal symptoms, depending on the drug used and the duration of treatment. These effects may be severe enough to cause non-compliance due to cessation of use in a significant proportion of patients. Most of the AEs is related to the estrogen deprivation^[7]. According to a systematic review, TAM was associated with more serious AEs in comparison with AIs, while exemestane caused more frequently musculoskeletal symptoms and hot flushes, but in some trials no

differences in AEs between SERMs and AIs was found^[67]. Another meta-analysis showed that patients treated with AIs had increased risk of gastrointestinal symptoms, such as nausea, vomiting and diarrhea, while the incidence of thromboembolic events and vaginal bleeding were decreased compared to other ETs^[66]. In several studies, AIs use was associated with a higher risk of arthralgia and decreased BMD compared to TAM, and the incidence of discontinuation ranges from approximately 12% to 14%^[16]. Effects on cognitive function related to the AIs use have also been reported, similar to the so-called “chemotherapy fog” observed following prolonged cancer CT^[71]. However, toxicity is related to individual treatment compliance and depends on the way in which the patient describes and reports the symptoms, and they usually resolve with discontinuation of treatment^[16]. Careful patient information is recommended before starting any treatment with ETs, especially in premenopausal women.

CONCLUSION

In patients with BC and ER+ tumors, ET has several advantages, including prolonging DFS and OS, and reducing the risk of recurrence, both in patients with early BC and in those with advanced or relapsed disease. In premenopausal women, OFS and TAM has similar efficacy and there is no evidence that a regimen based on OFS, SERMs, or AIs in combination can be the strategy of choice^[7,16]. In postmenopausal women, AIs are more effective than SERMs in reducing relapse of the disease, and extending the TAM administration for more than 5 year represents a useful strategy. In patients with advanced BC, molecular-target therapies are giving encouraging results, but further studies are required to confirm their real usefulness and a careful analysis of cost-benefits is also required.

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Basic Study

Effect of paricalcitol and enalapril on renal inflammation/oxidative stress in atherosclerosis

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Author contributions: Husain K performed the majority of the experiments, analyzed the data and wrote the paper; Suarez E performed the animal treatments, data recording and analysis; Isidro A performed physiological experiments; Hernandez W performed biochemical data and statistical analysis; Ferder L designed and coordinated the research.

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Institutional review board statement: The animal protocol was designed to minimize pain and discomfort to the animals. The animals were acclimatized to laboratory conditions (25 °C, 12 h/12 h light/dark, 50% humidity, ad libitum access to food and water) for one week.

Institutional animal care and use committee statement: All procedures involving animals were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of Ponce School of Medicine (Protocol #LF-07).

Conflict-of-interest statement: There is no conflict of interest associated with any of the senior author or other coauthors contributed their efforts in this manuscript.

Data sharing statement: This is a non-clinical study hence the technical appendix, statistical code, and data sets are not available.

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Abstract

AIM: To investigate the protective effect of paricalcitol and enalapril on renal inflammation and oxidative stress in ApoE-knock out mice.

METHODS: Animals treated for 4 mo as group (1) ApoE-knock out plus vehicle, group (2) ApoE-knock out plus paricalcitol (200 ng thrice a week), (3) ApoE-knock out plus enalapril (30 mg/L), (4) ApoE-knock out plus paricalcitol plus enalapril and (5) normal. Blood pressure (BP) was recorded using tail cuff method. The kidneys were isolated for biochemical assays using spectrophotometer and Western blot analyses.

RESULTS: ApoE-deficient mice developed high BP (127 ± 3 mmHg) and it was ameliorated by enalapril and enalapril plus paricalcitol treatments but not with paricalcitol alone. Renal malondialdehyde concentrations, p22^{phox}, manganese-superoxide dismutase, inducible nitric oxide synthase (NOS), monocyte chemoattractant

protein-1, tumor necrosis factor- α and transforming growth factor- β 1 levels significantly elevated but reduced glutathione, CuZn-SOD and eNOS levels significantly depleted in ApoE-knock out animals compared to normal. Administration of paricalcitol, enalapril and combined together ameliorated the renal inflammation and oxidative stress in ApoE-knock out animals.

CONCLUSION: Paricalcitol and enalapril combo treatment ameliorates renal inflammation as well as oxidative stress in atherosclerotic animals.

Key words: Atherosclerosis; Enalapril; Paricalcitol; Renal inflammation; Oxidative stress

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Core tip: Although the protective efficacy of vitamin D and angiotensin converting enzyme inhibitors (ACEIs) have been studied in the cardiovascular system of atherosclerotic mice. However this is the first report to investigate the renal protection by paricalcitol and enalapril, alone or in combination in ApoE-deficient atherosclerotic mice. This innovative study clearly shows that vitamin D, ACEI and their combo ameliorated the renal inflammation and oxidative stress in ApoE-knock out animals by depleting the inflammatory and oxidative stress markers as well as restoring the renal antioxidant defense system in atherosclerotic mice. The combination of paricalcitol and enalapril warrants the clinical usefulness in renal atherosclerotic patients.

Husain K, Suarez E, Isidro A, Hernandez W, Ferder L. Effect of paricalcitol and enalapril on renal inflammation/oxidative stress in atherosclerosis. *World J Biol Chem* 2015; 6(3): 240-248 Available from: URL: <http://www.wjgnet.com/1949-8454/full/v6/i3/240.htm> DOI: <http://dx.doi.org/10.4331/wjbc.v6.i3.240>

INTRODUCTION

Atherosclerosis is implicated of morbidity and mortality in patients with cardiorenal diseases worldwide. Epidemiological as well as clinical investigations demonstrated a strong relationship between vitamin D deficiency and cardiorenal risk factors such as hypertension, chronic kidney disease (CKD), diabetes mellitus, atherosclerosis, myocardial infarction, stroke, and congestive heart failure^[1,2]. It could be suggested that vitamin D deficiency contributes to the development of renal and cardiovascular diseases *via* its strong association with risk factors such as diabetes, hyperlipidemia and hypertension^[3-5]. However, direct effects of vitamin D on the renal and cardiovascular system may also be involved. Activation of vitamin D receptors not only regulates the parathyroid hormone but also alters the renin-angiotensin system (RAS), cardiac hypertrophy, inflammation, and calcium

levels^[6-12]. The molecular mechanism for how vitamin D may improve cardiorenal disease outcomes, specifically cardiorenal atherosclerotic lesions, is not well understood.

A number of studies documented an essential role of RAS in both cardiovascular pathophysiology, including atherosclerosis, and renal pathophysiology^[13-15]. RAS inhibitors and blockers have therapeutic potentials in chronic cardiorenal dysfunctions^[13,16-18]. Angiotensin II causes oxidants/antioxidants imbalance in the kidney, heart and vascular system inducing reactive oxygen species (ROS) production *via* the activation of NADPH oxidase^[19,20]. These ROS initiate vascular membrane lipid peroxidation leading to inflammation and the generation of inflammatory cytokines (TNF- α) through NF- κ B activation^[21,22] and other mediators such as vascular cell adhesion molecule-1 (VCAM-1), MCP-1, TGF- β 1, Matrix metalloproteinase 9 (MMP9), iNOS and Mn-SOD^[23-25]. ROS oxidize cellular biomolecules (lipids, proteins and nucleic acids) leading to renal, heart and vascular impairments^[26]. But the cellular ROS are scavenged by endogenous antioxidants including antioxidant enzymes such as SOD, catalase, and glutathione peroxidase (GSHPx), reduced glutathione (GSH) and vitamins A, C, and E^[27]. Most importantly, depletion of a major cellular antioxidant such as GSH has been reported to cause renal and cardiovascular dysfunction in rats^[28,29]. Antioxidant therapy has been shown to ameliorate renal and cardiovascular oxidative stress by scavenging excess ROS and upregulating the antioxidant defense system^[27,30]. However, since the protective mechanisms of angiotensin converting enzyme inhibitors (ACEIs) in the atherosclerotic lesions of mouse kidney are not completely understood, we set out to investigate this aspect in the present study.

Paricalcitol, a vitamin D analog, has been associated not only with the regulation of early stages of atherogenesis but also with vascular calcification. Treatments with vitamin D analogs at therapeutic dosages ameliorate the secondary elevation of parathyroid hormones and vascular calcium levels^[31-33]. In clinical studies, paricalcitol reduced proteinuria, inflammation, and the mortality rate in CKD patients^[34-36]. Our recent study demonstrated that when paricalcitol combined with enalapril ameliorated the oxidative cardiovascular injury by suppressing ROS-generating enzyme NADPH oxidase activity and by upregulating the antioxidant defense system in a uremic rat model and mouse model of atherosclerosis^[29,37-39]. It is not known, however, whether vitamin D analogs can protect against inflammatory and oxidative stress in the kidney of atherosclerotic mice. RAS is implicated in atherosclerosis and vitamin D suppresses it, the combination of vitamin D and RAS inhibitor most likely elevated the therapeutic potentials in the kidney of ApoE-deficient mice. Therefore, the present study aimed to investigate the protective efficacy of a vitamin D analog, *i.e.*, paricalcitol, and an ACE inhibitor, *i.e.*, enalapril on inflammation and oxidants/antioxidants

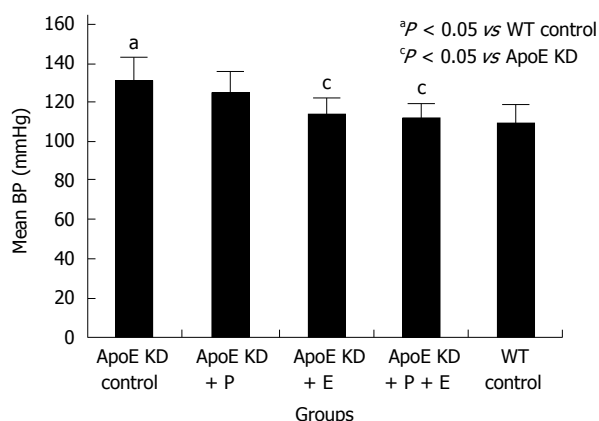


Figure 1 Effect of paricalcitol and enalapril alone and in combination for 16-wk on mean blood pressure (mmHg) in ApoE-deficient atherosclerotic mice. There was a significant ($P < 0.05$) increase in mean BP in atherosclerotic mice compared to wild type control ($n = 12$). Enalapril ($n = 12$) alone and in combination with paricalcitol ($n = 12$) significantly ($P < 0.05$) reduced mean blood pressure (BP) in atherosclerotic mice ($n = 12$). Paricalcitol ($n = 12$) slightly but not significantly decreased mean BP in atherosclerotic mice.

imbalance in the kidney of ApoE-deficient atherosclerotic mice.

MATERIALS AND METHODS

Animals

Sixty female mice (20-25 g) were procured from (Taconic Company, Hudson, NY, United States). Mice were given food and water ad libitum. They were maintained at a room temperature (25 °C with 50% humidity) and on 12/12-h light/dark cycle. After one week acclimatization they were separated into five groups and treated as follows:

Group 1 (ApoE deficient + vehicle): Mice were administered a vehicle (100 μ L of propylene glycol, *i.p.*) 3 times a week for 16 wk ($n = 12$).

Group 2 (ApoE deficient + paricalcitol): Mice were given paricalcitol (200 ng in propylene glycol, *i.p.*) 3 times a week for 16 wk ($n = 12$).

Group 3 (ApoE deficient + enalapril): Mice were given enalapril (30 mg/kg) in their drinking water for 16 wk ($n = 12$).

Group 4 (ApoE deficient + paricalcitol + enalapril): Mice were given paricalcitol + enalapril for 16 wk ($n = 12$).

Group 5 (wild-type normal control): Wild-type control mice were given vehicle only 3 times a week for 16 wk ($n = 12$).

The use of ApoE-deficient mice as a model of atherosclerotic lesions and drug treatment were reported earlier^[40,41]. BP was monitored after drug treatment using a Non-Invasive Blood Pressure System NIBP800 (Columbus Instruments, Columbus, Ohio, United States) as described earlier^[37-39]. After 16 wk, mice were euthanized by decapitation and kidneys were isolated. The kidneys ($n = 6$) from each group of mice were rinsed with PBS, immediately placed in

liquid nitrogen then preserved at -80 °C. The remaining kidneys ($n = 6$) from each group of mice were fixed in buffered formalin for histological study. All Procedures involving animals were reviewed including care to minimize the pain and discomfort by the Ponce School of Medicine's Institutional Animal Care and Use Committee (IACUC). The animal protocol #LF-07 was approved by the committee. The drugs were obtained from Sigma Chemical Company (St. Louis, Mo, United States) and Abbott Pharmaceuticals (Abbott Park, IL, United States).

Determination of glutathione

Glutathione (GSH) was determined as previously described^[37-39] using commercial kit (Cayman Company, Ann Arbor, MI, United States).

Lipid peroxidation assay

The end product of lipid peroxidation malondialdehyde (MDA) levels were estimated according to Ohkawa and colleague's method^[42]. For the standard, 1, 1, 3, 3-tetraethoxypropane was used.

Protein assay

Total protein levels were determined using Coomassie reagent according to the method of Read and Northcole^[43]. Bovine serum albumin (BSA) was used as the standard.

Protein extraction

Total protein extraction from frozen tissues was performed using M-PER buffer (Pierce, Rockford, IL, United States).

Western blot analysis

Total proteins (40 μ g) was resolved by 12.5% SDS polyacrylamide gel electrophoresis (SDS PAGE) running gel and a 5% stacking gel as described previously^[36-38].

Statistical analysis

The data are represented as mean $\pm$ SEM. The data were statistically analyzed by one-way analysis of variance (ANOVA) followed by a post-hoc, Schaffer test. $P < 0.05$ was considered significant. The statistical analysis of the data has been reviewed by Dr. Hernandez of Ponce School of Medicine.

RESULTS

As depicted in Figure 1, ApoE-deficient atherosclerotic mice in group 1 receiving vehicle alone developed significant hypertension (increased mean BP) compared to normal wild type controls in group 5 ($P < 0.05$) after 16 wk of treatment. Treatment with enalapril or enalapril in combination with paricalcitol for 16 wk prevented the increase in mean BP ($P < 0.05$). However, paricalcitol alone treatment for 16 wk did not

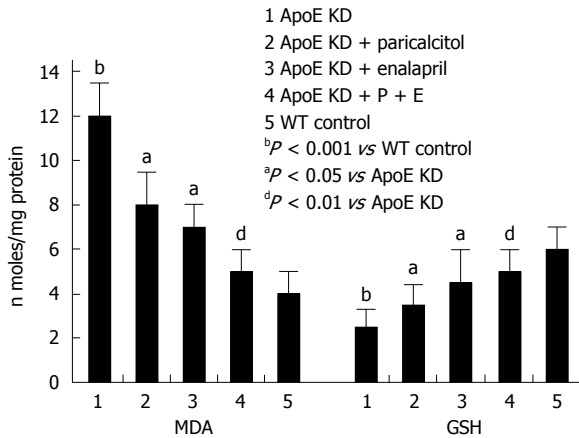


Figure 2 Effect of paricalcitol and enalapril alone and in combination for 16-wk on mono and combination for 16-wk on renal malondialdehyde and glutathione levels in ApoE-deficient atherosclerotic mice. There was a significant ($P < 0.001$) increase in the renal MDA levels in atherosclerotic mice compared to wild type control (group 5). Paricalcitol ($n = 6$) and Enalapril ($n = 6$) alone significantly ($P < 0.05$ and $P < 0.05$) reduced aortic malondialdehyde (MDA) levels. However combination of the two ($n = 6$) decreased greater and significant ($P < 0.01$) aortic MDA levels than either drug alone in atherosclerotic mice ($n = 6$). There was a significant ($P < 0.001$) decrease in aortic glutathione (GSH) levels in atherosclerotic mice compared to wild type control (group 5). Paricalcitol ($n = 6$) and enalapril ($n = 6$) alone significantly ($P < 0.05$) increased aortic GSH levels. However combination of the two ($n = 6$) increased greater and significant ($P < 0.01$) aortic GSH levels than either drug alone in atherosclerotic mice ($n = 6$).

lower the mean BP of mice in group 2.

Figure 2 shows that the levels of the renal lipid peroxidation end product, malondialdehyde (MDA), increased by 200% ($P < 0.001$) in atherosclerotic mice in group 1 compared to wild type controls in group 5, indicating the renal oxidative injury in atherosclerosis. Paricalcitol and Enalapril alone significantly reduced renal MDA levels (33% and 58%, respectively) compared to group 1 ($P < 0.05$). Combining these two drugs, however, decreased greater MDA levels by 67% ($P < 0.01$) than either drug alone in atherosclerotic mice.

There was a marked decrease in renal reduced glutathione (GSH) (58%, $P < 0.01$) in vehicle-treated atherosclerotic mice in group 1 compared to wild type controls in group 5 (Figure 2) indicating an oxidative injury. Paricalcitol and Enalapril alone significantly increased renal GSH levels (40% and 80%, respectively) compared to group 1 ($P < 0.05$). Combining of the two drugs, however, increased greater GSH levels by 100% ($P < 0.01$) than either drug alone in atherosclerotic mice. All three therapies protected against the reduction in renal GSH levels, indicating an up-regulation of the renal antioxidant system.

Renal p22^{phox} level was significantly elevated (150%) in ApoE-deficient mice compared to normal controls (Figure 3). Paricalcitol treatment decreased the subunit expression (38%) in the kidney compared to group 1. Enalapril and drug combo significantly decreased p22^{phox} (76% and 68% of group 1) thus lowers the

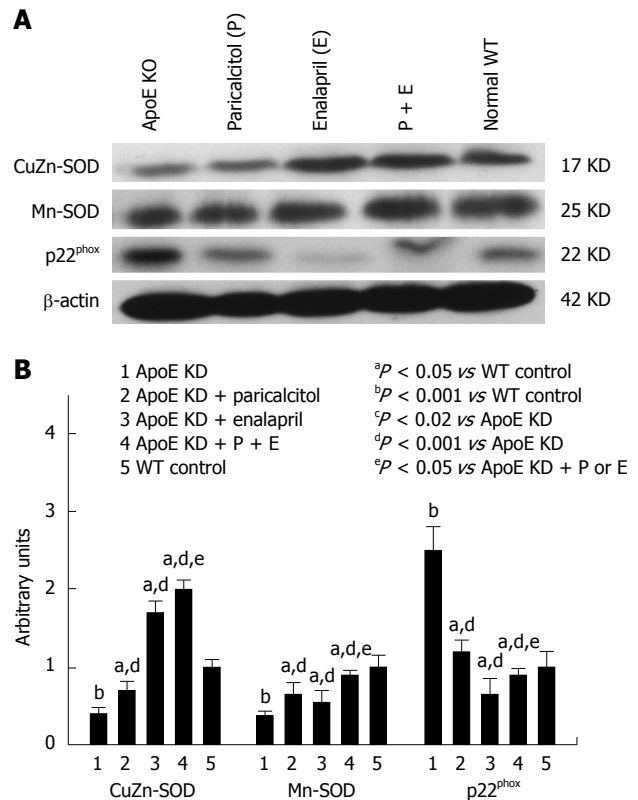


Figure 3 Western blot analysis and densitometry of protein band analysis to paricalcitol and enalapril alone and in combination for 16-wk on renal NADPH oxidase subunit p22^{phox}, manganese-superoxide dismutase (Mn-SOD) and copper/zinc-superoxide dismutase (CuZn-SOD) protein expression in ApoE-deficient atherosclerotic mice. A: Western blot analysis of the effect of paricalcitol and enalapril alone and in combination for 16-wk on renal NADPH oxidase subunit p22^{phox}, manganese-superoxide dismutase (Mn-SOD) and copper/zinc-superoxide dismutase (CuZn-SOD) protein expression in ApoE-deficient atherosclerotic mice; B: Densitometry of protein band analysis show that renal NADPH oxidase subunit p22^{phox} and Mn-SOD expression significantly increased ($P < 0.001$) whereas CuZn-SOD protein expression significantly decreased ($P < 0.001$) in atherosclerotic mice ($n = 3$) compared to controls ($n = 3$). Paricalcitol ($n = 3$), enalapril ($n = 3$) and combination of the two ($n = 3$) significantly ($P < 0.02$ and $P < 0.001$) ameliorated the oxidative stress by inhibiting the induction of NADPH oxidase and Mn-SOD expression (38%, 76% and 68%, respectively) and up-regulating the CuZn-SOD protein expression (52%, 342% and 398%, respectively) in atherosclerotic mice. The data represent mean $\pm$ SE of three independent Western blot experiments.

oxidative damage of the kidney.

As depicted in Figure 3 renal Mn-SOD level significantly elevated (98%) and CuZn-SOD level significantly depleted (55%) in atherosclerotic animals compared to group 5. Both drug separately as well as combined together depleted Mn-SOD levels (52%, 76% and 68%, respectively) and induced CuZn-SOD levels (52%, 342% and 398%, respectively) compared to group 1.

The renal inflammatory markers (TNF- α , MCP-1, TGF- β 1 and iNOS) were significantly elevated (190%, 198%, 158% and 992%, respectively) and eNOS levels significantly depleted (55%) in ApoE-deficient animals compared to group 5 (Figures 4 and 5). Both drugs alone as well as combined together significantly depleted iNOS (69%, 72% and 76%, respectively),

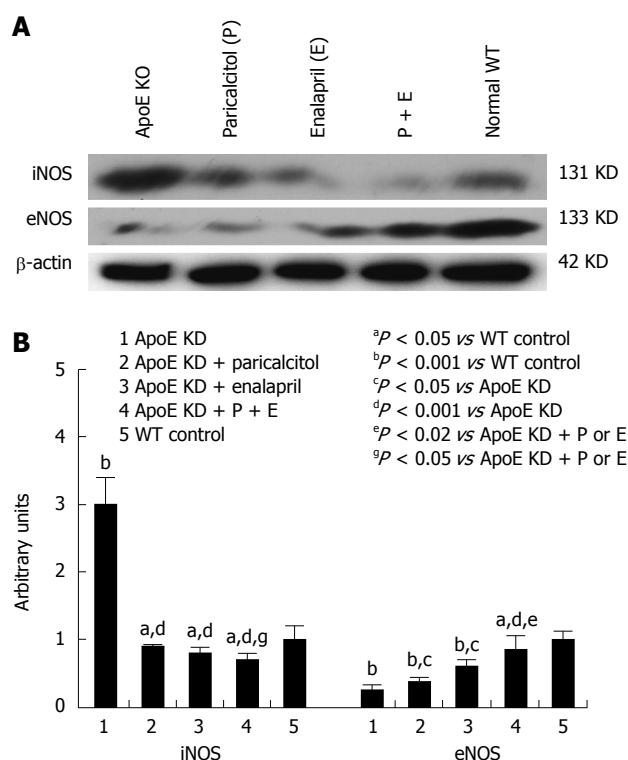


Figure 4 Western blot analysis and densitometry of protein band analysis to paricalcitol and enalapril alone and in combination for 16-wk on renal iNOS and eNOS protein expression in ApoE-deficient atherosclerotic mice. A: Western blot analysis of the effect of paricalcitol and enalapril alone and in combination for 16-wk on renal iNOS and eNOS protein expression in ApoE-deficient atherosclerotic mice; B: Densitometry of protein band analysis show that renal iNOS expression was significantly enhanced ($P < 0.001$) whereas eNOS protein expression was significantly decreased ($P < 0.001$) in ApoE-deficient atherosclerotic mice ($n = 3$) compared to controls ($n = 3$). Paricalcitol ($n = 3$), enalapril ($n = 3$) and the combination of the two ($n = 3$) significantly ($P < 0.05$ and $P < 0.001$) ameliorated the alterations in iNOS and eNOS expression in atherosclerotic mice. The data represent mean $\pm$ SE of three independent Western blot experiments.

MCP-1 (72%, 79%, 76%, respectively), TNF- α (79%, 88%, and 72%, respectively) and TGF- β 1 (24%, 82% and 93%, respectively) compared to group 1. Both drug separately as well as combined together ameliorated the renal endothelial damage *via* induction of eNOS (98%, 147% and 342%, respectively) compared to group 1.

DISCUSSION

This study reveals the protective efficacy of the vitamin D analog, paricalcitol, and ACE inhibitor, enalapril, either alone or in combination, on renal inflammatory and oxidative stress in ApoE-deficient atherosclerotic mouse model. A number of preclinical as well as clinical studies have demonstrated the beneficial effects of RAS blockade in renal and cardiovascular diseases including atherosclerosis^[13,16-18,29,37-39,44]. However, the mechanisms by which ACEIs provide protection against cardiovascular diseases are not fully understood. The role of inflammatory and oxidative injuries in the disorders of the kidney, heart and blood vessels such

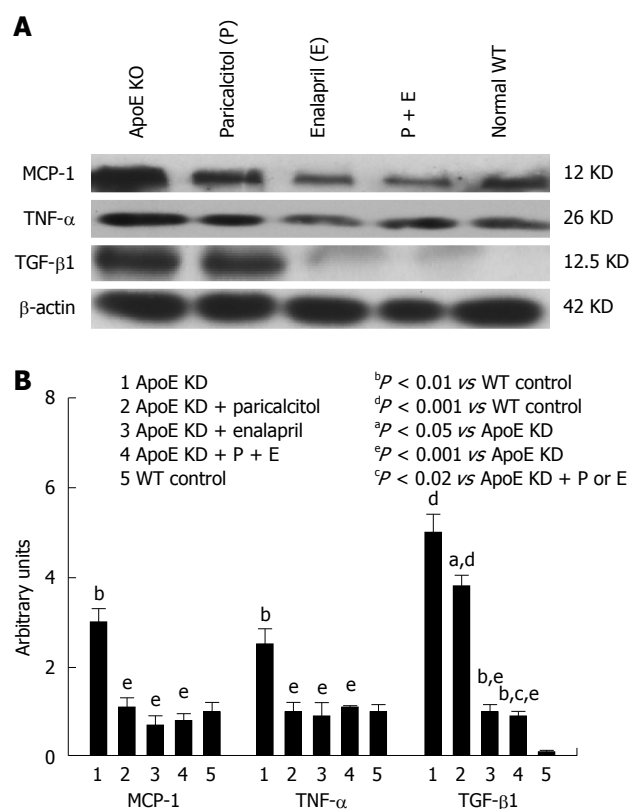


Figure 5 Western blot analysis and densitometry of protein band analysis to paricalcitol and enalapril alone and in combination for 16-wk on renal inflammatory proteins MCP-1, TNF- α and TGF- β 1 expression in ApoE-deficient atherosclerotic mice. A: Western blot analysis of the effect of paricalcitol and enalapril alone and in combination for 16-wk on renal inflammatory proteins MCP-1, TNF- α and TGF- β 1 expression in ApoE-deficient atherosclerotic mice; B: Densitometry of protein band analysis show that renal MCP-1, TNF- α and TGF- β 1 expression was significantly enhanced ($P < 0.01$, $P < 0.01$ and $P < 0.001$) in ApoE-deficient atherosclerotic mice ($n = 3$) compared to controls ($n = 3$). Paricalcitol ($n = 3$) and enalapril ($n = 3$) alone and in combination of the two ($n = 3$) significantly ($P < 0.05$ and $P < 0.001$) exerted anti-inflammatory response by down-regulating the MCP-1, TNF- α and COX-2 expression in atherosclerotic mice. The data represent mean $\pm$ SE of three independent Western blot experiments.

as CKD, renal and cardiac failure, atherosclerosis and hypertension is well known^[18,34,37-39,45]. Chronically increased angiotensin II activity has been implicated in promoting oxidative stress, inflammation and atherosclerosis^[46-48]. The inflammatory and oxidative stress response in atherosclerotic mice can induce the generation of oxidants and inhibit the endogenous antioxidants^[27,30,37,39]. Angiotensin II has been shown to induce vascular oxidative stress through the activation of NADPH oxidase, which leads to the generation of superoxide^[19,20,22,40]. The present data in an ApoE-deficient mouse model of atherosclerosis show that a profound increase in renal NADPH oxidase subunit p22^{phox} protein expression corresponds to enhanced MDA levels and elevated mean BP. The down-regulation of NADPH oxidase by enalapril, paricalcitol and the combination, clearly suggests a role for RAS in the progression of renal oxidative stress. Interestingly the vitamin D analog, paricalcitol, which has been shown to be efficacious in renal and cardiovascular

diseases^[12,31,34-36], has an antioxidant effect by decreasing the superoxide generating enzyme (NADPH oxidase) and MDA levels in the kidney. This action may be related to the negative regulation of RAS by vitamin D analogs^[21,49]. Earlier studies have shown that excess superoxide generation due to NADPH oxidase activation causes inflammation and further generation of inflammatory cytokines (TNF- α) through NF- κ B activation^[21,22,50]. The role of NF- κ B activation is also demonstrated in atherosclerosis^[21,51] and in renal disease^[52]. An earlier report demonstrated that 1, 25-dihydroxyvitamin D₃ blocks hyperglycemia-induced renal injury by blunting NF- κ B activation^[53]. Our recent studies demonstrate that renal and cardiovascular expression of pro-inflammatory substances (MCP-1, TNF- α , and COX-2) and macrophage infiltration are profoundly depressed when paricalcitol was combined with the ACE inhibitor, enalapril, in uremic rats and in atherosclerotic mice^[29,38]. In preclinical studies, the treatment of animals with vitamin D ameliorated fibrosis in the kidney by inhibiting TGF β and inducing hepatocyte growth factor^[5,54]. TGF β has been shown to inhibit interstitial fibrosis *via* down regulation of myofibroblast induction^[54,55]. Preclinical report demonstrated that paricalcitol administered intravenously inhibits interstitial fibrosis in the kidneys of animals with obstructive nephropathy^[35], and a clinical study has shown oral administration protected the kidney function in the CKD patients^[56]. Therefore, the current study demonstrating the depletion of renal inflammatory factors by drugs treatment in atherosclerotic mice, together with previous data, suggests that the renal protection provided by paricalcitol or enalapril and combination of the two is most likely due to their anti-inflammatory actions.

Inflammation and oxidative stress in the kidney of atherosclerotic mice may also be related to an alteration in the cellular antioxidant defense system. Glutathione (GSH), a major non-protein thiol antioxidant, maintains the cellular integrity^[28,57]. It directly scavenges excess ROS and also regenerates cellular antioxidant vitamins^[28,48]. Our data show a significant depletion of renal GSH in atherosclerotic mice, an indicator of oxidative stress^[48,57]. The depletion of GSH is known to cause failed antioxidant defense leading to lipid peroxidation^[28,48,57]. The suppression of cellular glutathione by an inhibitor of glutathione synthesis causes hypertension and cardiovascular oxidative stress in rats^[28]. Therefore, preservation of physiological levels of GSH is a vital cellular mechanism for the prevention of oxidative injury to the kidney. A lower level of antioxidants in the tissues of patients with renal and cardiovascular diseases has also been reported^[16,18,56,58]. The data further show that both paricalcitol and enalapril alone and in combination significantly elevates renal GSH levels suggesting the antioxidant actions of the treatments. The present

data show that renal mitochondrial Mn-SOD protein expression is significantly increased in ApoE-deficient atherosclerotic mice suggesting a defense mechanism for scavenge of excess superoxides. SODs are the primary scavengers of the endogenous ROS in the cells. There are three isozymes of SOD exist in mammalian cells: cytosolic CuZn-SOD, mitochondrial Mn-SOD and extracellular ecSOD and CuZn-SOD activity is inactivated by free radicals^[59]. The inflammatory and oxidative stress in the renal tissues of atherosclerotic mice induced accumulation of oxidant species leading to protein oxidation and degradation of enzyme protein. Importantly, activation or inhibition of SOD activities are known to be due to cellular accumulation of oxidant species^[60]. Mitochondrial Mn-SOD has been reported to be regulated by pro-inflammatory cytokine TNF- α ^[61] and both drugs paricalcitol and enalapril have anti-inflammatory actions^[16,29,33,34,37-39]. Hence the combination of the two drugs would be more effective in the amelioration of renal inflammatory injury in atherosclerosis. The data further show that renal eNOS protein expression down-regulated but iNOS protein expression up-regulated in ApoE-deficient atherosclerotic mice compared to controls suggesting oxidative and inflammatory injury to the renal vascular endothelium. Cellular nitric oxide (NO) is produced by eNOS in the endothelium and by iNOS in response to inflammation^[62]. Treatment with paricalcitol and enalapril attenuated the changes in eNOS and iNOS levels in atherosclerotic mice. The present data as well as our earlier studies^[29,33,37-39] clearly show that vitamin D and ACE inhibitor combo ameliorated the renal inflammation and oxidants/antioxidants imbalance in animal model of atherosclerosis than either drug alone.

In summary, we demonstrated that renal inflammatory and oxidative stress in ApoE-deficient mice is due to enhanced TNF- α , TGF β 1, MCP-1, p22^{phox}, Mn-SOD and iNOS levels and lipid peroxidation and to a reduction in GSH levels and CuZn-SOD protein expression. Treatment with either vitamin D and ACE inhibitor alone or their combo attenuates renal inflammation and oxidative damage in ApoE-deficient animals by depleting TNF- α , TGF β 1, MCP-1, p22^{phox}, Mn-SOD and iNOS levels and lipid peroxidation and by preserving GSH, CuZn-SOD and eNOS levels. In conclusion, the combination of paricalcitol and enalapril is more efficacious than either drug alone in ameliorating renal inflammatory and oxidative stress in atherosclerosis.

COMMENTS

Background

Atherosclerosis is the primary cause of renal, heart and vascular diseases worldwide. There is a strong association between vitamin D deficiency and renin-angiotensin system (RAS) in atherosclerosis. Although the cardiovascular protection by vitamin D and RAS inhibitors have been reported in the atherosclerosis. However the protection against renal inflammation and oxidative stress in atherosclerosis by these agents are not reported.

Research frontiers

The study aimed to investigate the protective efficacy of a vitamin D analog, *i.e.*, paricalcitol, and an ACE inhibitor, *i.e.*, enalapril, either separately or in combo, on inflammation and oxidant damage in the kidney of ApoE-deficient atherosclerotic mice.

Innovations and breakthroughs

This study demonstrates for the first time that renal inflammation and oxidative injury is protected by the combination of paricalcitol and enalapril in ApoE-deficient atherosclerotic mice.

Applications

The study hypothesized that renal inflammation and oxidative stress (oxidants/antioxidants imbalance) in atherosclerosis is ameliorated by the combination of vitamin D and RAS inhibitor in mice. This combination is applicable to be used in the prevention and treatment of atherosclerotic renal injury in patients.

Terminology

The renal inflammatory and oxidative stress in ApoE-deficient mice is due to enhanced inflammatory markers (MCP-1, TNF- α , TGF β 1) oxidants (p22^{phox}, Mn-SOD and iNOS and MDA) and depletion of antioxidants (GSH, CuZn-SOD and eNOS). Administration of vitamin D and ACE inhibitor either separately or in combo attenuates renal inflammation and oxidant damage in ApoE-deficient animals by suppressing inflammatory and oxidant markers and restoring the antioxidant defense system.

Peer-review

The paper "Effect of paricalcitol and enalapril on renal inflammation and oxidative stress in mouse model of atherosclerosis" is very well written research paper. In their study they concluded that paricalcitol and enalapril combination therapy affords protection against renal inflammation and oxidative stress in atherosclerosis.

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Basic Study

Characterization of two alkyl hydroperoxide reductase C homologs alkyl hydroperoxide reductase C_H1 and alkyl hydroperoxide reductase C_H2 in *Bacillus subtilis*

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Abstract

AIM: To identify alkyl hydroperoxide reductase subunit C (AhpC) homologs in *Bacillus subtilis* (*B. subtilis*) and to characterize their structural and biochemical properties. AhpC is responsible for the detoxification of reactive oxygen species in bacteria.

METHODS: Two AhpC homologs (AhpC_H1 and AhpC_H2) were identified by searching the *B. subtilis* database; these were then cloned and expressed in *Escherichia coli*. AhpC mutants carrying substitutions of catalytically important Cys residues (C37S, C47S, C166S, C37/47S, C37/166S, C47/166S, and C37/47/166S for AhpC_H1; C52S, C169S, and C52/169S for AhpC_H2) were obtained by site-directed mutagenesis and purified, and their structure-function relationship was analyzed. The *B. subtilis* *ahpC* genes were disrupted by the short flanking homology method, and the phenotypes of the resulting AhpC-deficient bacteria were examined.

RESULTS: Comparative characterization of AhpC homologs indicates that AhpC_H1 contains an extra C37, which forms a disulfide bond with the peroxidatic C47, and behaves like an atypical 2-Cys AhpC, while AhpC_H2 functions like a typical 2-Cys AhpC. Tryptic digestion analysis demonstrated the presence of intramolecular

Cys37-Cys47 linkage, which could be reduced by thioredoxin, resulting in the association of the dimer into higher-molecular-mass complexes. Peroxidase activity analysis of Cys→Ser mutants indicated that three Cys residues were involved in the catalysis. AhpC_H1 was resistant to inactivation by peroxide substrates, but had lower activity at physiological H₂O₂ concentrations compared to AhpC_H2, suggesting that in *B. subtilis*, the enzymes may be physiologically functional at different substrate concentrations. The exposure to organic peroxides induced AhpC_H1 expression, while AhpC_H1-deficient mutants exhibited growth retardation in the stationary phase, suggesting the role of AhpC_H1 as an antioxidant scavenger of lipid hydroperoxides and a stress-response factor in *B. subtilis*.

CONCLUSION: AhpC_H1, a novel atypical 2-Cys AhpC, is functionally distinct from AhpC_H2, a typical 2-Cys AhpC.

Key words: Cysteine-dependent peroxidase; Thioredoxin; Thiol peroxidase; Peroxiredoxin; Alkyl hydroperoxide; Ortholog; *Bacillus subtilis*; Oxidative stress

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Core tip: Two alkyl hydroperoxide reductase subunit C (AhpC) homologs (AhpC_H1 and AhpC_H2) were identified by searching the *Bacillus subtilis* database. Sequence homology and phylogenetic analyses revealed that AhpC_H1 is an ortholog of *Escherichia coli* (*E. coli*) AhpC, a representative of bacterial AhpC. AhpC_H1 forms dimers consisting of atypical 2-Cys subunits, while AhpC_H2 behaves like a typical 2-Cys AhpC. These AhpC homologs may perform their respective physiological functions at different peroxide levels. Structural and catalytic differences between the enzymes indicate that AhpC_H1 is not an ortholog of *E. coli* AhpC, but a novel type of atypical 2-Cys AhpC.

Cha MK, Bae YJ, Kim KJ, Park BJ, Kim IH. Characterization of two alkyl hydroperoxide reductase C homologs alkyl hydroperoxide reductase C_H1 and alkyl hydroperoxide reductase C_H2 in *Bacillus subtilis*. *World J Biol Chem* 2015; 6(3): 249-264 Available from: URL: <http://www.wjgnet.com/1949-8454/full/v6/i3/249.htm> DOI: <http://dx.doi.org/10.4331/wjbc.v6.i3.249>

INTRODUCTION

Peroxiredoxins (Prxs) are versatile and predominantly thiol-dependent peroxidases (EC 1.11.1.15) that detoxify hydrogen peroxide and various hydroperoxides^[1,2]. They are ubiquitously expressed, with multiple isoforms present in most organisms (e.g., three isoforms in *Escherichia coli*, five in *Saccharomyces cerevisiae*, six in *Homo sapiens*, and nine in *Arabidopsis thaliana*)^[3-13]. Prxs exhibit high reactivity with hydrogen peroxide and

organic hydroperoxides and are involved in peroxide detoxification and signal transduction^[14-20].

All Prxs share a common catalytic mechanism, in which an active-site cysteine (the peroxidatic cysteine) is oxidized to a sulfenic acid by a peroxide substrate, forming a disulfide linkage with a second resolving Cys, which is reduced back by the thioredoxin system consisting of thioredoxin 1 (Trx1), Trx reductase, and NADPH^[1,2]. According to the number of functional Cys residues, Prxs are divided into two families: 1-Cys Prxs and 2-Cys Prxs^[11,15].

The 2-Cys family contains typical and atypical groups of enzymes^[21]. The disulfide bridge formed in typical 2-Cys Prxs after hydroperoxide reduction is intermolecular, whereas in atypical Prxs, it is intramolecular^[22]. The founding members of the 2-Cys Prx family, the Prx1/alkyl hydroperoxide reductase C (AhpC) subfamily, including human Prxs I-IV and bacterial AhpC proteins are well-studied and broadly distributed enzymes that form inter-subunit disulfide bonds during catalysis. The amino acid sequence and physiological function of this type of thioredoxin-dependent peroxidases (thiol-specific antioxidants) were first determined in *Saccharomyces cerevisiae*^[23], and later in humans^[24] and *E. coli*^[3]. The members of these subfamilies are typically dimeric and form intra-subunit disulfide bonds. Another subfamily (the BCP/PrxQ group) is predominantly bacterial, although present in some eukaryotes and plants, but is absent in animals^[6,12]. Most enzymes of the BCP/PrxQ group are reportedly monomeric^[25], but there are two examples of dimeric proteins containing an A-type dimer (the TPx and Prx5 subfamilies). Current information suggests that BCP-PrxQ subfamily members can function as either atypical 2-Cys (α-group) or 1-Cys Prxs^[26].

Moreover, there are also high molecular weight multimeric enzymes^[27]. One atypical 2-Cys Prx exists as a hexamer in oxidized conditions, but dissociates to dimers upon reduction^[28]; the presence of the peroxidatic Cys is critical for hexamer formation, whereas substitution of the resolving Cys does not affect oligomerization^[29]. By analogy with the dimer-decamer transition of the typical 2-Cys Prx previously reported^[27], the dimer-hexamer transition in atypical Prx displays a functional switch, which could be involved in signaling^[28,30].

All aerobic bacteria have evolved a variety of antioxidant enzymes such as superoxide dismutases, catalases, and Prxs to cope with damaging endogenous reactive oxygen species (ROS) generated by the aerobic respiratory chain and metal-catalyzed oxidative reactions^[31]. *E. coli* contains three Prxs: AhpC (Prx1/AhpC subfamily)^[9], TPx (p20)^[4,5], and BCP (BCP/PrxQ subfamily)^[6,25].

Gene sequencing and phylogenetic studies have revealed that *B. subtilis* encodes four putative Prx enzymes with two conserved Cys residues, suggesting that they belong to different subgroups of the 2-Cys

Table 1 Oligonucleotides used for amplifying the genes expressing *Bacillus* peroxiredoxins proteins and its Cys residue-mutated proteins

	Sequence of primer for point mutation
AhpC_H1	
C37S F	AGCGTATTCTCTTTCTACCCA
C37S R	TGGGTAGAAAGAGAATACGCT
C47S F	TTCTCTTTTCGATCTCCAACGAGCTT
C47S R	AAGCTCAGTTGGAGATACGAAAGAGAA
C166S F	CCAGGTGAAGTTTCTCCGGCTAAATGG
C166S R	CCATTAGCCGGAGAACTTCACCTGG
AhpC_H2	
C52S F	TTCACCTTTGTTTCTCCGACAGAAATT
C52S R	AATTTCTGTCGGAGAAACAAAAGTGAA
C169S F	ACTGGCGGACTCTCTCCGGCTAACTGG
C169S R	CCAGTTAGCCGGAGAGAGTCCGCCAGT
	Sequence of primer for protein expression
AhpC_H1	
Forward	CCGCATATGCTTTAATCGGTAAAGAAGATC
Reverse	CCGGTCGACTTAGATTTTACCTACTAGATCAAGG
AhpC_H2	
Forward	CCGCATATGGCAGAACGTATGGTAGGTAA
Reverse	CCGGTCGACTTAAAGTGTTTTTGGCCTGGTTT
B_BCP	
Forward	CCGCATATGACTATAGAAATCGGACAAAAAC
Reverse	CCGGTCGACTTACTTTTTCAGACATTTTGAGG
B_TPx	
Forward	CCGCATATGGCTGAAATTACATTCAAAGGC
Reverse	CCGGTCGACTTACTTTCTAATGCAGCAGC

Prx family: there are two genes encoding putative AhpC-like proteins (AhpC_H1 and AhpC_H2), and two encoding TPx and BCP, respectively. AhpC_H1 has an additional N-terminal Cys residues at positions 37 (Cys37) besides two conserved Cys47 and Cys166, while ahpC_H2 possesses two conserved Cys residues (Cys52 and Cys169).

The present comparative study was designed to characterize two AhpC homologs and reveal the biological significance of the existence of two homologous AhpC enzymes in *B. subtilis*. This paper presents the first report of a detailed structure-functional analysis of the bacillus 2-Cys AhpC homologs AhpC_H1 and AhpC_H2.

MATERIALS AND METHODS

Materials

Bacterial culture media were purchased from Difco Laboratories (Detroit, MI, United States). Restriction enzymes and DNA ligase were obtained from Promega (Madison, WI, United States). Acrylamide-Bis (40% solution) was purchased from Bio-Rad (Hercules, CA, United States). Sodium dodecyl sulfate (SDS), ultrapure glycine, EDTA, dithiothreitol (DTT), Tris base, NADPH, ampicillin powder, isopropyl- β -D-thiogalactopyranoside (IPTG), H₂O₂, *tert*-Butyl hydroperoxide (*t*-BOOH) and cumene hydroperoxide (CMOOH) were from Sigma Aldrich (St. Louis, MO, United States). Iodoacetamide (IAA), N-ethyl maleimide (NEM), *tris* (2-carboxyethyl) phosphine (TCEP), 4-acetamide-4'-maleimidylstilbene-

2,2'-disulfonate (AMS), and sulfhydryl-reactive DyLight 405 maleimide were purchased from Thermo Scientific, Pierce (Rockford, IL, United States).

Sequence and phylogenetic analyses

The NCBI BLAST tool (<http://www.ncbi.nlm.nih.gov>) was used to search for Prx amino acid homologs in the updated GenBank/EMBL and Swiss-Prot databases. Multiple sequence alignments of *B. subtilis* and *E. coli* Prxs were performed using the ClustalW 2.1 program. Phylogenetic and molecular evolutionary analyses were conducted using MEGA (Molecular Evolutionary Genetics Analysis) version 6. A phylogenetic tree was generated by using the Maximum Likelihood method based on the JTT matrix-based model. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site (next to the branches).

Bacterial strains

B. subtilis Cu1065 (*subtilis* strain 168 *attSP β* *trpC2*, a derivative of *B. subtilis* subsp. *subtilis* str. 168) and *E. coli* XL1-blue and BL21 (DE3) strains were grown in Luria Broth (LB) medium under vigorous agitation at 37 °C. Bacterial growth was monitored by the absorbance at 600 nm (OD₆₀₀), and exponentially growing *B. subtilis* cultures were exposed to different stress conditions for 30 min.

Cloning, site-directed mutagenesis, expression, and purification of recombinant proteins

The entire coding regions of *B. subtilis* genes encoding AhpC_H1, AhpC_H2, B_BCP, and B_TPx were amplified by PCR using the High Expand Fidelity kit (Roche Life Science, United States) and primers listed in Table 1. The entire coding region of the *E. coli* *ahpC* gene was amplified by PCR using the following primers: forward, 5'-GGG ATC CCA TAT GTC CTT AAT TAA CAC-3'; reverse, 5'-CCT CGA GTT AGA TTT TAC CAA CCA GGT-3'. To obtain Cys→Ser substitutions in AhpC_H1 and AhpC_H2, point mutations in the *ahpC_H1* and *ahpC_H2* genes were generated by site-directed mutagenesis by using complementary primers (Table 1). Gel-purified *ahpC* genes were digested with *Nde*I and *Xho*I, ligated into the pET-21a(+) expression vector by using T4 DNA ligase (Promega), and used to transform competent *E. coli* XL1-blue. Bacteria were selected on LB agar containing ampicillin (100 μ g/mL), and purified plasmids were used for the sequencing of the cloned coding region by automated DNA sequencing.

The plasmids containing the confirmed sequences were used to transform *E. coli* BL21 (DE3) cells, which were then cultured at 37 °C overnight in 100 μ g/mL ampicillin-containing LB. For protein expression, bacteria were diluted 1:250 in fresh medium, grown until OD₆₀₀ = 0.4, and induced with 0.5 mmol/L isopropyl-L-D-thiogalactose (IPTG) for 4 h. The soluble recombinant proteins were produced in *E. coli*. Cells

Table 2 Oligonucleotides used for amplifying the DNA fusion construct to knockout *Bacillus* Prx genes

Prx null mutants	Primer names	Sequences of paired degenerated primers (5'→3')
ΔAhpC_H1	H1_Up F	CCTTATTTCACAGATAAGCTCCA
	Kan/H1 R'	CCTATCACCTCAAATGGTTCGCTG AATGTATATTCCTCTAAAAATGTAT
	Kan/H1 F'	CGAGCGCTACGAGGAATTTGTATGGAGTGCATTCAATTGGTACTTG
ΔAhpC_H2	H1_Down R	ACGTGCAGTGTAGATCGCTGC
	H2_Up F	AAGTCGCGAATTGAGTTGTCC
	Kan/H2 R'	CCTATCACCTCAAATGGTTCGCTGATGTATCCCTCCAATTTATTGTTTG
	Kan/H2 F'	CGAGCGCTACGAGGAATTTGTAT TTTTTCCTCAAGAACGAAAAGCGG
ΔB_BCP	H2_Down R	ATCTAAAATTGAGGAAAAACATCAA
	BCP_Up F	AATGGTACTGTGATCACTTCGTTT
	Kan/BCP R'	CCTATCACCTCAAATGGTTCGCTG TACGTTACCTCCGGATGTTTTTTT
	Kan/BCP F'	CGAGCGCTACGAGGAATTTGTAT ATCTCTATGAGCCTATGCTTACTT
ΔB_TPx	BCP_Down R	AGTAGTCGATATGTGCATGCATT
	TPx_Up F	TTTTTCGCTTTGGACATGCTAT
	Kan/TPx R'	CCTATCACCTCAAATGGTTCGCTGTATAATTCCTCCCTTTTGTATGTAT
	Kan/TPx F'	CGA GCGCTACGAGGAATTTGTAT GCAGGGAAAAAAGCTCCAGGC
	TPx_Down R	CGTCAGCTTTGAGCTCAAACG

Prx: Peroxiredoxins.

were harvested by centrifugation, suspended in 50 mmol/L Tris-HCl (pH 7.4) containing 2 mmol/L phenylmethylsulfonyl fluoride (PMSF) and 1 mmol/L EDTA, disrupted by sonication, and centrifuged at 16000 × g for 30 min. The clarified supernatant was subjected to ammonium sulfate fractionation, size exclusion chromatography, and anion exchange chromatography. Size exclusion chromatography was performed using a Pharmacia chromatography system (Pharmacia Biotech). Protein solution was loaded onto a Sephacryl S-200 column equilibrated with 200 mmol/L NaCl-containing Tris-HCl, pH 8.0, at a flow rate of 0.2 mL/min at 4 °C, and protein elution was monitored by the absorbance at 280 nm. Anion exchange chromatography was performed using DEAE-cellulose DE52 equilibrated with Tris-HCl, pH 8.0; protein was eluted with a linear salt gradient (0–500 mmol/L NaCl) in Tris-HCl, pH 8.0. Protein concentration was determined using the Bradford protein assay kit (Bio-Rad, United States).

Gene disruption by fusion PCR

The *B. subtilis* Prx-encoding genes were disrupted by the short flanking homology method^[32]. The 5' and 3' regions of the *prx* genes were amplified using primers designed to obtain 0.2-kb DNA fragments (Table 2). A kanamycin-resistance determinant used as a selection marker for ΔPrx strains was amplified from the pDG780 vector using specific primers (forward, 5'-CAG CGA ACC ATT TGA GGT GAT AG-3' and reverse, 5'-CGA GCG CCT ACG AGG AAT TTG TAT-3'). The marker was flanked by the two 0.2-kb terminal regions of the Prx-encoding genes using two-step fusion PCR. The first step was performed by fusing the 5'-terminal Prx gene fragment to the kanamycin-resistance gene. The amplified product was gel-purified and used as a template for the second round of PCR to fuse the 3' terminal Prx gene fragment. The final PCR product was gel-purified and used for the disruption of each Prx-encoding gene in *B.*

subtilis Cu1065. The null mutants were selected on 20 µg/mL kanamycin-containing LB agar, and chromosomal DNA was isolated and analyzed by PCR to confirm the replacement of the target gene.

Electrophoresis and Western blot analyses

Proteins were separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) in 14% acrylamide gels. In reducing conditions, proteins were dissolved in loading buffer containing 100 mmol/L DTT and boiled for 3 min prior to loading; in non-reducing conditions, proteins were loaded in DTT-free buffer without boiling. After electrophoresis, gels were stained with Coomassie Brilliant Blue (Sigma-Aldrich) or processed for Western blotting. For this, PAGE-separated proteins were transferred to nitrocellulose membranes (Bio-Rad), blocked with 5% (w/v) skim milk in PBS containing 0.1% Tween 20 (PBST) at room temperature for 2 h, and probed with a manufactured rabbit polyclonal anti-AhpC_H1 (1:3000) antibodies (Young In Frontier, Seoul, South Korea) for 3 h. After three washes with PBST, the membranes were incubated with horseradish peroxidase-conjugated anti-rabbit secondary antibody for 1 h at room temperature, washed three times with PBST, and developed using the TMB immunoblotting detection system (Kem-En-Tec).

Further protein characterization was performed by 2D electrophoresis. Briefly, the *B. subtilis* protein extract was loaded onto 7-cm immobilized pH gradient (IPG) strips (Bio-Rad, United States) with 4–7 pH gradient (left to right), and the first-dimension separation was conducted; in the second-dimension separation, 16% reducing SDS-PAGE gels were used. For protein detection, the gels were stained with Coomassie Brilliant Blue G-250 or subjected to immunoblotting.

High-performance liquid chromatography

AhpC_H1 was purified from *B. subtilis* and a 25-µg

sample in phosphate buffer, pH 7.4, containing 150 mmol/L KCl was loaded onto a ZORBAX® Pro 10/300 GF450 column equilibrated in phosphate buffer using an automatic injector (SIL-20AC, Shimadzu, Japan). Chromatography was performed on a Shimadzu high-performance liquid chromatography (HPLC) system at the flow rate of 0.5 mL/min at room temperature, and the absorbance was monitored using a diode array detector (SPD M10A, Shimadzu). The column was calibrated using protein molecular mass standards under the same conditions, and a calibration curve was obtained by plotting the V_e/V_o ratio (where V_e is the elution volume and V_o is the void volume) vs the logarithms of the molecular masses.

Chemical modification of cysteine sulfhydryl groups

For the reduction of protein disulfide groups, protein was reacted with chemical reducing agents such as DTT or TCEP or subjected to enzymatic reduction with *E. coli* Trx1, Trx1 reductase, and NADPH. After 30-min reaction at 30 °C, thiol-specific modification reagent 4-acetamide-4'-maleimidylstilbene-2,2'-disulfonate (AMS) was added for another 30 min at 30 °C.

Tryptic digestion and peptide identification

For HPLC analysis of Cys-containing peptides, sulfhydryl-reactive DyLight 405 maleimide (excitation and emission wavelengths 557 and 572 nm, respectively) was used to fluorescently label Cys residues in the intact or TCEP-denatured AhpC_H1. Proteins digested with trypsin treated with L-(tosylamido-2-phenyl) ethyl chloromethyl ketone (TPCK, an inhibitor of contaminating chymotryptic activity) in the presence of 2 mol/L urea were analyzed by reversed-phase HPLC. Briefly, proteins were incubated in 50 mmol/L NH_4CO_3 containing 2 mol/L urea and trypsin at the ratio 1:20 (w/w, trypsin/protein) at 30 °C for 12 h, and then loaded into a C18 HPLC column (2.1 mm × 25 cm). Peptides were eluted with a 5%-100% gradient of acetonitrile containing 0.2% trifluoroacetic acid (TFA) at the flow rate of 1 mL/min for 120 min. The elution of fluorescent peptides was monitored at 572 nm using a fluorescence detector (RF-10AXL, Shimadzu).

Enzyme assays

Enzymatic assays with the Trx system (NADPH, *E. coli* Trx reductase, and Trx1) and peroxides as substrates were conducted to detect peroxidase activity of the purified AhpC proteins by the decrease of absorbance at 340 nm. Briefly, the enzymes were added to the reaction mixture containing 0.14 mmol/L NADPH, 100 nmol/L *E. coli* Trx reductase, 5 $\mu\text{mol/L}$ *E. coli* Trx1, 50 mmol/L Tris-HCl (pH 7.4), 0.1 mmol/L EDTA, and hydroperoxide substrates (H_2O_2 and *t*-butyl hydroperoxide) at room temperature and the absorbance was continuously monitored at 340 nm using a diode array spectrophotometer (HP 7452A). Stock solutions of *t*-butyl hydroperoxide were prepared in DMSO prior to the reaction.

General methods

Plasmid and chromosomal DNA isolation, restriction enzyme digestion, *E. coli* transformation, and DNA ligation were performed according to standard protocols^[32]. The transformation of naturally competent *B. subtilis* cells was carried out as previously described^[33].

RESULTS

Bioinformatic analysis of *B. subtilis* putative Prx homologs

Four *prx*-homologous sequences in *B. subtilis* genome were identified by searching the Data Release R16.1 (the SubtiList World-Wide Web Server, <http://genolist.pasteur.fr/SubtiList/>). Figure 1 shows multiple sequence alignment of three *E. coli* Prxs (E_AhpC, E_BCP, and E_TPx) and four *B. subtilis* Prxs using the ClustalW 2.1 program, and a resulting phylogenetic tree. The hallmark of Prxs is a conserved peroxidatic Cys residue located near the N-terminus. The analysis of multiple protein alignment revealed that all *B. subtilis* Prxs possessed a highly conserved peroxidatic Cys, suggesting that these enzymes belong to the Prx family. The cladogram reveals that, with the exception of B_AhpC_H2, all *Bacillus* Prxs displayed sequence similarity with the corresponding *E. coli* Prxs and were therefore named after the respective *E. coli* enzymes. Thus, E_TPx and B_TPx showed 41.5% identity in 164 overlapping amino acids and E_BCP and B_BCP demonstrated 39.7% identity in 151 overlapping amino acids. E_AhpC and B_AhpC_H1 had 65.24% identity in a 187-amino acid region, while E_AhpC and B_AhpC_H2 had 38.4% identity in a 159-amino acid sequence. Between B_AhpC_H1 and B_AhpC_H2, there was 37.0% identity in a 181-amino acid sequence.

Sequence similarity analysis indicates that AhpC_H1 is a homologue of *E. coli* AhpC, a representative of the AhpC/Prx1 subfamily. Sequence alignment of AhpC homologs revealed two conserved Cys residues, catalytic and resolving, in the N-terminal and C-terminal regions, respectively, suggesting that B_AhpC_H2 also belongs to the AhpC/Prx1 family, although its similarity to E_AhpC (38.4%) is much lower than that of B_AhpC_H1 to E_AhpC (65.2%).

Taken together, these data suggest that *B. subtilis* encodes one member of the BCP/PrxQ, one member of the bacterial TPx, and two members of the AhpC/Prx1 subfamilies.

Structural analysis of *B. subtilis* recombinant AhpC proteins

AhpC_H2 and AhpC_H1 each contain a pair of conserved Cys residues (Cys52/Cys169 and Cys47/Cys166, respectively); in addition, AhpC_H1 possesses an extra Cys37 (Figure 1). The members of the AhpC/Prx1 subfamily (including *E. coli* AhpC) are typical 2-Cys Prxs, which exist as homodimers formed *via* an intermolecular disulfide bond between two subunits.

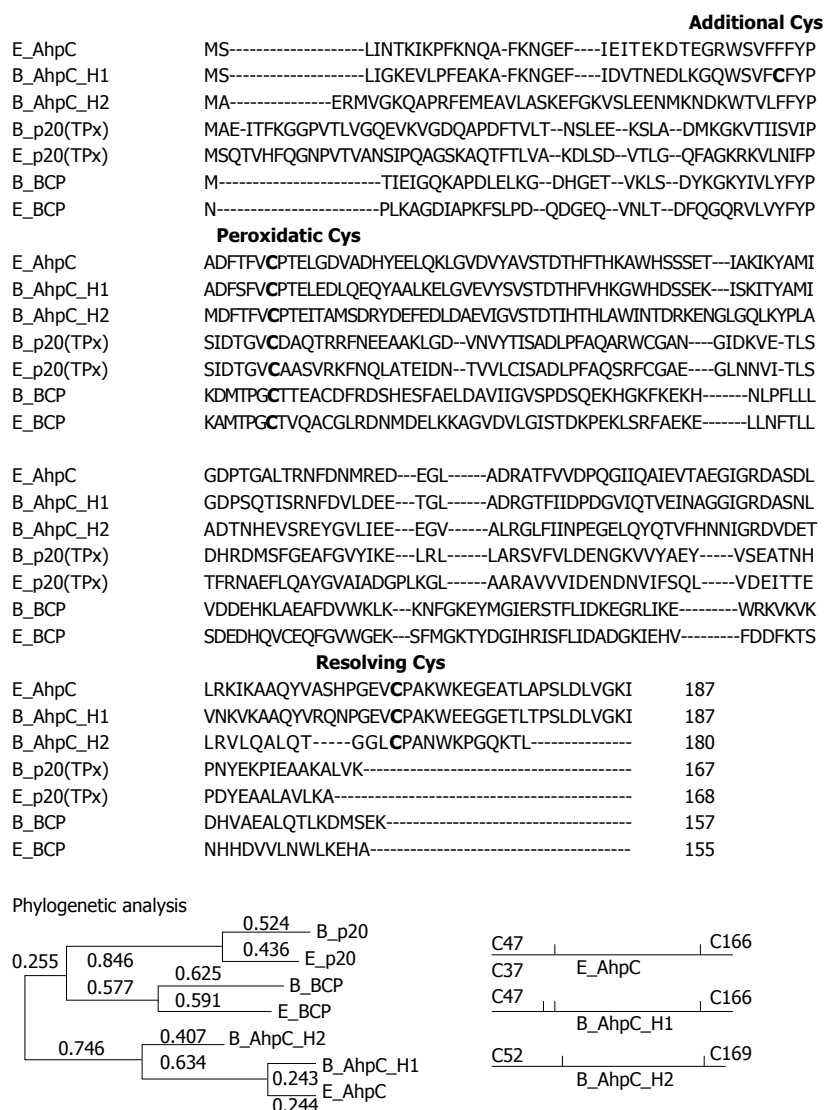


Figure 1 Amino acid sequence alignment of *Bacillus subtilis* and *Escherichia coli* peroxiredoxins. *B. subtilis* database was searched using amino acid sequences around conserved N-terminal Cys residues as a query. In addition to reported bacterial genes encoding BCP and TPx enzymes, two putative AhpC-encoding genes were identified, and four *B. subtilis* and three *E. coli* Prx sequences were aligned. The two conserved Cys residues, N-terminal peroxidatic and C-terminal resolving Cys, among the AhpC proteins are shown as shaded bold letters. Proteins homologous to *E. coli* AhpC were identified using the ClustalW 2.1 multiple sequence alignment. The phylogenetic tree was generated by using MEGA version 6. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site (next to the branches). The analysis involved 7 amino acid sequences. All positions containing gaps and missing data were eliminated. There were a total of 143 positions in the final dataset. Position of Cys residues in AhpC proteins is shown at the bottom right panel. E_BCP, *E. coli* BCP (accession number AAC75533); E_p20 (TPx), *E. coli* TPx (accession number EDV68400); E_AhpC, *E. coli* AhpC (accession NP_415138); B_ahpC_H1, *B. subtilis* AhpC homolog 1 (accession number BAA11268); B_AhpC_H2, *B. subtilis* AhpC homolog 2 (accession number WP_019258276); B_BCP, *B. subtilis* BCP (accession number AAC75533); and B_p20 (TPx), *B. subtilis* TPx (accession NP_390827).

To examine the possible formation of a disulfide bond between the two conserved Cys residues in *B. subtilis* AhpC enzymes, Cys was replaced with Ser by site-directed mutagenesis and the purified mutant AhpC_H1 and AhpC_H2 proteins were analyzed by their migration patterns in SDS-PAGE.

In non-reducing SDS-PAGE gels, wild-type AhpC_H2 and E_AhpC appeared as single bands with the molecular masses about 48 kDa and about 40 kDa, respectively (Figure 2A), whereas single approximately 24.5-kDa and 22-kDa bands, respectively, were observed in reducing gels (Figure 2B), suggesting that in natural conditions, these enzyme exist as dimers

likely held together by disulfide linkages. However, most AhpC_H1 appeared as a single band with a molecular mass of the monomer (approximately 23-kDa) under both non-reducing and reducing conditions (Figure 2A and B, respectively), suggesting the presence of monomers even in the oxidized state.

The migration pattern of AhpC_H2 mutant proteins (C52S, C169S, and C52/169S) in non-reducing SDS-PAGE gels were quite different from that of the wild-type counterpart (Figure 2A). C169S mostly existed in a monomeric form, presenting the evidence of intermolecular disulfide linkage between the two identical subunits. In non-reducing conditions, the

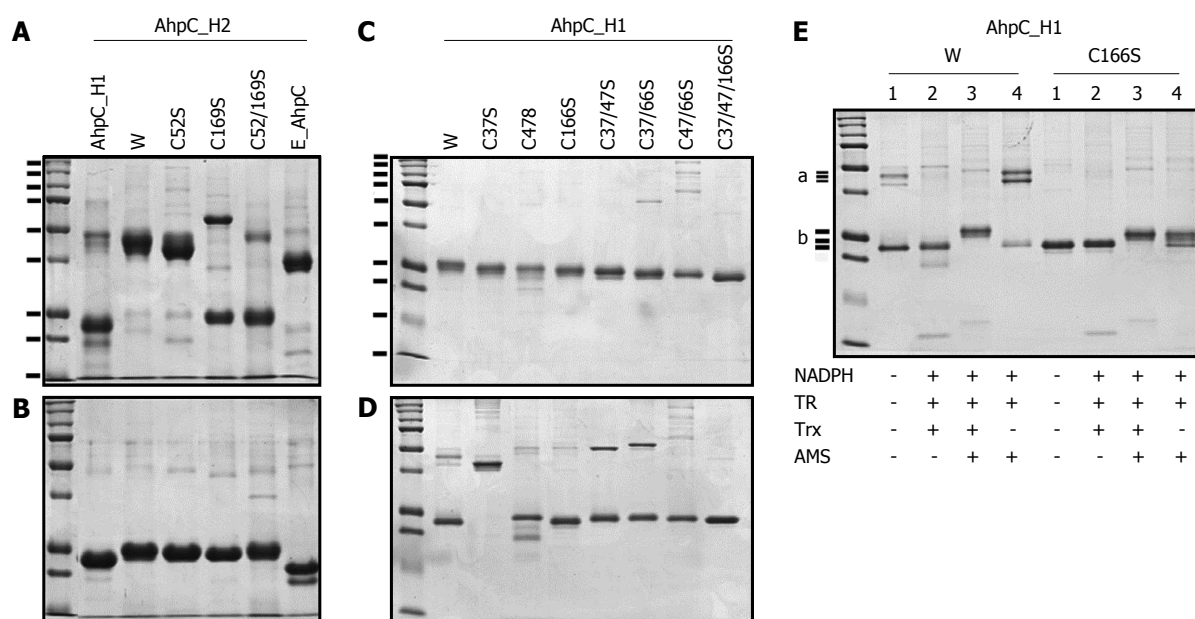


Figure 2 SDS-PAGE analyses of AhpC_H1 and AhpC_H2 proteins and their mutants carrying Cys→Ser substitutions. The wild-type (lane W) and mutated AhpC_H2 and AhpC_H1, and *E. coli* AhpC proteins were separated in non-reducing (A) and reducing (B) 12% SDS-PAGE gels; first lanes show molecular weight markers (15, 20, 25, 37, 50, 75, 100, 150, and 250 kDa). TCEP/AMS-treated (C) and non-treated (D) wild-type (lane W) and mutated AhpC_H1 proteins were separated in non-reducing 14% SDS-PAGE gels. AhpC_H1 and its C166S mutant reduced or not with the Trx system containing Trx 1, Trx reductase (TR), and NADPH were modified with a sulfhydryl group-specific reagent AMS and separated in non-reducing 14% SDS-PAGE gels (E). a marks the position of a single AhpC_H1 band (W, lane 1) and double bands (W, lane 4). b marks three positions of the AhpC_H1 band after the treatments indicated at the bottom. First lanes in B1, B2, and C show molecular weight markers (10, 15, 20, 25, 37, 50, 75, 100, 150, 250 kDa).

migration of the wild-type dimer appeared to be different from that of the mutant C52S and C169S dimers, suggesting the formation of inter-subunit disulfide bonds characteristic for typical 2-Cys Prxs such as E_AhpC.

In contrast to AhpC_H2 and E_AhpC, most of the AhpC_H1 proteins existed as monomers even in the oxidized state. The migration patterns of AhpC_H1 Cys→Ser mutants (C37S, C47S, C166S, C37/47S, C37/166S, C47/166S, and C37/47/166S) indicated that the distance of protein migration in reducing conditions depended on the number of substituted Cys residues (Figure 2C), thus confirming that all three Cys residues are susceptible to AMS modification. In non-reducing conditions, the C37S protein migrated as a dimer (Figure 2D). Considering that, in non-reducing conditions, the wild-type protein was predominantly monomeric (lane W in Figure 2D), dimerization of the C37S mutant suggests that Cys37 could participate in the formation of an intramolecular disulfide bond. In non-reducing gels, most of the proteins carrying C47S and C166S substitutions were detected at the positions slightly higher than that of the wild-type protein; however, a single C166S mutant migrated similarly to the wild-type protein, suggesting the existence of intramolecular disulfide linkage between Cys37 and Cys47 in both proteins.

Taken together, these results indicate a possibility that AhpC_H1 is an atypical 2-Cys enzyme, which forms the intramolecular disulfide linkage between Cys37 and Cys47 rather than between Cys47 and

Cys166. To examine the presence of Cys37-Cys47 intramolecular disulfide bond, the wild-type AhpC_H1 and its C166S mutant were reduced using the Trx system, and analyzed by non-reducing SDS-PAGE. As shown in Figure 2E, Trx-treated C166S occupied a slightly higher position than the wild-type protein; AMS modification shifted the position of both proteins toward higher molecular weight (Figure 2E, lane 3). These results reveal that the disulfide bond can be specifically reduced by the Trx system. Furthermore, AMS modification of the wild-type AhpC_H1 in the absence of Trx1 (Figure 2E, lane 4) or TCEP (data not shown) resulted in the formation of two different dimers, but did not cause dimerization of C166S, which confirms the presence of free Cys166 in AhpC_H1. Other sulfhydryl-modifying agents such as NEM and IAA also induced AhpC_H1 dimerization (data not shown). Overall, the effect of thiol-specific modification of free Cys166 on the tertiary structure of AhpC_H1 is worth noting, although such modification may not occur *in vivo*.

Finally, we directly demonstrated the presence of free Cys166 residue in the oxidized AhpC_H1. AhpC_H1 was modified with fluorescent sulfhydryl-reactive maleimide and digested with trypsin in the presence or absence of 2 mol/L urea. The reversed-phase HPLC chromatogram of trypsin-digested native AhpC_H1 demonstrates a unique peak of a fluorescent peptide (a in Figure 3, W), which was significantly increased in the trypsin-digested denatured (2 mol/L urea-treated) AhpC_H1 (a in Figure 3, W/Urea). The

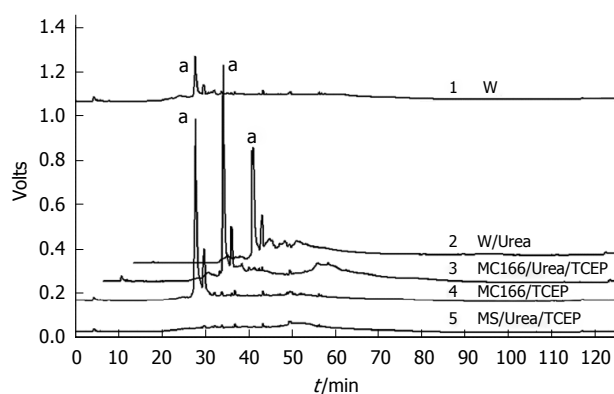


Figure 3 Reversed-phase high-performance liquid chromatography analysis of fluorescently labeled trypsin-digested AhpC_H1. AhpC_H1 was modified with sulfhydryl-reactive DyLight 405 maleimide, digested with TPCK-treated trypsin in the presence or absence of 2 M urea, and analyzed by reversed-phase HPLC; fluorescence was monitored at 527 nm. Fluorescent peak (a) corresponds to a peptide containing free Cys residue conjugated with the fluorescent dye. W: Wild-type AhpC_H1; MC166: C37/47S double mutant; MS: Cys37/47/166S triple mutant.

same peak appeared in the trypsin-digested denatured and native C37/47S double mutant (Figure 3, MC166/Urea/TCEP and M166/TCEP, respectively), which has only one free Cys166 residue. The triple mutant C37/47/166S (MS) did not produce any fluorescent peak. Taken together, these data suggest that the peak represents the peptide with free Cys166 residue, indicating the existence of free Cys166 and the intramolecular disulfide linkage between Cys37 and Cys47.

Enzymatic reduction of the intramolecular disulfide bond between Cys37 and Cys47

The modification of Trx-preincubated AhpC_H1 and AhpC_H2 with AMS, a thiol-specific reagent, resulted in the shift of the monomer to a slightly higher position (Figure 4A and B, respectively), suggesting that the Trx system (Trx1, Trx reductase, and NADPH) is able to reduce protein disulfide bonds in both AhpC_H1 and AhpC_H2.

Pre-incubation of AhpC_H1 with the Trx system containing increasing concentrations of *E. coli* Trx1 resulted in an increase of the AMS-modified AhpC_H1 in a Trx 1 concentration-dependent manner (Figure 4C), indicating that the intramolecular disulfide bond can be reduced by the Trx treatment. The dimerization of AMS-modified AhpC_H1 was also confirmed (Figure 4C). Similarly, the dimeric AhpC_H2 was gradually converted to a corresponding AMS-modified monomer in a Trx1 dose-dependent manner (Figure 4D).

To ensure functional activity of the Trx1 system to reduce disulfide bonds, we tested the Trx system in the reaction with *Bacillus* B_BCP and B_TP_x as well as with Trx-supported peroxidases from other species such as *E. coli* AhpC and human Prx1 and Prx2 (Figure 4E-I, respectively). The results indicate that the reduced forms of all Prxs increased in a

Trx1 concentration-dependent manner, indicating that all the tested enzymes were sensitive to the Trx system. More importantly, in non-reducing conditions, AhpC_H2, but not AhpC_H1, demonstrated a pattern very similar to that of *E. coli* AhpC (E_AhpC) (Figure 4G), suggesting that AhpC_H2 is structurally similar to E_AhpC in that they exist as intermolecular dimers linked by a disulfide bond, although amino acid sequence similarity between AhpC_H1 and E_AhpC is much higher than that between AhpC_H2 and E_AhpC (Figure 1). Taken together, these results indicate the presence of a redox-sensitive disulfide linkage between Cys37 and Cys47 of AhpC_H1, characterizing it as a member of the atypical 2-Cys subfamily.

Identification of Cys37-Cys47 intramolecular disulfide linkage in the native AhpC_H1 protein

In the previous experiments, we demonstrated that, unlike AhpC_H2, AhpC_H1 formed the intramolecular Cys37-Cys47 disulfide bond. However, it may be possible that it is characteristic only for the recombinant protein and not for the native *B. subtilis* AhpC_H1. To test this possibility, we examined the presence of the disulfide linkage in the native *B. subtilis*-purified AhpC_H1. Crude protein extract was fractionated using ammonium sulfate gradient (20% to 60%). Figure 5A shows that a major protein band of approximately 22-kDa was detected in the 40% and 45% fractions. Western blot analysis with the AhpC_H1 antibody demonstrated that the predominant band was AhpC_H1 (Figure 5B). In the 40% fraction, AhpC_H1 was reduced with TCEP, and the resulting product was specifically modified with AMS (Figure 5C). The analysis of the modified proteins by non-reducing SDS-PAGE and western blotting (Figure 5D) revealed that AMS could modify only TCEP-reduced AhpC_H1 (Figure 5D). Taken together, these results demonstrate that *B. subtilis* expresses a significant amount of oxidized AhpC_H1 with the Cys37-Cys47 intramolecular disulfide linkage.

AhpC_H1 oligomerization

Previous studies have demonstrated that the Cys37-Cys47 bond in AhpC_H1 is redox-sensitive. AhpC_H1 molecular mass was determined by HPLC. Figure 6A demonstrates that AhpC_H1 was eluted between ovalbumin (OVA, 44 kDa) and carbonic anhydrase (CA, 29 kDa); therefore, the apparent AhpC_H1 molecular mass was estimated to be approximately 38.6 kDa. Given that a theoretical AhpC_H1 molecular mass is approximately 20.5 kDa, this result indicates that AhpC_H1 exists as a homodimer. Considering that AhpC_H1 mostly appears as a 22-kDa band in non-reducing SDS-PAGE gels, this result suggests that the dimer is likely formed *via* non-covalent interactions. On the other hand, AhpC_H2 was eluted at 9.116 mL (data not shown). Given that the elution volume of apoferritin (AF, 443 kDa) was 9.514 mL, it suggests

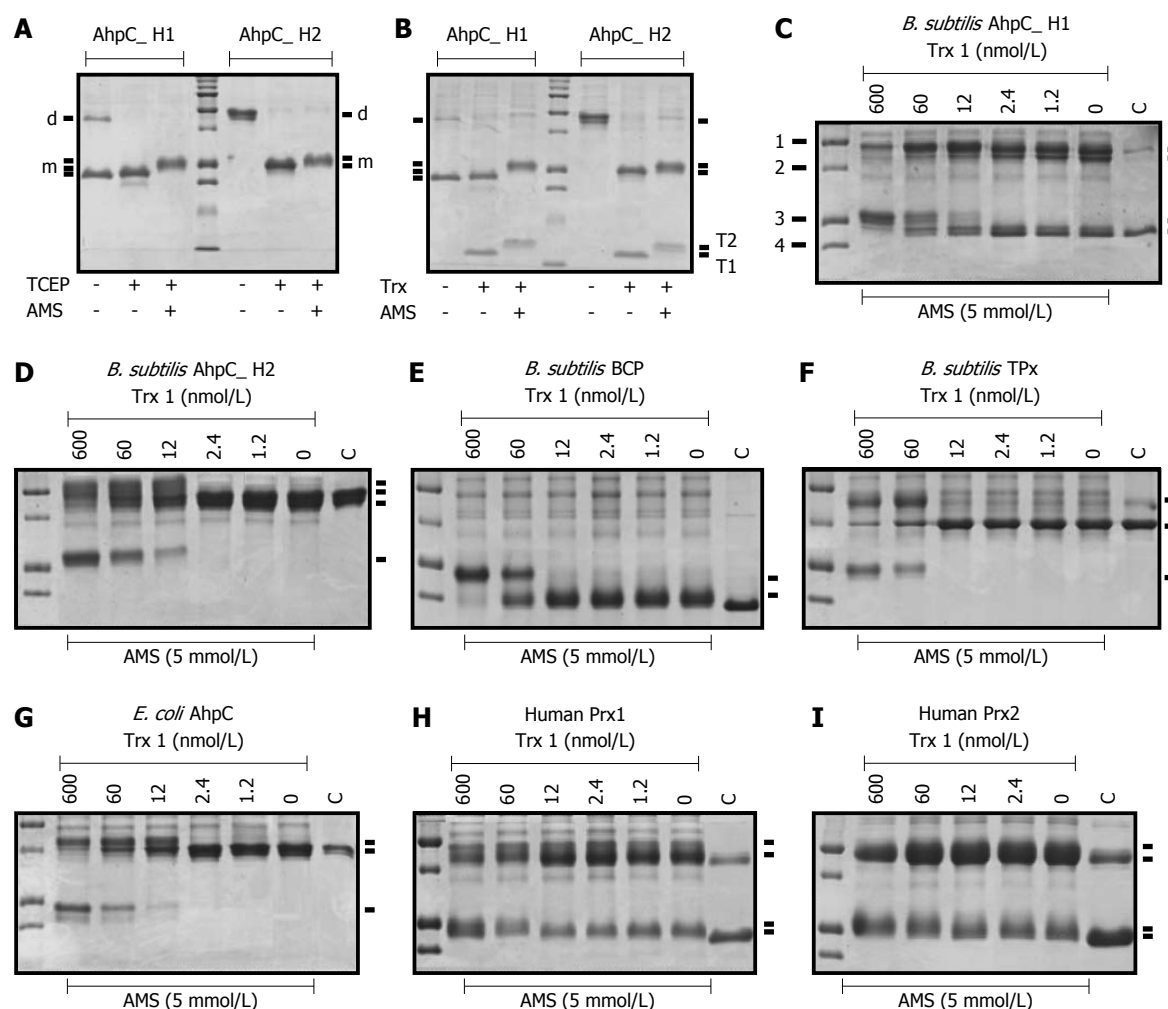


Figure 4 Non-reducing SDS-PAGE of peroxiredoxins reduced with the Trx system. (A, B) AhpC_H1 and AhpC_H2 were incubated without or with 2 mmol/L TCEP (A) or Trx system (B) for 30 min at 30 °C, followed by incubation with AMS for 30 min at 30 °C. The reaction was terminated by adding SDS sample buffer, and proteins were separated in 16% non-reducing SDS-PAGE gels. Protein migration pattern in A is the same as in B, suggesting that the Trx system reduced disulfide bonds in both proteins; d and m mark the position of dimeric and monomeric, respectively, AhpC_H1 and AhpC_H2. T1 and T2 denote the oxidized and reduced/modified Trx 1 proteins, respectively. Middle lanes show molecular weight markers (10, 15, 20, 25, 37, 50, 75, 100, 150, and 250 kDa). Trx, the Trx system; (C-F) Conversion of different Prxs from the oxidized to reduced form by the Trx system. 1, 2, 3, and 4 indicate molecular weight markers of 50, 37, 25, and 20 kDa, respectively.

that AhpC_H2 oligomer has a higher molecular mass compared to AhpC_H1.

It has been reported that in some cases, Prx oligomerization is reversible and depends on the redox status of Cys residues^[27-30]. To examine AhpC_H1 redox-dependent oligomerization, the molecular mass of TCEP-reduced AhpC_H1 was determined by HPLC. Figure 6B shows that the elution volume of reduced AhpC_H1 was 9.379 mL (Figure 6B, AhpC_H1/TCEP), which, considering the elution volume of 443-kDa apoferritin (9.514 mL) indicates that the apparent molecular mass of reduced AhpC_H1 was above 443 kDa. AhpC_H1 incubation with H₂O₂ did not significantly affect the molecular mass (Figure 6B, AhpC_H1/H₂O₂).

Taken together, these results suggest that AhpC_H2 exists as a high molecular mass oligomer, while AhpC_H1 is a homodimer. Oxidized AhpC_H1 monomers are dimerized *via* noncovalent interactions; however, after

reduction, the dimers tend to assemble into high molecular mass oligomers.

Substrate-mediated inactivation of AhpC_H1 and AhpC_H2

Because some Prxs, especially of eukaryotic origin, are known to be inactivated by high substrate concentrations^[1,2], we tested the response of AhpC_H1 and AhpC_H2 to elevated concentrations of their preferred substrate H₂O₂ by using the Trx1 system. Peroxidase activity was continuously monitored by the absorbance at 340 nm indicating NADPH concentration. Figure 7A and B shows a time-dependent decrease in NADPH concentration due to the peroxidase activity of AhpC_H1 and AhpC_H2, respectively. At low H₂O₂ concentrations (< 100 μmol/L), AhpC_H2 was more active than AhpC_H1, but was significantly inhibited by higher H₂O₂ concentrations, whereas AhpC_H1 was resistant even to high H₂O₂ levels, which completely

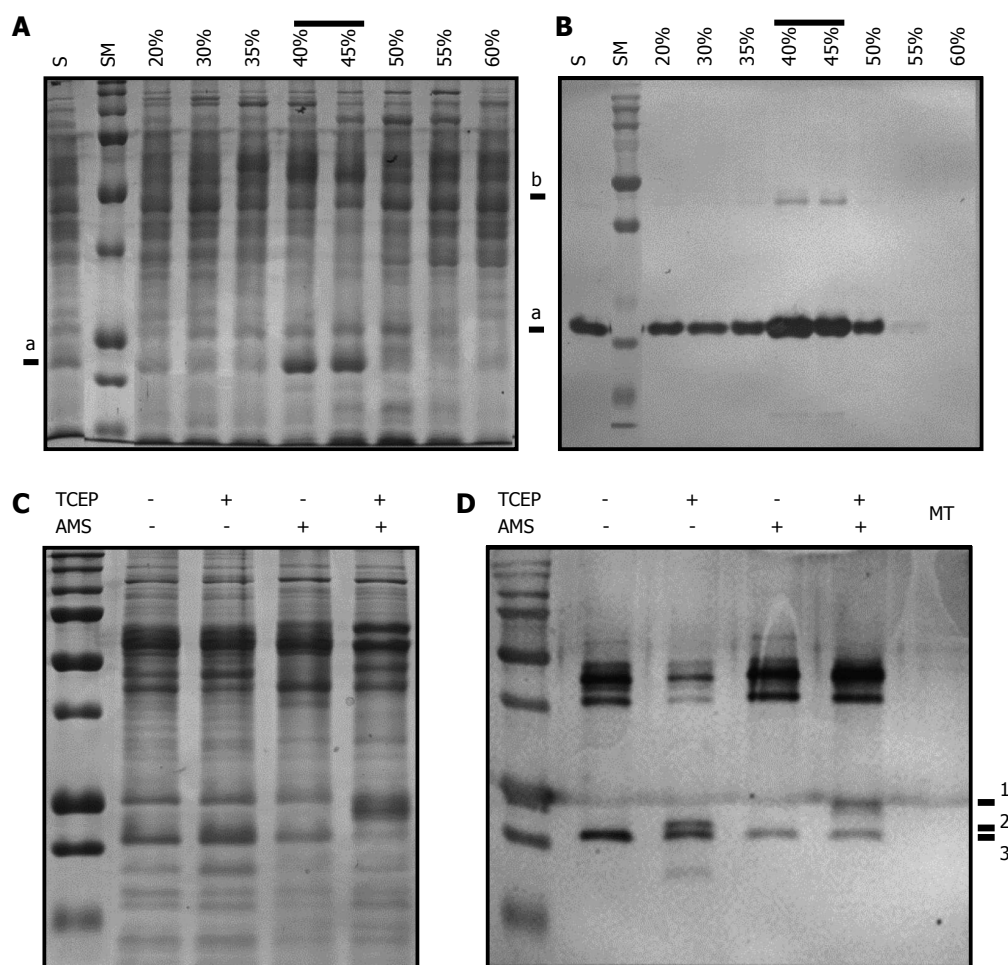


Figure 5 Identification of intramolecular disulfide linkage in AhpC_H1 from *B. subtilis*. A, B: Soluble fraction of *B. subtilis* overnight cultures was precipitated with 30%, 35%, 40%, 45%, 50%, 55%, and 60% ammonium sulfate. After dialysis of solubilized precipitates, 20 μ g of each fraction was separated in 14% reducing SDS-PAGE gel, and stained with Coomassie brilliant blue (A) or processed by western blotting using anti-AhpC_H1 antibody (for western blotting, 1 μ g protein was separated). Correlation of band intensities in (A) and (B) indicates that the major band marked as a is an AhpC_H1 monomer; b marks an AhpC_H1 dimer; C: Proteins (20 μ g) of the 40% fraction were incubated in the presence or absence of 2 mM TCEP, reacted with 5 mmol/L of AMS for 30 min at 30 $^{\circ}$ C, separated by non-reducing SDS-PAGE, and stained with Coomassie Brilliant Blue; D: Similarly treated proteins (100 ng) were subjected to western blotting. Triple bands (1, 2, and 3) around the position of the monomer indicate AMS modification of Cys residues after the reduction by TCEP. MT, AhpC_H1-deficient *B. subtilis*; the absence of the AhpC_H1 band indicates antibody specificity. SM, molecular weight markers (15, 20, 25, 37, 50, 75, 100, 150, 250 kDa).

inhibited AhpC_H2 activity (Figure 7C), indicating that AhpC_H1 is a much more robust enzyme compared to AhpC_H2.

Trx-dependent peroxidase activity of AhpC_H1 mutants

Figure 8 shows Trx-dependent peroxidase activity of the wild-type AhpC_H1 and its C37S, C47S, C166S mutants toward *t*-butyl hydroperoxide substrate. Compared to almost inactive C47S and C166S mutants, the C37S protein showed a substantial enzymatic activity (approximately 19.6% of that of the wild-type protein), indicating that, in addition to Cys47 and Cys166, Cys37 is also required for the peroxidation activity of AhpC_H1.

AhpC_H1 expression in *B. subtilis*

B. subtilis genome encodes four Prxs (B_BCP, B_TPx, B_AhpC_H1, and B_AhpC_H2) (Figure 1), and their expression as antioxidant proteins should be regulated

by growth conditions in a gene-specific manner. SDS-PAGE analysis of *B. subtilis* crude extracts indicates high expression of AhpC_H1 (Figure 5). To further evaluate the regulation of Prx enzymes in *B. subtilis*, mutant strains deficient in B_BCP (Δ BCP), B_TPx (Δ TPx), B_AhpC_H1 (Δ AhpC_H1), or B_AhpC_H2 (Δ AhpC_H2) were constructed and analyzed by 2D electrophoresis. Protein patterns visualized by Coomassie Brilliant Blue staining indicate that one of the predominant spots represented the AhpC_H1 monomer (Figure 9A, a), whereas the other spot detected in the cumene hydroperoxide (CMOOH)-stressed wild-type strain (Figure 9A, W + CMOOH, b) is suggested to be the AhpC_H1 dimer.

Except for AhpC_H1, other Prxs were not detected by 2D electrophoresis (Figure 9A). As expected, AhpC_H1 (Figure 9A, a) was seen in all mutants but Δ AhpC_H1. It is worth noting that another spot corresponding to the AhpC_H1 dimer was detected

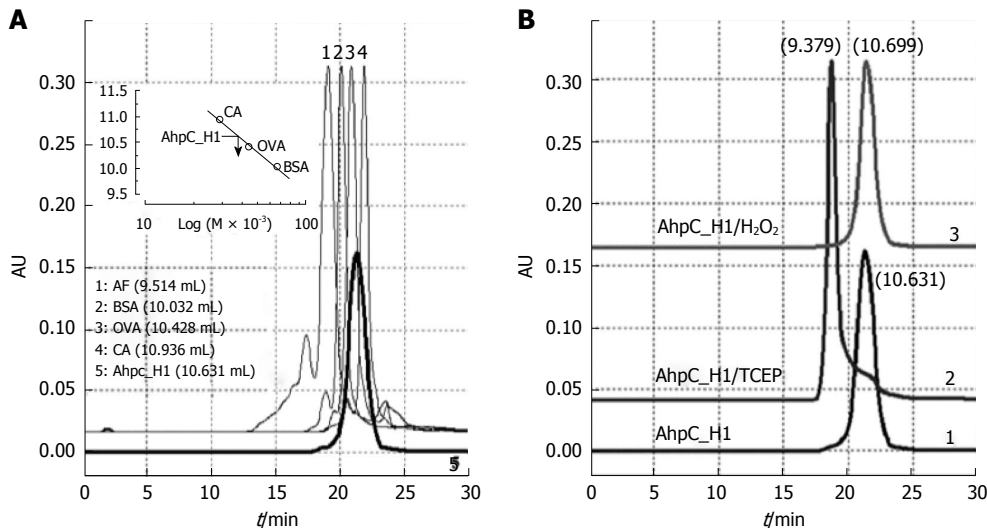


Figure 6 High-performance liquid chromatography gel-filtration chromatograms of AhpC_H1 purified from *B. subtilis*. AhpC_H1 was loaded onto a ZORBAX® Pro 10/300 GF450 column and eluted with 50 mmol/L phosphate buffer, pH 7.4, containing 150 mmol/L KCl. A: AhpC_H1 elution profile (bold line, 5); protein molecular mass standards: 1, apoferritin (AF, 443 kDa); 2, bovine serum albumin (BSA, 66 kDa); 3, ovalbumin (OVA, 44 kDa); 4, carbonic anhydrase (CA, 29 kDa). Inset shows a calibration curve used to determine AhpC_H1 molecular mass; B: The profiles of TCEP-reduced AhpC_H1 (AhpC_H1/TCEP) and AhpC_H1 treated with 2 mmol/L H₂O₂ for 30 min at 30 °C (AhpC_H1/H₂O₂) were compared with that of untreated AhpC_H1. Elution volume (mL) is in parentheses. Oligomeric AhpC_H1 was eluted at 9.379 mL.

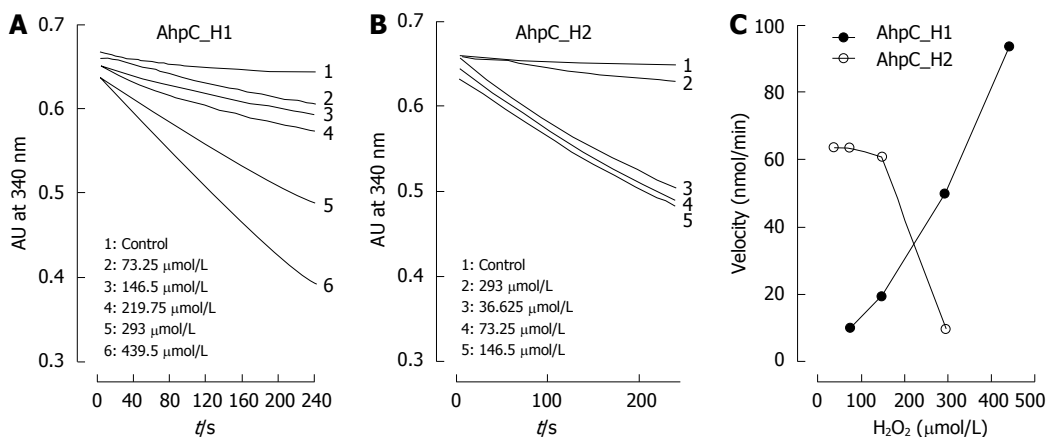


Figure 7 Susceptibility of AhpC_H1 with AhpC_H2 to inactivation by hydrogen peroxide. Peroxidase activity of AhpC_H1 (A) and AhpC_H2 (B) was continuously monitored by the decrease in 340-nm absorbance due to NADPH oxidation at 25 °C in the presence of 50 mmol/L of HEPES-NaOH buffer, pH 7.4, containing 1 mmol/L EDTA, 100 nmol/L of *E. coli* Trx reductase, 0.14 mmol/L of NADPH, 5 μmol/L of *E. coli* Trx, 2 μmol/L of AhpC_H1 and AhpC_H2 and varying concentrations of H₂O₂. Control, absorbance without H₂O₂; C: Changes in initial peroxidase activity rate depending on H₂O₂ concentration. AhpC_H1 was resistant, while AhpC_H2 was sensitive to inactivation by H₂O₂.

by western blotting analysis using anti-AhpC_H1 antibodies (Figure 9B, W/2D Western, b). Taken together, these results suggest that the expression of AhpC_H1 in *B. subtilis* is much higher than that of B_BCP, B_TPx, and AhpC_H2.

Hyperoxidation of AhpC_H1

In oxidative stress-exposed *B. subtilis*, the position of the AhpC_H1-corresponding spot at a more acidic area (Figure 9A, W+CMOOH, b) suggests that Cys residue(s) could be hyperoxidized to Cys-SO₂H or Cys-SO₃H^[34]. To further investigate AhpC_H1 response to hyperoxidation, exponentially growing *B. subtilis* was treated with increasing concentrations

of CMOOH, and protein migration was analyzed by 2D electrophoresis. Figure 9C shows that CMOOH treatment stimulated, at a concentration-dependent manner, the appearance of two different protein spots (b and c) at more acidic positions; at the same time, the original protein spot (a) gradually disappeared, while spot c increased. Considering that sulfinic acid (Cys-SO₂H) is more negatively charged than sulfenic acid (Cys-SO₂H), spots b and c seem to correspond to the sulfenic and sulfinic forms of AhpC_H1. The predominant appearance of the more acidic AhpC_H1 form in the oxidative stress-exposed *B. subtilis* might be considered as evidence that Cys166 residue is the primary site for hyperoxidation, provided that only

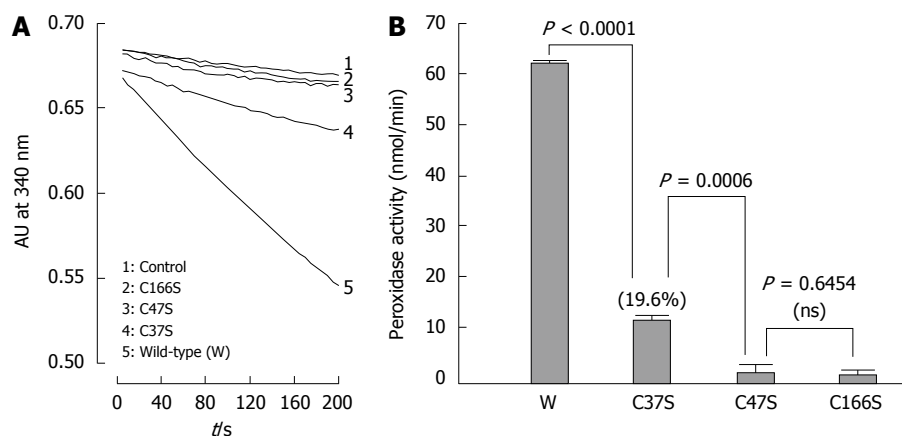


Figure 8 Trx system-supported alkyl hydroperoxide peroxidase activity of the wild-type and mutant AhpC_H1 proteins. A: Peroxidase reaction was initiated by adding 2 $\mu\text{mol/L}$ of the wild-type (W) or C37S, C47S, and C166S mutated AhpC_H1 enzymes to the reaction mixture containing 50 mmol/L Hepes-NaOH, pH 7.4, 1 mmol/L EDTA, 100 nmol/L of *E. coli* Trx reductase, 5 $\mu\text{mol/L}$ of *E. coli* Trx, and 0.14 mM NADPH; the activity was continuously monitored by the decrease in 340-nm absorbance at 25 $^{\circ}\text{C}$; B: Initial peroxidase activity rate of the wild-type and mutant AhpC_H1. The data are expressed as the mean $\pm$ standard error of three independent measurements.

Cys166 is reduced among the three Cys residues of AhpC_H1.

AhpC_H1 induction in *B. subtilis* exposed to alkyl hydroperoxides

AhpC_H1 expression in *B. subtilis* in response to oxidative stress was analyzed in exponentially growing cultures treated with different concentrations of H_2O_2 , *t*-butyl hydroperoxide (tBOOH), CMOOH, paraquat (PQ), and diamide for 45 min. Western blotting analysis (Figure 10) indicates that AhpC_H1 levels were not significantly affected by H_2O_2 , paraquat, and diamide; however, organic peroxides, especially CMOOH, markedly increased AhpC_H1 expression in *B. subtilis*, suggesting the function of AhpC_H1 as an antioxidant scavenger of lipid hydroperoxides.

Phenotype of the AhpC_H1-deficient mutant strain

To determine functional significance of four *Bacillus* Prxs, we compared the growth of the $\Delta\text{AhpC_H1}$, $\Delta\text{AhpC_H2}$, $\Delta\text{B_BCP}$, and $\Delta\text{B_TPx}$ deletion mutants with that of the parental wild-type strain. All of the mutants exhibited gradual reduction of cell density in the stationary phase (Figure 11A), which could be caused by a decrease of cell viability. Figure 11B shows that among the four null mutants, $\Delta\text{AhpC_H1}$ exhibited better survival in the stationary phase, despite much higher levels of AhpC_H1 expression in the wild-type *B. subtilis* compared to other Prxs.

DISCUSSION

Sequence homology and phylogenetic analysis performed in this study reveal that AhpC_H1 is closely related to *E. coli* AhpC (E_AhpC), a typical representative of bacterial AhpC, suggesting that AhpC_H1 may be an E_AhpC ortholog. AhpC_H1 has the highest sequence similarity to E_AhpC among the four analyzed *B. subtilis*

Prxs (65.24% identity in a 187-amino acid region), although it possesses an extra Cys37 in addition to two conserved residues, Cys47 and Cys166. AhpC_H2, another AhpC enzyme in *B. subtilis*, showed 38.4% identity with E_AhpC in a 159-amino acid sequence. Our results suggest that unlike AhpC_H1, AhpC_H2 is a member of the typical 2-Cys AhpC/Prx1 subfamily, which includes *E. coli* alkyl hydroperoxide reductase. Comparative structural analysis and evaluation of peroxidase activity, resistance to inactivation by peroxide substrates, and phenotypes of null mutants have demonstrated that AhpC_H1 is a new type of bacterial AhpC, with the structure and functional activity distinct from those of AhpC_H2. Thus, our data indicate that AhpC_H2, not AhpC_H1, is an ortholog of *E. coli* AhpC. AhpC_H1 (GenBank accession number BAA11268) was first suggested to be an AhpC subunit of the *B. subtilis* alkyl hydroperoxide reductase induced in response to stressful conditions such as heat and high salinity or growth inhibition at the stationary phase^[35-37]. We found a gradual decrease in the viability of Prx-deficient mutant strains in the stationary phase, which is consistent with previous reports, suggesting a functional role of these enzymes in *B. subtilis* stress response during the stationary growth phase. Together with AhpC_H1, other three *B. subtilis* Prxs are likely to be involved in bacterial defense system against toxic conditions created by increased oxidative stress^[38].

Bacterial AhpC enzymes are known as electron acceptors from the Trx system, although they show much higher reactivity with their specialized flavoprotein reductase AhpF^[9]. AhpC proteins from *Mycobacterium tuberculosis*^[39] and *Helicobacter pylori*^[40] have been identified as Trx-dependent alkyl hydroperoxide reductases. In this study, we observed that, regardless of the nature of disulfide linkages (intra- or intermolecular), all four *B. subtilis* Prxs could be easily reduced by the Trx system consisting of Trx1, Trx reductase, and NADPH,

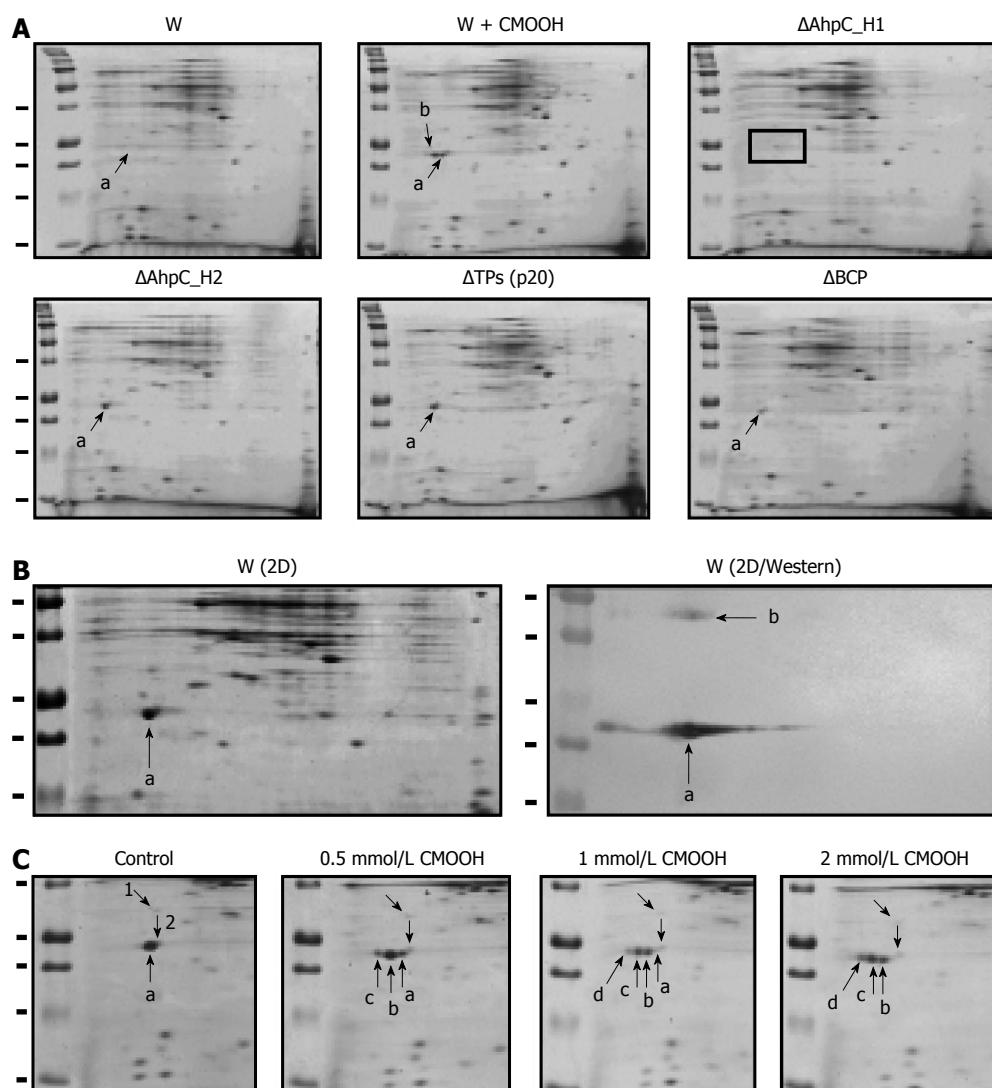


Figure 9 Expression of peroxiredoxins in the wild-type and Prx-deficient *B. subtilis* strains. A: Two-dimensional (2D) electrophoresis of soluble proteins from untreated (W) and cumene hydroperoxide (CMOOH)-treated (W + CMOOH) wild-type *B. subtilis*, and Prx-null mutant strains. Soluble protein fraction (20 μg) obtained from exponential *B. subtilis* cultures treated with or without 1 mM CMOOH for 30 min was separated in 2D gels and analyzed for AhpC_H1 expression after staining with Coomassie Brilliant Blue. a indicates AhpC_H1; b indicates AhpC_H1 shift to acidic area; in AhpC_H1 deletion mutant ($\Delta\text{AhpC_H1}$), AhpC_H1-specific signal is absent; B: *B. subtilis* was grown and treated as in (A), and soluble proteins (1 μg) were separated by 2D electrophoresis and stained with Coomassie Brilliant Blue (W/2D) or analyzed by western blotting using polyclonal antibodies against AhpC_H1 (2D/Western); a and b indicate the position of AhpC_H1 monomeric and dimeric forms, respectively; C: *B. subtilis* cultures were grown as in (A), treated with the indicated concentrations of CMOOH for 30 min, and soluble proteins were separated by 2D electrophoresis followed by Coomassie Brilliant Blue staining. a, indicates original and b, c, and d indicate oxidized AhpC_H1 proteins; 1 and 2 denote internal standards. 2D electrophoresis was performed using 4–7 pH gradients (left to right); molecular weight markers (50, 37, 25, 20, and 15 kDa) are shown in the left lines.

although in the oxidized AhpC_H1, the intramolecular disulfide bond was formed between Cys37 and Cys47 and not between the conserved Cys47 and Cys166 residues. Conversion of the reduced AhpC_H1 from a dimeric form to a high-molecular mass oligomer raises a possibility that the disulfide bond in AhpC_H1 has a more complex role as a functional motif, which promotes conformational transitions unrelated to disulfide linkage. In the case of typical 2-Cys Prx members belonging to the AhpC/Prx1 subfamily, hyperoxidation of the conserved Cys residue(s) has been known as a covalent modification, which stabilizes higher order structures^[33,41]. We have also demonstrated the existence of the hyperoxidized AhpC_H1 as a predominant form in oxidative-stress-exposed *B. subtilis*. Based on the

observation of oxidation-induced dimerization (Figure 6B), we could speculate that the reduction of the disulfide bond between Cys37 and Cys47 may be the first step in the oligomerization process. Covalent modification of Cys residues could stabilize higher order structures as is the case with typical 2-Cys Prx members that lack the intramolecular disulfide bond.

Similar to AhpC_H1, *M. tuberculosis* AhpC has been reported to possess three Cys residues, two at positions 174 and 176 in the C-terminal region, and the conserved Cys61 at the N-terminus. Both Cys174 and Cys176 can form an intermolecular disulfide bond with the conserved N-terminal Cys61 during cyclic catalysis, and all three Cys residues are shown to be important for enzymatic activity^[42]. In AhpC_H1, the

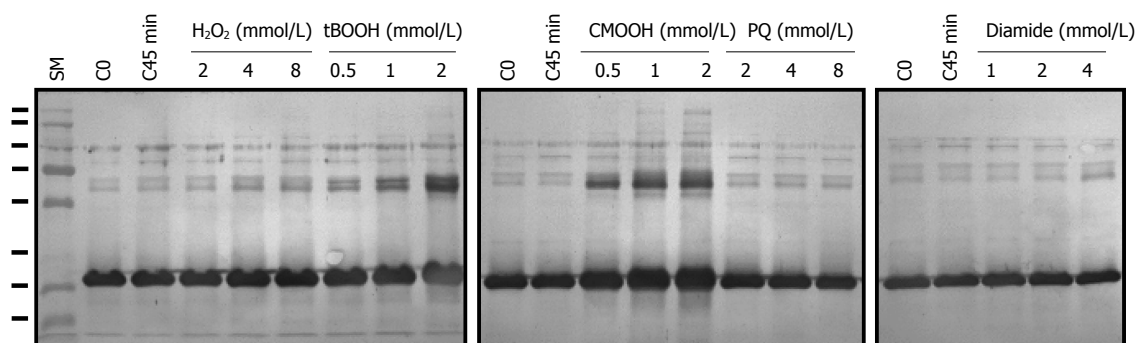


Figure 10 Effects of various oxidants on AhpC_H1 expression in *B. subtilis*. Exponentially grown bacterial cultures were treated with indicated concentrations of H₂O₂, t-butyl hydroperoxide (tBOOH), cumene hydroperoxide (CMOOH), paraquat (PQ), and diamide for 45 min at 30 °C. Soluble proteins (5 µg) were separated in 14% reducing SDS-PAGE gels, transferred to nitrocellulose membrane, and reacted with the polyclonal AhpC_H1 antibody. C0 and C45min indicate untreated *B. subtilis* at time 0 and after 45-min growth, respectively. SM, molecular weight markers (15, 20, 25, 37, 50, 75, 100, and 150 kDa).

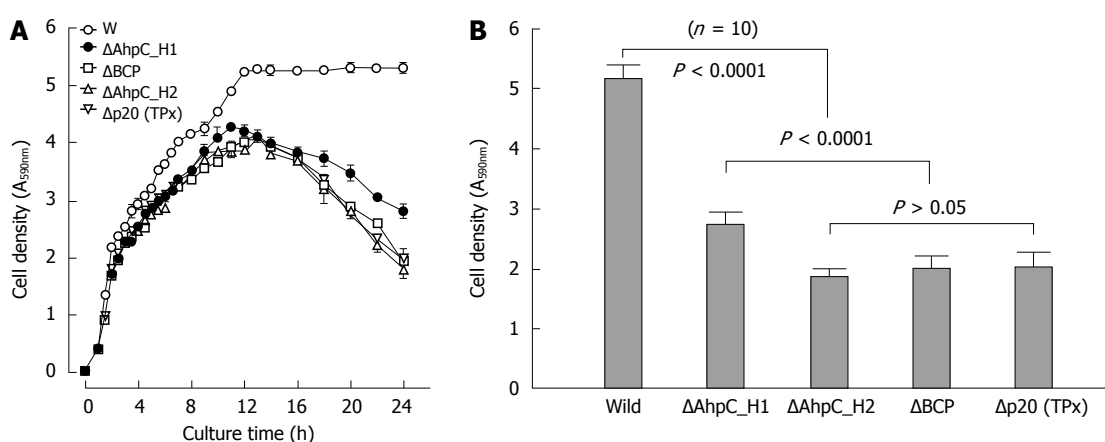


Figure 11 Growth of the wild-type and Prx-deficient Δ AhpC_H1, Δ AhpC_H2, Δ BBCP, and Δ BTPx *B. subtilis* strains. A: Bacterial growth was monitored by the optical density at 600 nm (OD₆₀₀) for up to 25 h after addition of the same cell density of exponentially grown culture in aerobic condition; the data are expressed as the mean $\pm$ standard error of five independent measurements; B: The growth of the wild-type and Prx-deficient *B. subtilis* strains after 1 d of culture. The data are expressed as the mean $\pm$ standard deviation of 10 independent measurements.

additional Cys37 was also observed to be involved in peroxidation catalysis. Distinct catalytic activities of AhpC_H1 and AhpC_H2 toward H₂O₂ (Figure 7C) may provide a rationale for the existence of two different AhpC enzymes in *B. subtilis*, which may function at different concentrations of H₂O₂.

Peroxiredoxins, a class of ubiquitously expressed cysteine-dependent peroxidases, serve not only as ROS detoxifiers but also as regulators of signal transduction pathways and peroxide responses. Thus, an intriguing feature of AhpC_H1 is redox-dependent oligomerization, which regulates enzyme activity. Other functional consequences of this dimer-oligomer conversion remain to be solved, but we hypothesize that the reduction of the intra-subunit disulfide linkage between Cys37 and Cys47 by the Trx system or other electron donors serves to stabilize the oligomeric structure, and further regulates some physiological processes. Given that AhpC_H1 is the most abundant protein among the four types of *B. subtilis* Prxs, the detailed *in vivo* function of AhpC_H1 is worth further investigation.

In summary, structural and functional differences

between AhpC_H1 and AhpC_H2 revealed in this study clearly demonstrate that AhpC_H1 is not an ortholog of *E. coli* AhpC, but a novel type of atypical 2-Cys AhpC. The present data also suggest that AhpC_H2, as a typical 2-Cys enzyme, is a true member of the AhpC/Prx1 subfamily, although AhpC_H1 has the highest sequence similarity to *E. coli* AhpC among the four *B. subtilis* Prxs.

COMMENTS

Background

Peroxiredoxins (Prxs) are versatile and predominantly thiol-dependent peroxidases that detoxify hydrogen peroxide and various hydroperoxides. They are ubiquitously expressed, with multiple isoforms present in most organisms. Prxs exhibit high reactivity with hydrogen peroxide and organic hydroperoxides and are involved in peroxide detoxification and signal transduction. The present comparative study was designed to characterize two alkyl hydroperoxide reductase (AhpC) homologs and reveal the biological significance of the existence of two homologous AhpC enzymes in *B. subtilis*.

Research frontiers

This paper is the first report of a detailed structure-functional analysis of the bacillus 2-Cys AhpC homologs AhpC_H1 and AhpC_H2.

Innovations and breakthroughs

AhpC_H1 is not an ortholog of *E. coli* AhpC, but a novel type of atypical 2-Cys AhpC, and AhpC_H2, as a typical 2-Cys enzyme, is a true member of the AhpC/Prx1 subfamily, although AhpC_H1 has the highest sequence similarity to *E. coli* AhpC among the four *B. subtilis* Prxs.

Applications

Peroxiredoxins, a class of ubiquitously expressed cysteine-dependent peroxidases, serve not only as ROS detoxifiers but also as regulators of signal transduction pathways and peroxide responses. Thus, an intriguing feature of AhpC_H1 is redox-dependent oligomerization, which regulates enzyme activity. The reduction of the intra-subunit disulfide linkage between Cys37 and Cys47 by the Trx system or other electron donors serves to stabilize the oligomeric structure, and further regulates some physiological processes. Given that AhpC_H1 is the most abundant protein among the four types of *B. subtilis* Prxs, the detailed *in vivo* function of AhpC_H1 is worth further investigation.

Terminology

Peroxiredoxins (Prxs): a ubiquitous family of antioxidant enzymes that convert a harmful peroxide to water and oxygen. Peroxiredoxins are frequently referred to as alkyl hydroperoxide reductase (AhpC) in bacteria. Other names include thiol specific antioxidant (TSA). This family contains AhpC and TSA, as well as related proteins; Phylogenetic analysis: An analysis of relationships between collection of genes and proteins that are derived from a common ancestor; Orthologous proteins: any proteins found in two or more species that can be traced to a common ancestor; specif. one of two homologous genes that is descended from a common ancestor, but which has evolved in a different way; Homologous proteins: proteins having a very similar primary, secondary, and tertiary structure.

Peer-review

The paper examines the two proteins in an attempt to discover the function of these two homologous enzymes in the same organism, both of which are postulated to protect against oxidative damage in cells. These two proteins were initially identified by sequence homologies, cloned, expressed, and purified. The authors have also generated a number of site-directed mutants of the proteins and examined the role of Cys residues in the proteins. Overall, the work in the manuscript carefully examines both the biochemistry and genetics of the AhpC H1 and H2 proteins. AhpC H1 levels increase in response to alkyl peroxides. The authors have indicated that at least AhpC H1 protects against oxidative stress. It appears that there is a significant drop in cell density. Using the mutant proteins and complementation with the AhpC H1 or H2 would have provided further proof of the role of these proteins. Also, showing actual survival of the cells in response to alkyl peroxides or hydrogen peroxide would have been useful.

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Basic Study

Effect of agitation speed on the morphology of *Aspergillus niger* HFD5A-1 hyphae and its pectinase production in submerged fermentation

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Author contributions: Ibrahim D designed and coordinated the research; Weloosamy H performed the majority of experiments; Lim SH analyzed the data; Ibrahim D, Weloosamy H and Lim SH wrote the paper.

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Abstract

AIM: To investigate the impact of agitation speed on pectinase production and morphological changing of *Aspergillus niger* (A. niger) HFD5A-1 in submerged fermentation.

METHODS: A. niger HFM5A-1 was isolated from a rotted pomelo. The inoculum preparation was performed by adding 5.0 mL of sterile distilled water containing 0.1% Tween 80 to a sporulated culture. Cultivation was carried out with inoculated 1×10^7 spores/mL suspension and incubated at 30 °C with different agitation speed for 6 d. The samples were withdrawn after 6 d cultivation time and were assayed for pectinase activity and fungal growth determination. The culture broth was filtered through filter paper (Whatman No. 1, London) to separate the fungal mycelium. The cell-free culture filtrate containing the crude enzyme was then assayed for pectinase activity. The biomass was dried at 80 °C until constant weight. The fungal cell dry weight was then expressed as g/L. The 6 d old fungal mycelia were harvested from various agitation speed, 0, 50, 100, 150, 200 and 250 rpm. The morphological changing of samples was then viewed under the light microscope and scanning electron microscope.

RESULTS: In the present study, agitation speed was found to influence pectinase production in a batch cultivation system. However, higher agitation speeds than the optimal speed (150 rpm) reduced pectinase

production which due to shear forces and also collision among the suspended fungal cells in the cultivation medium. Enzyme activity increased with the increasing of agitation speed up to 150 rpm, where it achieved its maximal pectinase activity of 1.559 U/mL. There were significant different (Duncan, $P < 0.05$) of the pectinase production with the agitation speed at static, 50, 100, 200 and 250 rpm. At the static condition, a well growth mycelial mat was observed on the surface of the cultivation medium and sporulation occurred all over the fungal mycelial mat. However with the increased in agitation speed, the mycelial mat turned slowly to become a single circular pellet. Thus, it was found that agitation speed affected the morphological characteristics of the fungal hyphae/mycelia of *A. niger* HFD5A-1 by altering their external as well as internal cell structures.

CONCLUSION: Exposure to higher shear stress with an increasing agitation speed could result in lower biomass yields as well as pectinase production by *A. niger* HFD5A-1.

Key words: *Aspergillus niger*; Agitation; Submerged culture; Pectinase; Microbial growth; Dissolved oxygen

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Core tip: We report the influence of agitation speed on pectinase production on our newly isolate *Aspergillus niger* (*A. niger*) HFM5A-1 in submerged fermentation. The agitation speed was found to influence pectinase production in a batch cultivation system. However, higher agitation speeds than the optimal speed (150 rpm) reduced pectinase production which due to shear forces and also collision among the suspended fungal cells in the cultivation medium. It was found that agitation speed affected the morphological characteristics of the fungal hyphae/mycelia of *A. niger* HFD5A-1 by altering their external as well as internal cell structures.

Ibrahim D, Weloosamy H, Lim SH. Effect of agitation speed on the morphology of *Aspergillus niger* HFD5A-1 hyphae and its pectinase production in submerged fermentation. *World J Biol Chem* 2015; 6(3): 265-271 Available from: URL: <http://www.wjgnet.com/1949-8454/full/v6/i3/265.htm> DOI: <http://dx.doi.org/10.4331/wjbc.v6.i3.265>

INTRODUCTION

Pectinases constitute is a unique group of enzymes that cleave the glycosidic bonds of galacturonic acid residues of pectic substances, which are the complex structural polysaccharides of plant cells. Major pectinases are refer to polygalacturonase, pectin lyase, pectate lyase and pectin esterase^[1]. Pectinases are

useful industrial enzyme and are largely used in various industrial processes such as for solubilization of the cell wall of plants, wood, fruit or paper^[2,3].

There are several parameters influencing the maximal enzyme production in submerged fermentation, usually it consist of physical and chemical parameters. Physical parameters involving the optimization of initial medium pH, cultivation temperature, inoculum sizes and also agitation speed. Microorganisms differ in their oxygen requirement. Oxygen acts as a terminal electron acceptor for oxidative reactions to provide energy for cellular activities. The rate of the agitation speed could influenced the extent of mixing in the shake flasks system and affected the nutrient availability as well^[4]. Agitation speed has been affected many enzymes activities in different strains of bacteria and fungi^[5,6] as well as microalgae^[7].

Agitation provides adequate mixing, mass, heat transfer and also improving dissolved oxygen in the culture medium. At lower agitation speed insufficient oxygen in the culture medium usually affects the microbial growth, whereas higher agitation speeds sometimes also lowering the enzymes production^[8]. Higher agitation speed develop shear forces among the suspended microbial cells in the culture medium and the production drops due to cell damages which results from cell collision. Shear forces also can give several effects on the fungal cell. It can cause the morphological changes to the fungal by damaging the external and internal cell structures, variation in fungal growth and yield formation^[9]. Therefore, the optimal agitation speed is necessary to be determined in order to obtain maximal enzyme production. The aim of this study was to determine the influence of agitation speed on pectinase production and morphology of *Aspergillus niger* (*A. niger*) HFD5A-1 in submerged fermentation using a flask system.

MATERIALS AND METHODS

Microorganisms, culture maintenance and inoculum preparation

A. niger HFM5A-1 which was isolated from a rotted pomelo was supplied by the Industrial Biotechnology Research Laboratory, School of Biological Sciences, Universiti Sains Malaysia, Penang, Malaysia. The fungal culture was maintained on potato dextrose agar (Merck, Germany) slant supplemented with 1.0% citrus pectin (w/v) (Sigma, Denmark) at 30 °C for five days aerobically (until sporulation) before storing them at 4 °C until further use. The cultures were sub-cultured regularly at every month to maintain viability.

The inoculum preparation was performed by adding 5.0 mL of sterile distilled water containing 0.1% Tween 80 to a sporulated culture. The spores were dislodged by using a sterile inoculation loop and shaking vigorously. The spore suspension was adjusted to 1×10^7 spores/mL using haemocytometer chamber

(Neubauer Germany) and 1.0 mL was taken as the inoculum.

Cultivation medium and fermentation

The cultivation medium used for fermentation was a modified medium of Maldonado and Strasser de Saad^[10]. The medium consisted of (w/v): KH₂PO₄, 0.4%; Na₂HPO₄, 0.2%; FeSO₄·7H₂O, 0.02%; CaCl₂, 0.001%; peptone, 0.4%; MnSO₄·7H₂O, 0.007%; H₃BO₃, 0.001% and citrus pectin, 1.8%. The initial pH of the medium was adjusted to 4.5. Cultivation was carried out after inoculated with 1×10^7 spores/mL suspension and incubated at 30 °C with different agitation speed for 6 d. The samples were withdrawn after 6 d cultivation time and were assayed for pectinase activity and fungal growth determination.

Enzyme recovery and fungal biomass determination

The culture broth was filtered through filter paper (Whatman No. 1, London) to separate the fungal mycelium. The cell-free culture filtrate containing the crude enzyme was then assayed for pectinase activity.

The filter paper containing biomass was dried at 80 °C until constant weight and the fungal cell dry weight was obtained by deducting the weight of filter paper and the fungal biomass from the weight of filter paper alone. The fungal cell dry weight was then expressed as g/L. All the experiments were performed in triplicate and the values were reported as standard deviations.

Assay for pectinase activity

Pectinase assay was carried out by measuring reducing sugars release from pectin hydrolyzation. One aliquot of sample was added to a solution containing one aliquot of 1% pectin in 0.1 mol/L citrate buffer with pH 4.5. After incubation at 45 °C for 30 min, reducing sugars were determined by the dinitrosalicylic acid (DNS) method^[11] using galacturonic acid as a reference. The enzymatic activity was expressed in terms of Unit (U). One unit of enzyme activity was defined as the amount of enzyme that catalyzes the release of 1 µmol of galacturonic acid per mL of culture filtrate per minute under the assay conditions.

Scanning electron microscopy studies

The 6 d old fungal mycelia were harvested from various agitation speed, 0, 50, 100, 150, 200 and 250 rpm, washed three times with sterile distilled water and then blotted dry in Whatman filter papers. The samples for scanning electron microscopy (SEM) were prepared as described previously by Darah *et al.*^[5] and Darah and Ibrahim^[12] and observed under FESEM LEO Supra 50VP, Carl Zeiss, Germany.

Statistical analysis

In order to evaluate and determine the significant of the findings and also to compare the differences

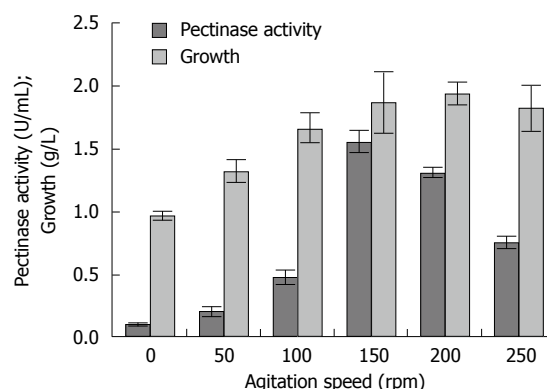


Figure 1 Effect of agitation speeds on pectinase, fungal growth and protein content by *Aspergillus niger* HFD5A-1 at 6 d cultivation time.

among the findings, the statistical analysis was used. One way Analysis of Variance (ANOVA) and Duncan Multiple Range Test (DMRT) with PASW Statistics 18 version were used to analyze the significant different of the mean of experimental data. A 5% confidence level or $\alpha = 0.05$ were used to test all experimental data. All enzyme activities and fungal growth were made in triplicates. The error bars showed the standard deviation of triplicates.

RESULTS

Enzyme activity rises up with the increasing of agitation speed up to 150 rpm (Figure 1), where it achieves its maximum pectinase activity of 1.559 U/mL. There were significant different (Duncan, $P < 0.05$) of the pectinase production with the agitation speed at static, 50, 100, 200 and 250 rpm. However, its highest fungal growth was showed at 200 rpm with 1.938 g/L. The agitation speed which not at 150 rpm resulted in low pectinase production. The lowest pectinase production was from the non-agitated culture (static condition) with about 0.111 U/mL and of 0.972 g/L of fungal growth.

The influence of agitation speed was found had a great impact on the morphology of *A. niger* HFD5A-1 (Figure 2). At static condition, a mycelial mat was discovered on the top of the cultivation broth (Figure 2A) and sporulation occurred all over the fungal mycelial mat. Nevertheless increased in agitation speed, the mycelial mat turned to form a single spherical pellet. It was noted that at 50, 100 and 150 rpm of agitation produced slimy mycelial clumps that were small (2.8 cm; Figure 2B), medium (3.6 cm; Figure 2C) and big (4.7 cm; Figure 2D) sizes, respectively. There were no sporulation observed at 50, 100 and 150 rpm. However, mycelial clumps formed at 150 rpm were in intense yellow in coloration. At 200 rpm, a few spherical and elongated pellet of fungal growth with the sizes between 0.6-1.0 mm were formed (Figure 2E). Besides, at 250 rpm a mixture of small and bigger sizes of fungal pellets were formed, and the small fungal pellet were those



Figure 2 Mycelial morphology of *Aspergillus niger* HFD5A-1 observed with naked eyes at different agitation speeds at 6 d cultivation time. A: Static condition; B: 50 rpm; C: 100 rpm; D: 150 rpm; E: 200 rpm; F: 250 rpm.

that detached from big pellets (Figure 2F).

In order to have a closer view of the fungal pellets, they were further analyzed under stereo light microscope at different agitation speeds (Figure 3). Figure 3A (static condition) shows loosely packed mycelium with sporulation, but the density of the mycelium were observed to increase with increasing agitation speeds from 50 rpm to 150 rpm (Figure 3B-D). Conversely, at 200 and 250 rpm (Figure 3E and F) there were decreasing in density of mycelium.

Observations under scanning electron microscope were also carried out to have a clearer view of Figure 3. SEM micrographs (Figure 4) show the mycelia of fungal are cylindrical in shape and branched with fruiting bodies. This condition occurred to the fungal culture agitated at low agitation speeds, *i.e.*, at static (Figure 4A), 100 rpm (Figure 4B) and 150 rpm (Figure 4C), whereas at higher agitation speeds of 200 rpm (Figure 4D) the fungal culture exhibited lesser branching

mycelia. There was no formation of fruiting bodies at 200 rpm. In addition, fungus grown in static condition showed branched mycelia and numerous formations of fruiting bodies (Figure 4A). At 100 rpm (Figure 4B) fungal growth more on the formation of hyphae with less fruiting body. This condition can be seen in the formation of tight packing structures. However, agitation speed of 150 rpm (Figure 4C) exhibited dense and branched mycelia with increasing formation of fruiting bodies. The fungal growth was best at the agitation of 200 rpm (Figure 4D) which exhibited smoother, not well rounded of hyphae and dense with high branched mycelia. The formations of chlamydospores also observed at 200 rpm.

DISCUSSION

Generally, filamentous fungi have a typical polarized growth pattern in the form of cannular filaments name

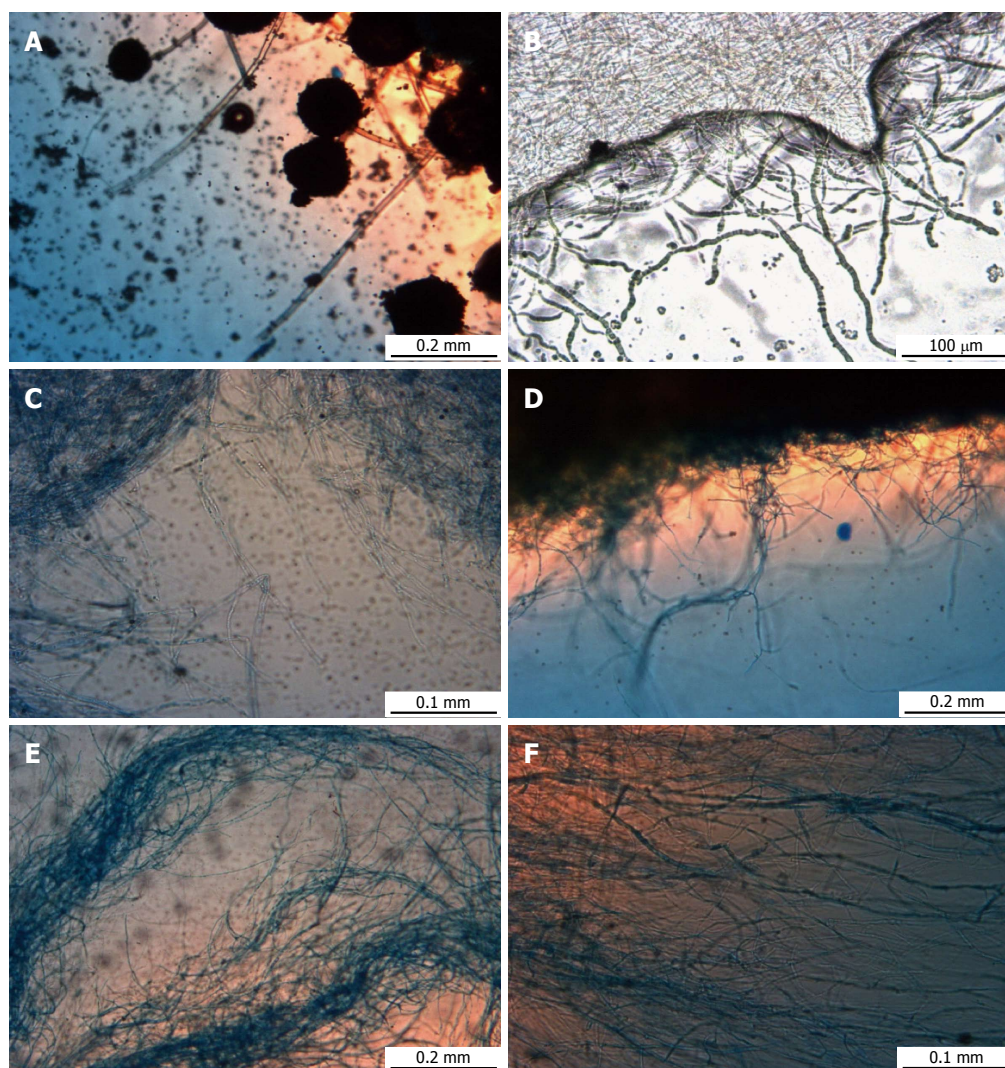


Figure 3 Mycelial morphology of *Aspergillus niger* HFD5A-1 observed under light microscope at different agitation speeds at 6 d cultivation time. A: Static condition; B: 50 rpm; C: 100 rpm; D: 150 rpm; E: 200 rpm; F: 250 rpm.

as hyphae^[9]. The hyphae then grow in branches to form mycelium. In submerged cultures, the fungal culture exhibits two major morphologies observed as pellets and free mycelium. The pellets are clumps and dense agglomerates of hyphae which may be spherical or ellipsoidal agglomerates^[13,14], whereas the free mycelia, are freely spread throughout the culture medium^[9]. These morphologies are very much influenced by some environmental and genetic factors such as type of organism, pH and composition of the media, inoculation ratio, agitation speed and aeration rate, feeding rate and genetic factors of the culture^[15,16]. The morphology of filamentous fungi in submerged fermentation has great effect on metabolite production as well as productivity. These two morphologies exhibit significant differences during the cultivation period^[17].

Agitation speed has been shown to affect many enzyme productions by microorganisms. In the present investigation, *A. niger* HFD5A-1 showed highest pectinase activity at 150 rpm after 6 d of cultivation.

This is probably due to aeration of the culture medium was increased and dissolved oxygen in the media was sufficient. Nutrient uptake by fungus increased resulting in increased pectinase production. At 200 rpm, the enzyme production was dropped even though the fungal growth increased considerably. The adequate aeration and nutrient might caused the fungus to grow well but shear forces and cell damage finally had negative effect on enzyme production^[18]. However, the different strains of fungus demonstrated different optimal agitation speeds for maximize the enzyme production yield in submerged fermentation. Darah *et al.*^[19] reported that *A. niger* FETL FT3 has shown the maximum production of tannase activity at 200 rpm agitation speed instead of 150 rpm agitation speed.

Agitation speed and dissolved oxygen concentration on pectinase production are important factors leading to successful progress of fermentation. Sufficient dissolved oxygen in medium is an important for microbial cells in mass transfer characteristics example substrate and product^[5]. Agitation provides adequate

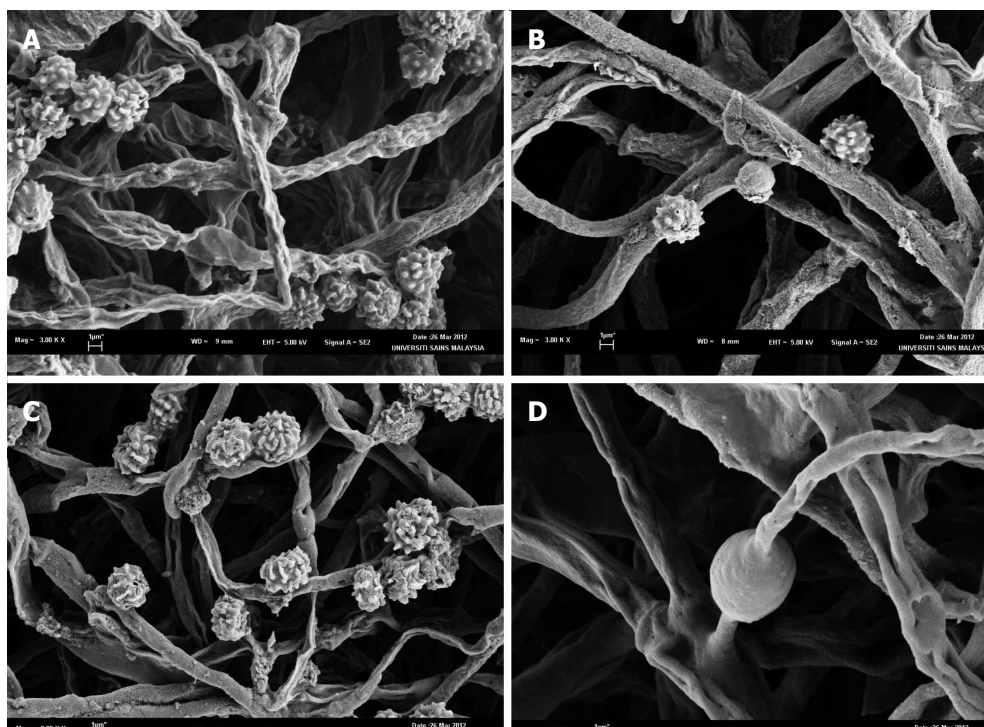


Figure 4 Scanning electron microscopy micrographs of the mycelial morphology *Aspergillus niger* HFD5A-1 at different agitation speed at 6 d cultivation time. A: 0 rpm; B: 50 rpm; C: 150 rpm; D: 200 rpm.

mixing, mass and heat transfer. However, agitation also creates shear forces, causing morphological changes, and eventually damage to the cell structure^[5,20].

In industrial applications submerged cultures of filamentous fungi are widely used to produce commercially products and pellet form is usually preferred in downstream fermentation process due to the non viscosity of fermentation medium^[14]. Hence, the mass transfer of oxygen and nutrients is much easier and also the separation of the pellets from the medium is noncomplex^[21]. Furthermore agitation and aeration are simple technique in fermentation system, therefore the operating cost is not costly. Thus, agitation rate need to be controlled in order to obtain maximum production.

Agitation speed is an important factor in enhancing pectinase yield produce by *A. niger* HFD5A-1 in submerged fermentation. However, agitation speeds beyond than the optimum speed (150 rpm) resulted in low pectinase production.

COMMENTS

Background

Agitation speed is a crucial factor affected the enzyme production in submerged fermentation system. Therefore, the optimal agitation speed is necessary to be determined in order to obtain maximal enzyme production. The purpose of this study was therefore to evaluate the influence of agitation speed on pectinase production as well as morphology of *Aspergillus niger* (*A. niger*) HFD5A-1 in submerged fermentation using a flask system which has not been studied yet.

Research frontiers

The author aimed to to evaluate the influence of agitation speed on pectinase production as well as morphology of *A. niger* HFD5A-1 in submerged

fermentation using a flask system.

Innovations and breakthroughs

This study demonstrates agitation speed plays an important role in enhancing pectinase production by *A. niger* HFD5A-1 in submerged fermentation. However, higher or lower agitation speeds than the optimum speed (150 rpm) resulted in low pectinase production. Even though agitation increases the dissolved oxygen level in the cultivation medium, higher agitation speed can cause damage to the fungal cells that was due to shear forces and collision among the fungal pellets. Therefore, the relationship among morphology and rheology as well as the factors influencing them has to be fully investigated.

Applications

The authors hypothesize that agitation speed plays an important role in enhancing pectinase production by *A. niger* HFD5A-1 in submerged fermentation. The information obtained should assist progress towards the large scale production of pectinase by submerged cultivation of *A. niger* HFD5A-1.

Peer-review

In this paper, Darah *et al* report the effect of agitation speed on the morphology of *A. niger* HFD5A-1 hyphae and its pectinase production in submerged fermentation. The findings are interesting.

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