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## Impact and risk factors of post-stroke bone fracture

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### Abstract

Bone fracture occurs in stroke patients at different times during the recovery phase, prolonging recovery time and increasing medical costs. In this review, we discuss the potential risk factors for post-stroke bone fracture and preventive methods. Most post-stroke bone fractures occur in the lower extremities, indicating fragile bones are a risk factor. Motor changes, including posture, mobility, and balance post-stroke contribute to bone loss and thus increase risk of bone fracture. Bone mineral density is a useful indicator for bone resorption, useful to identify patients at risk of post-stroke bone fracture. Calcium supplementation was previously regarded as a useful treatment during physical rehabilitation. However, recent data suggests calcium supplementation has a negative impact on atherosclerotic conditions. Vitamin D intake may prevent osteoporosis and fractures in patients with stroke. Although drugs such as teriparatide show some benefits in preventing osteoporosis, additional clinical trials are needed to determine the most effective conditions for post-stroke applications.

**Key words:** Bone fracture; Recovery; Bone mineral density; Stroke; Osteoporosis

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**Core tip:** Post-stroke bone fracture negatively impacts stroke recovery, prolongs hospital stays, and increases economic cost. Stroke, osteoporosis and bone fracture share common risk factors. The main risk factors for post-stroke bone fracture include aging, osteoporosis, and loss of posture control. Bone mineral density measurement may identify patients who are at risk of post-stroke bone fracture. Drugs and supplements, such as vitamin D and teriparatide, can be tested in clinical settings for prevention of post-stroke bone fracture. Although bisphosphonates incur side effects, they are considered first-line drugs to prevent post-stroke bone fracture.

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## INCIDENCE OF POST-STROKE BONE FRACTURE

Aging, smoking, hypertension and lack of physical activity contribute to osteoporosis bone fracture and stroke. Although strokes can occur at any age, they mostly occur in older age, which may due to reduction in bone density<sup>[1]</sup>. Only one fourth of strokes occur in people under the age of 65<sup>[2]</sup>. The prevalence of stroke more than doubles each decade after the age of 55.

Stroke and bone fracture share common risk factors. Individuals with a greater risk of stroke are also more prone to osteoporosis and fractures. Risk factors for post-stroke bone fracture include osteoporosis, aging, and loss of posture control. Osteoporosis is the main risk factor of bone fracture, with increasing prevalence worldwide. In the United Kingdom, over half of women, and one-fifth of men over age 50, will sustain a fragility fracture<sup>[3]</sup>.

The risk of bone fracture post stroke is underestimated because it is often considered secondary to falling, especially among elderly. Previously, 534 elderly persons were assessed over 2 mo, results revealed that stroke was influenced by physical factors including falls<sup>[4]</sup>. A fracture post-stroke puts the patient at a greater disadvantage during rehabilitation, and greatly affects the quality of life after recovery.

## RISK FACTORS OF POST-STROKE BONE FRACTURE

Stroke patients are highly susceptible to bone fracture<sup>[5]</sup>. They have a 2 to 4-fold increased risk of hip fractures, which carries a high mortality rate in the elderly<sup>[6]</sup>. Changes in walking posture, skeletal weight load, and bone maintenance have been studied as risk factors for poor post-stroke bone health<sup>[7]</sup>. Other factors include low bone mineral density (BMD), loss of balance, inappropriate gait, and muscle deformation<sup>[8]</sup>. In order to predict the risk of falls and fractures in stroke patients, we must take into account not only structural changes, but also functional disability.

Post-stroke fractures are of particular concern given the impact on functional outcomes of stroke rehabilitation. Functional impairment of stroke is commonly measured using two scales, the barthel index (BI) and modified Rankin scale (mRs). Both assessment scales place stroke patients with differing functional capabilities into categories, describing a level of independence or need for medical assistance. The BI is a measure of functional independence on ten activities of daily living (ADL). The

mRs measures global disability of a patient. Harrison *et al*<sup>[9]</sup> studied the effectiveness and drawbacks of each scale and drew the conclusion that while all scales had weaknesses, certain types of disability were reliably assessed by these scales.

The level of functional impairment may be useful in estimating risk of falls. In a study of 135 elderly stroke patients, Nyberg *et al*<sup>[10]</sup> developed a fall prediction index that classifies patients into low-, intermediate-, and high-risk groups. The index includes male sex, poor ADL performance, urinary incontinence, bilateral motor impairment, impaired postural stability, visuospatial hemi-neglect, and use of diuretics, anti-depressants or sedatives as factors in increased falling<sup>[10]</sup>. A comprehensive index for assessing the risk of falls is vital to reliably predict the possibility of post-stroke bone fracture.

In addition, weak bones are a risk factor for post-stroke bone fracture. Hospitalization of stroke patients suffering adverse changes in motor function and body composition leads to long-term increase in fracture risk<sup>[11]</sup>. Although BMD decrease is common in stroke patients > 65 years, studies have demonstrated that BMD loss in paretic limbs are greater than their non-paretic counterparts, such as the arms. Interestingly, vitamin D deficiency causing osteopenia seems to be a possible mechanism<sup>[12]</sup>. Bone loss progresses rapidly in the acute stages of stroke<sup>[13]</sup>, with the most dramatic changes seen in the first few months<sup>[14]</sup>. By measuring the magnitude of changes to volumetric BMD after stroke in a prospective study, Borschmann *et al*<sup>[15]</sup> found a 7% difference in total volumetric BMD between paretic and non-paretic distal tibiae 6 mo post-stroke, with lower density on the paretic side. Another study showed that while immobility does increase bone loss, stroke patients who maintained their ability to walk had a 3% decrease in BMD. This suggests that stroke itself is a risk factor for bone loss and fragile bones. Therefore, more studies are needed to better understand the influence and underlying mechanisms of stroke on BMD loss, as well as to develop preventive methods.

Biochemical markers related to bone turnover can be used to evaluate bone health and risk of bone fracture. Haddaway *et al*<sup>[16]</sup> showed that stroke patients older than 60 years displayed much earlier bone resorption (a process of bone breakdown) when compared with normal individuals, supporting the idea that bone remodeling is correlated with post-stroke bone fragility. Bone turnover in elderly women was inversely correlated with bone density at the hip on both hemiplegic and normal sides, which may adversely influence bone fracture. Serum biomarkers, such as osteocalcin (OC), bone alkaline phosphatase combined with BMD analysis better predict than physical exam alone. Early evaluation of bone turnover could help identify bone loss in stroke patients<sup>[17]</sup>.

Motor changes, including posture, mobility and balance are also factors associated with bone loss. Chang *et al*<sup>[18]</sup> compared the femoral neck bone loss in hemi-

paretic patients with high or low weight-bearing on the paretic lower limb. The study showed that patients with lower standing weight placed on their paretic limb (< 50%) had faster bone loss and BMD reduction during the follow-up period ( $\geq 6$  mo) compared with those who bore more weight (> 50%). The proportion of weight bore by the paretic leg was related to the BMD, indicating that reduced weight bearing is a predictor of bone loss in stroke patients<sup>[18]</sup>. Although results from a study by Melton *et al.*<sup>[19]</sup> suggested that skeletal weight load is not an independent factor of bone loss, the effect of reduced skeletal weight load may be more pronounced in paresis. Immobility is also associated with increased bone loss in stroke patients, with wheelchair-bound patients showing a 4-fold loss in BMD (13% to 3%) compared with ambulating patients<sup>[20]</sup>. Together, these factors amplify the fragility of bones in patients undergoing rehabilitation, leading to osteoporosis<sup>[21]</sup>.

Of course, some stroke patients are at a higher risk of having bone fractures than others. For example, a study by Ashburn *et al.*<sup>[22]</sup> revealed that patients who were physically unstable (experienced near-falls), with upper-limb functional impairment, were at higher risk of falls. This suggests that proper arm function can prevent falls, as patients are able to support themselves using their arms. Interestingly, stroke survivors without functional impairment had significantly higher risk of fragility fractures in legs compared with arms. However, stroke survivors with functional impairment had no prevalence of fracture on any specific site of their body<sup>[23]</sup>.

Prevention of fractures is of utmost concern, as post-fracture care can be long-term and strenuous, especially for stroke patients. Physical rehabilitation is essential for maximum motor recovery. Post-stroke rehabilitation includes re-learning of basic movement skills and other common activities. To perform daily activities, patients must recover balance, muscle strength, and movement. Early and consistent physical rehabilitation is key in achieving the most favorable results<sup>[24]</sup>. Having a fracture post or pre-stroke can put the patient at a greater disadvantage in their rehabilitation, and negatively affects their quality of life<sup>[25]</sup>. Bone fractures on the paretic side can be especially detrimental, as damage to the structure of the paretic limb complicates motor recovery.

There are many factors affecting the outcome of rehabilitation, including, but not limited to, the type of stroke lesion, age of the patient, previous lifestyle, rehabilitation, exercise and mobility<sup>[26]</sup>. Recent systematic reviews and meta-analyses show that in rat models, exercise was associated with decreased lesion volume and improved functional outcomes<sup>[27,28]</sup>. Studies also showed that early mobilization had a strong positive correlation with favorable outcomes in patients<sup>[29-31]</sup>. Despite the acceptance among studies on the positive effect of early mobilization, analyses of randomized-controlled trials comparing first-day and second-day mobilization on functional outcomes (good functional

outcomes were mRS scores  $\leq 2$ ) showed little difference between the groups<sup>[32]</sup>. Future trials should define proper mobilization times at different functional impairment levels.

Patients with conditions that affect bone loss pre-stroke suffer more complications post-stroke. The effect of stroke on BMD has been independently observed in patients with pre-stroke osteoporosis<sup>[33]</sup>. Older women often experience bone loss after menopause, which is further exacerbated by stroke. In men, subjects with pre-stroke osteoporosis (due to aging or otherwise) suffer similar consequences of stroke, but given that most clinical trials have focused on menopausal women, male subjects are often neglected in studying osteoporosis.

The independent interaction of stroke on bone density may be difficult to observe due to the presence of multiple factors. Therefore, the multi-factorial character of post-stroke bone fracture needs to be studied deeper to determine possible interventions.

It is also interesting to note that there is a two-way interaction between stroke and bone health. Stroke leads to low BMD, and low BMD increases the risk of stroke<sup>[34,35]</sup>. A prospective investigation conducted between 1997 and 2000 evaluated the relationship between BMD and incidence of stroke in 14290 participants. The study showed that the risk of stroke increased in low BMD individuals. The findings indicated that BMD predicted the risk of stroke, especially in women<sup>[36]</sup>. It has also been shown that vertebral fracture occurs more often in first time-stroke patients that have low pre-stroke BMD. Therefore, low pre-stroke BMD poses a greater risk of fracture, resulting in additional functional loss<sup>[37]</sup>. Hypertension is also a major risk factor for stroke. Elderly persons with shorter stature and lower BMD have been associated with increased arterial stiffness and hypertension. Therefore, this population could be at higher risk compared with the general population for post-stroke bone fracture<sup>[38]</sup>.

Low estrogen level is correlated with reduced BMD. At menopause, decreased estrogen leads to a rapid loss of BMD and increased incidence of bone fracture in women. Estrogen plays a key role in regulating bone mass and strength by controlling the activity of bone-forming osteoblasts and bone-resorbing osteoclasts<sup>[39]</sup>. Therefore, post-menopausal women are more likely to develop osteoporosis, and thus have an increased risk of bone fractures. According to statistical data obtained from the International Osteoporosis Foundation, osteoporosis causes more than 8.9 million fractures worldwide annually, resulting in an osteoporotic fracture every three seconds<sup>[40]</sup>.

Using anti-depressants on stroke patients may increase the possibility of post-stroke bone fracture. Stroke patients commonly suffer from secondary disorders, such as depression, that may affect bone health. Anti-depressants, in particular selective serotonin reuptake inhibitors (SSRIs), have been correlated with an increased risk for osteoporosis<sup>[41]</sup>. The association of fracture incidents and

antidepressants was studied using data from the Canadian Multicenter Osteoporosis Study in a prospective randomly selected population-based community cohort<sup>[42]</sup>. Among 6645 subjects, 192 (2.9%) were using SSRIs and/or serotonin and noradrenaline reuptake inhibitors (SNRIs) at baseline. During the 10-year study period, SSRI/SNRI use was associated with increased risk of fragility fracture (HE = 1.88; 95%CI: 1.48-2.39). Therefore, more attention and care should be given when prescribing antidepressant drugs to patients.

Although current evidence on the impact of stroke on bone health seems contradictory, what is clear is that risk of bone fracture and stroke are strongly correlated. However, whether stroke is an independent factor for BMD loss, due to motor function changes, is unknown. To prevent bone fracture, methods and treatments to reduce bone loss in stroke patients should be researched. A clear time frame for physical therapy, especially early mobilization, is important in long-term recovery. Patient fall prediction indexes can be developed further, and rehabilitative measures would function better when tailored to different risk grades. Ultimately, while evidence strongly supports the effectiveness of general rehabilitation, a deeper understanding of treatment response should be prioritized.

## ASSESSMENT OF POST-STROKE BONE FRACTURE

In this section, we discuss treatment options available today as well as assessments to minimize the risk of bone fractures and to optimize post-stroke recovery. Given that osteoporosis is a predictor for stroke and bone fractures, the majority of the following discussion is focused on management of this condition.

Calcium is a major component of bone, supplementation of calcium is regarded as a possible solution for preventing post-stroke bone fracture. However, there is conflicting data on the effectiveness of calcium supplementation. Some evidence shows calcium intake has adverse cardiovascular effects, raising widespread concern. However, a recent study showed dairy products do not increase the risk of cardiovascular disease, particularly low fat versions. Therefore, dairy products could be a good resource for calcium intake<sup>[43]</sup>.

Recent studies have shown that increasing the intake of calcium does not reduce osteoporotic fracture rates<sup>[44]</sup>. A prospective longitudinal cohort study revealed that gradual increases in dietary calcium intake above the first quintile in female population were not associated with further reductions to the risk of fracture or osteoporosis<sup>[45]</sup>. If these findings are true, then taking calcium will not prevent osteoporosis. Instead, it may increase the risk of developing cardiovascular problems long term.

Alternatively, other studies have shown that calcium supplementation does not increase carotid artery intimal medial thickness or carotid atherosclerosis<sup>[46]</sup>. Thus, the

impact of calcium intake on osteoporosis is not clear. Until more confirmatory data are available, physicians should not stop prescribing calcium supplements to their patients<sup>[47]</sup>. The appropriate intake amount varies, ranging 800-1500 mg/d<sup>[48]</sup>. Some sources recommend calcium supplementation not exceed 1200-1500 mg/d. A study by Khan *et al.*<sup>[49]</sup> found that to decrease fracture, non-fatal CVD, stroke, and all-cause mortality risks in both men and women, a specific dose of 1348 mg/d is optimal.

Given no clear consensus on proper calcium dosage, early screening and active management of osteoporosis at the acute stages of stroke are critical<sup>[37]</sup>. One of the best predictive factors for osteoporosis is BMD, and it should be measured on all stroke patients. Dual-energy X-ray absorptiometry (DXA) is a primary tool that accurately evaluates BMD. According to the United States Preventive Services Task Force, women over the age of 65 should get a DXA scan, men are recommended to get the scan at age 70<sup>[50]</sup>. At risk individuals should also get the scan performed. Risk is determined using the World Health Organization's Fracture Risk Assessment Tool, a diagnostic that evaluates the 10-year probability of bone fracture risk. Clinical risk factors assessed include prior fragility fracture, parental history of hip fracture, current tobacco smoking, long-term use of glucocorticoids, rheumatoid arthritis, other causes of secondary osteoporosis, and daily alcohol consumption<sup>[51]</sup>.

There are 2 DXA scan methods, a central DXA that scans the hip and lower spine, and a peripheral DXA that most often scans the heel. A study showed the heel BMD can serve as a surrogate for hip BMD<sup>[52]</sup>. BMD measurements, used concomitantly with the Tinetti Test (TT), or Performance Oriented Mobility Assessment, provide a useful indication for those needing early prophylaxis against bone loss. The TT is a solid predictor to assess balancing ability of post-stroke patients. It was created to screen for balance and mobility skills (gait) in older adults, and determines the likelihood of falls. This is especially important in hemiplegic stroke patients. Using a 3-point scale, a score less than 19 indicates a high risk for falls, and scores between 19 and 24 indicate a moderate fall risk<sup>[53]</sup>. However, it is difficult to assess with specificity on a 3-point scale<sup>[54]</sup>. This test is not useful on post-stroke patients that are bed-ridden.

A more reliable assessment is the Timed "Up and Go Test" (TUG). It is the shortest, simplest clinical balance test. A stop-watch timer is more objective compared with a rating scale used in the TT<sup>[55]</sup>. In a cross-sectional study performed by Ng and Hui-Chan, the TUG test showed excellent reliability (ICC > 0.95). Subjects with chronic stroke were found to have more significant spastic and weaker plantar flexors, slower walking speeds, and poorer walking endurance when compared to healthy elderly subjects (all  $P < 0.003$ )<sup>[56]</sup>.

With the balancing ability assessed by either the TT or TUG, the severity of disuse on the hemiplegic side of

post-stroke patients can be determined. This disuse of the hemiplegic side in stroke patients results in bone-mass reduction, especially in the presence of vitamin D deficiency<sup>[57]</sup>. By comparing BMD, serum concentrations of intact parathyroid hormone (PTH), OC, tartrate-resistant acid phosphatase, 25-hydroxyvitamin D (25-OHD), and calcium levels between healthy and post-stroke patients, BMD values were lower on the hemiplegic side compared with the non-hemiplegic side. Vitamin D deficiency and compensatory secondary hyperparathyroidism stimulated skeletal turnover is an important cause of osteopenia in the hemiplegic limbs of stroke patients. This suggests that administration of vitamin D supplements is beneficial in stroke patients to reduce fracture risk.

In addition to increasing risk of bone fractures, vitamin D deficiency in post-stroke patients may also cause a variety of issues in non-stroke patients. Vitamin D deficiency impairs gastrointestinal absorption of calcium and bone mineralization, muscle strength, and is also associated with muscle mass loss, which contributes to an increased risk of fall<sup>[58]</sup>. Furthermore, vitamin D deficiency is a risk factor for strokes due to the associations of low 25-OHD levels, particularly in the presence of arterial hypertension<sup>[59]</sup>. Vitamin D also exhibits neuroprotective, as well as neuromuscular and osteo-protective effects, which may reduce cognitive and functional impairments in stroke patients. However, current evidence is too scarce to draw any conclusions. Further evaluations are needed to confirm the effect of vitamin D treatment in reducing stroke associated mortality and morbidity.

While vitamin D supplementation is important in patients who have osteoporosis, there is conflicting evidence showing that vitamin D does not prevent osteoporosis in healthy individuals. In a meta-analysis from 2014, the effects of vitamin D supplements on BMD were found to have no significance<sup>[60]</sup>. However, some studies showed that vitamin D with co-administration of calcium significantly improved BMD and thus, reduced the risk of falling in elderly patients<sup>[61]</sup>.

Another strong indicator for osteoporosis is serum OC, which is associated with bone turnover. By examining serum total osteocalcin (TOC), carboxylated osteocalcin (COC), and their ratio (COC/TOC), data show serum COC concentrations, especially COC/TOC, predicted the occurrence of fractures in the elderly<sup>[62]</sup>. Thus, low COC/TOC is directly correlated with risk of fracture. Bone turnover can be further evaluated using serum urinary cross-linked N-telopeptide of type I collagen (NTX), a reliable bone resorption marker in patients with metabolic bone disease. According to a study performed by Maeno *et al.*<sup>[62]</sup>, patients in the highest quartile of serum NTX concentrations exhibited rapid bone loss rates, with a sensitivity and specificity for detecting the rate of 48% and 83%, respectively.

Therefore, assessment instruments, such as the TT, TUG test, DXA scan, serum NTX, and COC/TOC levels, as well as supplements such as calcium and vitamin D, are important in identifying and preventing osteoporosis

and fractures in patients with stroke. If preventive measures against osteoporosis are not well managed, or if a person already has osteoporosis, the treatment goals should be to minimize further bone loss and prevent osteoporotic fractures.

## PREVENTION AND ADDITIONAL MANAGEMENT

Osteoporosis is a devastating disorder that impairs bone strength, causing an increased risk of fracture. It is usually diagnosed by assessing BMD via the DXA scan, and is defined by the World Health Organization criteria as a BMD T-score of 2.5 standard deviations or more below the average of a young, healthy person<sup>[63]</sup>.

Most studies show that mitigation of osteoporosis prevents post-stroke bone fracture. Hemiplegic patients and severe stroke patients will experience bone remodeling. A study by Ramnemark *et al.*<sup>[64]</sup> in 1999, with 24 extensive paresis stroke patients, found that during the first year after severe stroke, patients developed pronounced hemi-osteoporosis. This was not associated with general changes in lean or fat mass. The development of hemi-osteoporosis was independent of weight changes after stroke<sup>[65]</sup>.

Currently, bisphosphonates are used to treat osteoporosis because these drugs slow bone breakdown due to their strong affinity to skeleton, low toxicity to other tissues and organs in the body, and ease of frequency of administration<sup>[66]</sup>. Alendronate, risedronate, ibandronate, and zoledronate were found to reduce fracture risk by 50% within the first year of therapy in both men and women<sup>[67]</sup>. However, some studies suggest that bisphosphonates may increase the risk of esophageal cancer and cardiovascular diseases<sup>[68]</sup>, osteonecrosis of the jaw, and subtrochanteric fractures<sup>[69,70]</sup>. These side effects seem likely to hinder the use and benefits of bisphosphonates. Thus, bisphosphonates use should be determined on a case-to-case basis.

Recent studies show that teriparatide, a synthetic peptide that mimics the action of PTH, rebuilds bone and reverses osteoporosis. A large, randomized, placebo-controlled clinical trial found that postmenopausal women with severe osteoporosis had reduced spinal and non-vertebral fractures by more than 50% when using teriparatide<sup>[71]</sup>. However, this trial was stopped after controversy that teriparatide caused osteosarcoma, a type of bone cancer, when injected into laboratory rats<sup>[72]</sup>. Retrospective studies have found no significant association between osteosarcoma and teriparatide, and only 2 cases of osteosarcoma in PTH-treated patients have been reported. The prevalence of osteosarcoma in rats is likely due to the ever-growing epiphysis in rats. Thus, teriparatide is contraindicated in children and young adults whose epiphysis has not closed. Adults that are pregnant, have risk of renal failure, or have a history of radiation therapy should also refrain from using teriparatide. Currently, teriparatide should be limited to 2 years, and is not encouraged to prevent osteoporosis,

to treat mild osteoporosis, or by people who can take other osteoporosis treatment<sup>[73]</sup>. The post-treatment effect of bone loss is prevented by adding an anti-resorptive drug after stopping teriparatide. Based on the researches we already have, benefits of bisphosphonates surpass disadvantages for not using them. Regardless of bisphosphonate's side effects, it is still a first-line drug choice to prevent post-stroke bone fracture. However, large scale randomized double blind clinical trials still should be conducted in the further to explore a safe preventive method for reducing bone fracture on post-stroke patients.

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## Inflammatory diseases modelling in zebrafish

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### Abstract

The ingest of diets with high content of fats and carbohydrates, low or no physical exercise and a stressful routine are part of the everyday lifestyle of most people in the western world. These conditions are triggers for different diseases with complex interactions between the host genetics, the metabolism, the immune system and the microbiota, including inflammatory bowel diseases (IBD), obesity and diabetes. The incidence of these disorders is growing worldwide; therefore, new strategies for its study are needed. Nowadays, the majority of researches are in use of murine models for understand the genetics, physiopathology and interaction between cells and signaling pathways to find therapeutic solutions to these diseases. The zebrafish, a little tropical water fish, shares 70% of our genes and conserves anatomic and physiological characteristics, as well as metabolical pathways, with mammals, and is rising as a new complementary model for the study of metabolic and inflammatory diseases. Its high fecundity, fast development, transparency, versatility and low cost of maintenance makes the zebrafish an interesting option for new researches. In this review, we offer a discussion of the existing genetic and induced zebrafish models of two important Western diseases that have a strong inflammatory component, the IBD and the obesity.

**Key words:** Zebrafish; Western diseases; Inflammatory disorders; Obesity; Inflammatory bowel diseases

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**Core tip:** The western lifestyle with a high fat and carbohydrates diet, lack of physical activity and stress, is a trigger for different diseases with complex interactions between the host genetics, the metabolism, the immune system and the microbiota, as the inflammatory bowel

disease (IBD), obesity and diabetes. The zebrafish has 70% homology with our genes, shares anatomic and physiological characteristics with mammals, and emerges as a new model for the study of metabolic and inflammatory diseases. In this review, we examine the existing genetic and induced zebrafish models of two important Western diseases with strong inflammatory component, IBD and obesity.

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## INTRODUCTION

In the last decades, the standard of living of the western world has had consequences that affect human health. Factors such as a diet that is high in carbohydrates and fats, a sedentary lifestyle, and stress trigger a state of chronic systemic low-grade inflammation, insulin resistance, and changes in the microbiota<sup>[1-3]</sup>, which lead to the so-called Western diseases. Some of these diseases include inflammatory bowel disease (IBD), obesity, type 2 diabetes and heart disease, among others, and they are an issue of global significance, because of the high incidence of such disorders in western society. It is estimated that IBD affects approximately 1-1.3 million people in the United States<sup>[4]</sup> and in the same country 9.3% of the population has diabetes<sup>[5]</sup>. Additionally, more than two-thirds (68.8%) of United States adults are considered as overweight or obese<sup>[6]</sup>.

Given the worldwide importance of these diseases, much research is currently in progress seeking answers to unresolved questions about their physiopathology, the pathways involved and new therapies to treat these conditions. Although mainly mammalian models, such as rabbits, rats and mice, are used for these studies<sup>[7-10]</sup>, other models that have been gaining ground in the field of inflammatory diseases do exist<sup>[11-13]</sup>. The zebrafish is a small tropical freshwater fish primarily used as a vertebrate model in developmental biology because of its characteristic high fecundity, short *ex vivo* development time, ease of observation in the embryonic and larval states, relative ease of genetic manipulation, and low cost of production<sup>[14]</sup>. Additionally, the zebrafish genome is fully mapped (<http://www.sanger.ac.uk/resources/zebrafish/>), having approximately 70% of orthologs with the human genome<sup>[15]</sup>. Zebrafish have specific technical advantages as model for the *in vivo* analysis and knock-down technology<sup>[12,16]</sup>. They have anatomical features commonly found in mammals, including a central, autonomic and enteric nervous system, a multi-chambered heart, a liver, an intestinal system, a pancreas, and a kidney responsible for the production of hematopoietic cells, as well as immunological maturation sites such as the thymus and the spleen<sup>[13]</sup>. Zebrafish have a functional innate immune

system at 48 h post-fertilization (hpf) and a mature adaptive system approximately 4-6 wk post-fertilization (wpf)<sup>[17]</sup>, with many of the same immune cells, cytokines and chemokines known in humans<sup>[18]</sup>. Furthermore, in the last decade, zebrafish have become a model for different human diseases and a tool for drug screening<sup>[11,13,19]</sup>. All of these characteristics make the zebrafish an excellent model for the study of inflammatory pathologies.

In this review, we discuss and summarize the current larval and adult zebrafish models of Western diseases with an inflammatory component, including IBD and obesity.

## IBD MODELS

In humans, the IBD are a group of chronic inflammatory conditions of the small intestine and colon, appearing as a result of deregulated interactions between the immune system and the commensal microbiota, triggered by a genetic predisposition of the affected individual and external factors<sup>[20-22]</sup>. In mice, there is a wide range of genetic, spontaneous and chemical models<sup>[23-25]</sup> that have been used in an attempt to find answers to different about IBD issues. Beginning some years ago, an increasing variety of zebrafish IBD chemical models have arisen, which are based on models previously tested in mice.

The zebrafish intestine has been described by many authors<sup>[26-28]</sup> as very similar in anatomy and architecture to the mammalian small intestine. It is a compartmentalized tubular structure with three intestinal segments defined by histological morphology of the epithelial folds and the distribution of different cell types. It has a mucosal layer of simple folded epithelium formed by columnar absorptive enterocytes, goblet cells and enteroendocrine cells; it lacks Paneth cells and a lamina propria beneath the epithelium. The mucosa is directly surrounded by circular and longitudinal smooth muscle layers, and small groups of enteric nerves can be observed between the two muscular layers with the nerve fibers innervating the connective tissue beneath the epithelium. Because of these simple anatomical characteristics, the zebrafish has proven to be an excellent model to study intestinal inflammation.

### Genetic susceptibility

Nowadays, there's little evidence of genetic spontaneous colitis models in zebrafish as exist in mice [*e.g.*, NEMO KO, STAT3 KO in myeloid cells, interleukin (IL)-10 KO]<sup>[29-31]</sup>, however, there have been discovered many zebrafish genes related to genetic susceptibility in human IBD. The loss of *myd88*<sup>[32,33]</sup>, an adaptor molecule, central in innate immune signaling<sup>[34]</sup>, induce predisposition to bacterial infections and compromised expression of immune transcription factors (*nfb*, *ap-1*) and molecules (*il-1β*, *mmp9*), proving to be an important molecule in the development of the inflammatory process in zebrafish. NOD proteins are intracellular pattern recognition receptors involved in innate immune response and have been associated with genetic vulnerability to IBD<sup>[35]</sup>.

In zebrafish, *nod1* and *nod2* genes are expressed in intestinal epithelial cells (IECs) and neutrophils. In a model of infection with *Salmonella enterica*, morpholino (MO) knock-down (KD) of *nod1* and *nod2* have decreased survival after infection, and *nod1* KD also had a decreased expression of dual oxidase (*duox*), which is responsible for the synthesis of reactive oxygen species and has role in neutrophils migration, since its depletion slows down the repopulation of the caudal hematopoietic tissue<sup>[36]</sup>. In mammals, the cytokine IL-22, which is produced by T-helper cells and innate lymphocytes, has important functions in host defense at mucosal surfaces and in tissue repair<sup>[37]</sup>. In zebrafish, IL-22 expression was detected predominantly in the myeloid innate lineage during early developmental stages, and proved to have participation in host-microbe interaction since its knock-down present high susceptibility to bacterial infections and an increased pro-inflammatory cytokine expression. IL-22 is increased in patients with Crohn's disease (CD) but is decreased in ulcerative colitis (UC) patients<sup>[38,39]</sup>, thus would be interesting investigate the role of this cytokine during zebrafish intestinal inflammation. Furthermore, IL-10 and IL-23 expression have been related to an upregulated response to LPS and bacterial infection in zebrafish<sup>[40,41]</sup>, and both have proven roles in IBD, since the *il10*<sup>-/-</sup> mice develops a spontaneous colitis<sup>[31]</sup> and IL-23 is essential for T cell-mediated colitis<sup>[42]</sup>.

Endoplasmic reticulum (ER) stress is a defense mechanism triggered by a variety of conditions that disturb folding of proteins in the ER. To alleviate this stress the unfolded protein response (UPR) is activated, restoring ER homeostasis, promoting cell survival and adaptation. Modifications in genes that are centrally involved in the UPR appears as a risk factor for both forms of IBD, UC and CD<sup>[43]</sup>. In zebrafish, two mutants present ER stress in IECs, the *sec13*<sup>sq198</sup> and *cdipt*<sup>hi559</sup>, with defects in intestinal development in the first one and alteration of the villi, disorganization in the proliferation of IECs, apoptosis of goblet cells, abnormal mucosecretion, bacterial overgrowth and leucocyte infiltration, in the second one, characteristics resembling the IBDs<sup>[44,45]</sup>. All these examples, demonstrates conserved genes and pathway in zebrafish, that makes it an interesting model for the research of new IBD related genes.

### Chemically induced adult models

Disruption of the intestinal epithelial barrier and exposition to luminal bacterial antigens into the mucosa is one of the key characteristic of mammalian IBD<sup>[21]</sup>, and is the main accomplishment of most of the chemicals used to induce colitis, closely resembling morphological, histopathological and symptomatic features of human IBD. The first chemical model of intestinal inflammation studied in adult zebrafish<sup>[46]</sup> was based on the mouse oxazolone model of UC<sup>[47]</sup>. A concentration of 0.2% Oxazolone/50% ethanol was intrarectally injected inducing an inflammation characterized by the intestinal infiltration of granulocytes, eosinophils, macrophages and lymphocytes, as well as

changes in the intestinal architecture such as bowel-wall thickening, loss of intestinal folds, and depletion of goblet cells. An increase of *tnfα*, *il1β*, and *il10* transcripts was also observed and was reversed by the use of antibiotics prior the induction of the colitis. A marked influence of the microbiota was evidenced by an enhanced susceptibility to inflammation due to an increase in the bacterial load when fishes were kept in stand-alone/static tanks than in continuous flow-tanks. Treatment with vancomycin, an antibiotic active against gram-positive bacteria, resulted in the reduction of the enterocolitis score and the infiltration of neutrophils, as well as an outgrowth of the *Fusobacteria* phylum; while treatment with colistin, targeting gram-negative bacteria, did not affect the total enterocolitis score but reduced eosinophilic and lymphocytic infiltration and increase in Proteobacteria group. This indicates that the oxazolone model in zebrafish can be used as a complementary model for the study of experimental UC.

A second model of intestinal inflammation in adult zebrafish used 2,4,6-trinitrobenzenesulfonic acid (TNBS) in a 30% ethanol solution injected intrarectally<sup>[48]</sup>. In mice, this model is widely used to study IBD because of clinical and histopathological findings resembling those seen in CD<sup>[49,50]</sup>. Using a wide range of TNBS concentrations, the authors observed a reduction of fish survival in a dose-dependent way with a recovery in survival rate when fishes were treated with vancomycin. A histological analysis showed disruption of the epithelial integrity with presence of ulcerations, swelling, thickening and the detachment of villi. No changes in goblet cells were observed in TNBS treated fishes. Inflammatory events peaked at 6 h post-induction (hpi), with an increase in infiltrated neutrophils in Tg(*mpx:eGFP*) animals and a significant increase in mRNA expression of *il1β*, *il8* and *il10*, in TNBS-exposed fishes compared with controls. These results are similar to a murine TNBS model in which an increase in neutrophilic infiltration in damaged tissue associated with a high myeloperoxidase activity<sup>[51]</sup> and an increase in TNFα levels<sup>[10]</sup> can be observed. Melanin-Concentrating Hormone (MCH) is a conserved neuropeptide involved in appetite regulation that recently has been related with intestinal inflammation<sup>[52]</sup>. The *mch2* gene isoform, equivalent to mammalian *mch*<sup>[53]</sup>, was upregulated in the intestine in TNBS-exposed zebrafish; however the *mch1* isoform did not show any change. The MCH receptor MCHR1b was also upregulated, while MCHR2 was downregulated after TNBS treatment. This downregulation of MCHR2 is different than the expression of MCHR2 in humans, which was shown to increase during intestinal inflammation. Further studies are necessary to discover the contribution of MCH to intestinal inflammation.

The oxazolone and TNBS adult zebrafish enterocolitis models are comparable with the respective murine models in some aspects and provide a complementary tool for the study of IBD. However, because these models have been recently developed, studies that delineate all their inflammatory and pathologic features in order to

enhance their use are scarce in the literature, and more studies are necessary to accomplish this goal.

### Chemically induced larval models

Contrary to adult models, the larval model lacks a functional adaptive immune system<sup>[17,54]</sup>; therefore it allows the observation of the isolated participation of innate immunity in inflammatory intestinal conditions. Additionally, the use of a larval model permits exploitation of transgenic lines to visualize *in vivo* changes in digestive organs and immune cells, such as gutGFP<sup>[55]</sup>, Tg(*mpx:EGFP*)<sup>[14]</sup>, Tg(*ptprc:DsRed*)<sup>sd3[56]</sup>, MO microinjections<sup>[57]</sup> and the CRISPR/Cas9 system<sup>[58]</sup>, along with mutagenesis screening to discover novel candidate genes involved in diseases, among other applications.

The most used colitis model in zebrafish larva was developed by two different research groups<sup>[59,60]</sup> using TNBS in concentrations between 50-75 µg/mL diluted directly in the swimming water of embryos at 3 dpf to 6-8 dpf, higher concentrations resulted in less than 50% survival 3 d post-exposure<sup>[60]</sup>. Different histological characteristics were observed depending on the time and concentration of the TNBS exposition. Several changes were shown for a treatment of 5 d and 75 µg/mL TNBS including an expansion of the intestinal lumen, a smoothing of the epithelial line, a loss of villi and epithelial clefts, and an increase in the number of goblet cell throughout the mid and posterior intestine<sup>[59]</sup>, with first changes appearing at 6 dpf<sup>[61]</sup>. Whereas, a 3 d exposition of 50 µg/mL TNBS does not induce any change in intestinal cell morphology or increase in goblet cell number<sup>[60]</sup>. We think that discordance on these characteristics in different research groups could also be influenced by the variability of the microbiota in the different facilities<sup>[62]</sup>, nevertheless, more studies need to be conducted to test this hypothesis. Subcellular changes in TNBS-exposed larvae included the accumulation of lysosomes in the apical region of the epithelial cells and the loss of tight and gap junctions between IECs<sup>[59]</sup>, as observed in human IBD<sup>[63,64]</sup>. A well preserved microvilli were present in both TNBS and controls larvae, suggesting a direct action of the TNBS on the physiology of the IECs and not an erosive action. TNBS-exposed fishes showed increases expression of *il1β*, *tnfα*, *il8*, *il12a*, *ifng1-2*, *il10* at 6 dpf in the intestinal tissue<sup>[60]</sup> (Morales Fénero CI *et al.*, unpublished data). Interestingly, some of these molecules are Th1 type cytokines, and though the larvae lacks circulating lymphocytes, they are probably produced by epithelial and/or infiltrating myeloid cells<sup>[65,66]</sup>, as observed in the intestine of Tg(*mpx:EGFP*; *ifabp:RFP*)<sup>[60]</sup> and Tg(*lys:DSRED*) TNBS-exposed larvae (Morales Fénero CI *et al.*, unpublished data). Moreover, an increase in TNFα expression in the intestinal lumen was directly related to the TNBS dose and could be reverted with a prednisolone treatment<sup>[59,61]</sup>. Decrease in RNA expression of ileal fatty acid binding protein (*fabp6*)<sup>[67]</sup> and increased intestinal lipids accumulation visualized by Nile red (NR) lipophilic stain, reflect alterations in fatty acid metabolism in TNBS-treated fishes, as well as, loss

in the endocytic function in the mid-intestinal region<sup>[68]</sup>. Furthermore, a slight disruption of the intestinal vasculature was evidenced by a reduction of intestinal capillary branches and a decrease in the expression of vascular endothelial growth factor, in colitis induced larvae.

The microbiota is a source of pathogen-associated molecular patterns against which the immune cells can react and is a principal factor in IBD pathology<sup>[22]</sup>. Broad-spectrum antibiotics increased the low survival rate obtained with high concentrations of TNBS, and even recover the low survival rate of TNBS-exposed *myd88* morphants to control levels<sup>[60]</sup>. In addition, TNBS exposition in larva induced a lesser diversity of bacteria, with an increase in the phylum Proteobacteria (*Hydrocarboniphaga daqingensis*, *Limnobacter sp.*, *Citrobacter freundii*, *Comamonas sp.*, *Salmonella sp.*) and a decrease in the phylum Firmicutes (*Lactococcus plantarum* and *Streptococcus sp.*) compared with the control group<sup>[61]</sup>. The same bacteria phyla have been previously observed altered in human IBD<sup>[69,70]</sup>. Finally, treatment with 5-aminosalicylic acid co-administered with TNBS, prevented the disease alterations induced by the hapten, decreased the expression of *il1β*, *tnfα*, *ccl20* and *il8*, and inhibited the increase of myeloid cells in (*lys:EGFP*) transgenic larva, as well as reduced the recruitment of leucocytes to the intestine and skin in Tg(*mpx:EGFP*) larvae<sup>[59,60]</sup>. Similar results were obtained with prednisolone which was also effective against the generation of disease changes, including reduced expression of *il1β*, *tnfα* and *il8*<sup>[60]</sup>, a decrease in TNFα and reduction of the number of goblet cells to normal levels<sup>[59]</sup>. Treatment with NOS inhibitors, rescued the *in vivo* and histological disease phenotype, while treatment with the immunomodulatory drugs thalidomide and parthenolide failed to rescue the changes induced by TNBS despite a decrease in the expression of TNFα in the intestinal tissue. These results indicate that the zebrafish can be an excellent pharmacological tool for drug screening in the search for new treatments for inflammatory diseases.

An additional murine version of UC model uses Dextran Sulfate Sodium (DSS) in the drinking water in an acute protocol of 5-7 d<sup>[9,71]</sup> or with the induction of chronic inflammation interspersed with periods of DSS-water and periods of recuperation with normal water<sup>[72,73]</sup>. In another attempt to use the zebrafish as a model for intestinal diseases, researchers exposed larvae at 3 dpf to 0.5% DSS for 3 d, generating a phenotype of marked mucus production with no changes observed in the number of goblet cells in the mid-intestine and the esophagus<sup>[74]</sup>. Nevertheless, exposure to DSS did not affect the expression levels of the *muc* gene, an ortholog of the human MUC5 gene family that is expressed in the esophagus. However, other "*muc* genes", such as *muc2.1*, which is highly expressed in the gut can also be analyzed for changes in this model<sup>[75]</sup>. An analysis of Tg (*kita:GAL4*, *UAS:EGFP*) larvae that labels fin fold mucus producing cells revealed a slight increase in the number of positive

cells when the larvae were treated with DSS, compared with controls<sup>[76]</sup>. Suggesting that intestinal goblet cells and not esophagus goblet cells, are altered and that they are responsible for the production of excess mucus. An augmented bacterial load and an increased number of intestine-infiltrating neutrophils was observed in *Tg(mpx:EGFP)*<sup>[114]</sup> DSS-exposed larvae compared with controls. This was reversed by antibiotic and dexamethasone treatment, however, depletion of the microbiota prevented the appearance of the DSS-induced mucosecretory phenotype while dexamethasone did not have the same effect. Exposition to DSS also increased the levels of *cd20*, *il1 $\beta$* , *il23*, *il8*, *mmp9* and *tnfa*, and decreased the proliferating cell nuclear antigen (*pcna*) gene. Curiously, using DSS at lower concentrations (0.25%) caused a loss of the inflammatory characteristics but a persistence of the mucosecretory phenotype, which was protective against TNBS induced colitis, and could be suppressed with a retinoic acid treatment, resulting in a worse survival rate and increased neutrophil infiltration.

Based on the premise that the use of non-steroidal anti-inflammatory drugs (NSAIDs), could lead to the impairment of mucosal barrier function<sup>[77,78]</sup>, researchers used the NSAID glafenine as an inducer of intestinal inflammation<sup>[79]</sup>. An overnight treatment of 12  $\mu$ mol/L glafenine in 5 dpf larvae produced an obstruction of cells and debris in the lumen of the mid-intestine and posterior intestine of the zebrafish, which consisted of dead IECs. Analysis of transversal sections showed intestinal shedding and an obstructed lumen with hypertrophic and hyperplastic IECs, with apical-pyknotic nuclei, signs of cellular damage and apoptosis, which were confirmed by an increase in activated caspase-3 positive cells. The visualization of glafenine-treated fish with transmission electron microscopy showed apoptotic IECs, cellular debris and microvesiculation of IECs, as well as, a pitted ER and organelles enveloped by membranes, which are signs of ER stress<sup>[80]</sup>. This characteristics were reverted by treatment with the  $\mu$ -opioid receptor agonist (D-Arg2,Lys4) dermorphin-(1,4)-amide (DALDA), which decreased the formation of debris and apoptotic cell obstruction, improved the survival of glafenine-exposed larvae, decreased the expression of caspase-3, and increased the number of proliferative 5-ethynyl-2'-deoxyuridine (EdU) positive cells. Furthermore, DALDA-treated larvae showed decreased ER stress in IECs and a conserved epithelial architecture, as well as, upregulation of the UPR mediators spliced-xbp1 (*s-xbp1*) and activating transcription factor 6 (*atf6*), which were normal in glafenine-exposed animals. For other side, *atf6*-MO suppressed the rescue mediated by DALDA. This is anomalous with the function of the mammalian ATF6, which has apoptotic effects<sup>[81]</sup>. A lack of study on immune parameters such as innate cells infiltration or the quantification of cytokines and chemokines, leaves us with little knowledge about this model, which could be an excellent model to study of ER stress during intestinal inflammation. Modifications in genes that are centrally involved in the UPR appear to be a risk factor for both

forms of IBD, UC and CD<sup>[43-45]</sup>.

The aforementioned models of intestinal inflammation, summarized in Table 1, encompass the current options to analyze different aspects of IBD, including the possibility of study in conditions of isolated innate immune system. Other tools, as the generation of gnotobiotic zebrafish<sup>[82,83]</sup> make this good model system for the study of the microbiota that is central to the pathology of IBD.

## OBESITY AND METABOLIC SYNDROME

Another western-lifestyle disease that has worldwide impact and involves chronic inflammation is obesity and the associated metabolic syndrome. Subjects related to lipid metabolism in zebrafish are relatively new but have begun to gain ground in the study of adipogenesis, metabolic alterations and obesity.

Zebrafish, as other teleosts, are poikilothermic animals and they only have white adipose tissue (WAT) and lack brown fat, which is more characteristic of homeothermic organisms. The first visceral adipocytes appear to form in proximity to the pancreas after exogenous feeding is initiated, and they increase in number and distribution as the zebrafish grow, with the participation of the markers of adipocyte lineage peroxisome-proliferator activated receptor  $\gamma$  (*ppary*) and fatty acid binding protein 11a (*fabp11a*)<sup>[84]</sup>. Lipid absorption in zebrafish is very similar to the process in mammals. Bile is synthesized in the liver, stored in the gall bladder and brought to the intestine through the bile duct, where it emulsifies lipids that are broken down by luminal lipases and absorbed like fatty acids and triacylglycerols. Lipids are transported in the plasma as unbound fatty acids or bound to carrier proteins, as triacylglycerols (TAG)-rich chylomicrons, and then they are delivered to the liver and stored in visceral, intramuscular and subcutaneous reservoirs, mainly as TAG<sup>[85-87]</sup>. Currently, a variety of techniques exists that allows visualization of the lipids in zebrafish without sacrificing the animal, including the fluorescent compounds NR and BODIPY® - conjugated lipids and Oil Red O, which is suitable for fixed fish and is an excellent tool for the study of lipid metabolism<sup>[86]</sup>.

### Genetic models of obesity

Energy homeostasis in the zebrafish is conserved and regulated by peripheral signals like PYY, GLP-1, ghrelin, adiponectin, leptin and insulin<sup>[88-93]</sup>, originated in the gastrointestinal tract and adipose tissue, processed in the brain by the central melanocortin system (CMS), as in mammals<sup>[86,94,95]</sup>. The CMS circuits include the pro-opiomelanocortin (*POMC*) gene, melanocortin peptides and its receptors (MC1R-MC5R), as well as the melanocortin antagonist agouti-related protein (AgRP)<sup>[96,97]</sup>. AgRP mRNA is upregulated by fasting in humans, mice and zebrafish<sup>[97-100]</sup>, and its overexpression has been related to obesity in mice<sup>[101]</sup>. Song *et al.*<sup>[102]</sup> created a transgenic animal that overexpressed the *AgRP* gene under the control of the  $\beta$ -actin promoter. AgRP transgenic animals were demonstrated to gain more weight, present

**Table 1 Zebrafish models of inflammatory bowel diseases**

| Model     | Age             | Induction                                                                         | Characteristics                                                                                                                                                                                                                                                |
|-----------|-----------------|-----------------------------------------------------------------------------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Oxazolone | Adult           | Intrarectal administration of 0.2% oxazolone in 50% ethanol. Stand-alone tanks    | Epithelial damage; infiltration of neutrophils and eosinophils in intestine; depletion of goblet cells; upregulation of IL1 $\beta$ , TNF $\alpha$ and IL10 <sup>[46]</sup>                                                                                    |
| TNBS      | Adult           | Intrarectal administration of TNBS (160 mmol/L) in 30% ethanol. Stand-alone tanks | Dose-dependent fish survival; disruption of the epithelial integrity; ulcerations; swelling, thickening and detachment of villi; no changes in goblet cells; upregulation of IL1 $\beta$ , IL8 and IL10 <sup>[48]</sup>                                        |
|           | Larva (3-8 dpf) | 50-75 $\mu$ g/mL TNBS in swimming water (E3 medium)                               | Dose-dependent survival; expansion of intestinal lumen; loss of villi; increased number of goblet cell; upregulation of IL1 $\beta$ , TNF $\alpha$ , IL8, and MMP9; increased TNF $\alpha$ expression in lumen; infiltrate of myeloid cells <sup>[59,60]</sup> |
| DSS       | Larva (3-6 dpf) | 0.5% DSS in swimming water (E3 medium)                                            | Mucosecretory phenotype; neutrophilic infiltration microbiota - dependent; upregulation of CCL20, IL1 $\beta$ , IL23, IL8, MMP9 and PCNA; increased proliferating cells <sup>[76]</sup>                                                                        |
| Glafenine | Larva (5 dpf)   | 25 $\mu$ mol/L glafenine for 12 h in swimming water (E3 medium)                   | Apoptosis in intestinal epithelial cells; ER stress in IECs <sup>[79]</sup>                                                                                                                                                                                    |

TNBS: 2,4,6-trinitrobenzenesulfonic acid; DSS: Dextran sulfate sodium.

increased total triglycerides, present larger visceral adipocytes and increased linear growth, compared with wild type (WT) animals. However, though they could not demonstrate the direct action of the zebrafish AgRP protein as a competitive antagonist of the melanocortin receptors, the authors showed that the positive response to  $\alpha$ -melanocyte stimulating hormone ( $\alpha$ -MSH) by zebrafish MC3R, MC4R and MC5bR transfected cells could be antagonized with mouse-AgRP. Further research on this pathway revealed an *in vivo* interaction of MC4R with two forms of melanocortin receptor accessory protein 2 (MRAP2) in zebrafish<sup>[103]</sup>. In cell culture, MRAP2a binds to MC4R and reduces its ability to bind to its ligand  $\alpha$ MSH, and *in vivo*, MRAP2a is expressed during larval stages and stimulates growth by blocking the action of MC4R. The MC4R antagonist AgRP is also highly expressed in larvae and collaborates with MARP2 to maintain MC4R in a stable inactive state. On the other hand, MRAP2b is highly expressed in the adult zebrafish brain and it causes a moderate increase in the expression of MC4R in transfected cells, and even increase MC4R affinity to its ligand  $\alpha$ MSH, suggesting that MRAP2b is the homologous isoform of the mammalian MRAP2. Given the chronic inflammatory state in obese mammals, it would be interesting to analyze the inflammatory state resulting from genetic modifications of this pathway, in order to find a genetic relationship to inflammation.

An interesting transgenic of exogenous human constitutively active Akt1 (*myrAkt1*) expressed in the skin protein Keratin-4 (*krt4*) presented a severe obese phenotype in adult zebrafish<sup>[104]</sup>. Tg(*krt4:Hsa.myrAkt1*)<sup>cy18</sup> animals exhibit hypertrophic and hyperplastic growth of the epidermis during the larval stages, caused by the upregulation of the activated Akt1 downstream targets glycogen synthase kinase 3  $\alpha/\beta$  (GSK3 $\alpha/\beta$ ), mammalian target of rapamycin (mTOR) and 70-kDa S6 protein kinase (70S6K). The adult Tg(*krt4:Hsa.myrAkt1*)<sup>cy18</sup> had an increased body weight but not body length and also an augmented conditional factor, equivalent to human BMI, compared to WT siblings. Analysis with Oil Red O revealed pronounced lipid accumulation in the entire body that

primarily arose from an excess of triglycerides with normal cholesterol accumulation. Sagittal sections of the entire body of obese transgenic adult zebrafish showed adipocyte hyperplasia rather than hypertrophy, as well as ectopic adipocytes in the muscles of the dorsal body and the gill arch that also infiltrated and replaced bone and skeletal muscle cells. This seemed to be triggered by upregulation of the ectopic expression of *myrAkt1* in liver, muscle and bone and activation of the mTOR pathway in adipose tissue. Exploring the mRNA expression in tail samples of Tg (*krt4:Hsa.myrAkt1*) cy18, Rasouli *et al.*<sup>[105]</sup> found downregulation of the transcripts of myogenic factor 5 (*myf5*), myogenic factor 6 (*myf6*) myogenic differentiation 1 (*myod1*) and myosin light polypeptide 2 (*mylz2*), which are myogenic regulatory factors and structural proteins. Factors participating of skeletogenesis as runt-related transcription factor 2 (*runx2*) and collagen type II  $\alpha$ -1a (*col2a1a*) were also down-regulated, while genes related to lipid metabolism such as *ppary* and CCAAT/enhancer binding protein  $\alpha$  (*cebp $\alpha$* ) were intensely upregulated, in addition to fatty acid-binding proteins (*fabp11a* and *fabp11b*), sterol regulatory element binding transcription factor 1 (*srebf1*), lipoprotein lipase (*lpl*) and stearoyl-CoA desaturase (*scd*). Analysis of the inflammatory state of this transgenic animal revealed high expression of adiponectin (*adipoql* and *adipoql2*), of the adiponectin receptors *adipor1a* and *adipor1b*, the leptin receptor (*lepr*), and *lipin1*, known as adipocytokines. Inflammatory molecules such as *tnf $\alpha$* , *il1 $\beta$* , *mmp2* and *mmp9* were also upregulated, and although no differences in the number of whole body neutrophils were found, neutrophil aggregation could be seen in the obese animals' tails. It would be interesting to see if these neutrophils aggregate in WAT. Other characteristics of this transgenic animal were a "sedentary" swimming behavior because muscles were replaced by fat, a lower survival rate compared with WT, and reduced glucose clearance after feeding, suggesting impaired glucose tolerance in these animals.

### Diet induced obesity models

In addition to the genetic mutations that could lead to

an obesogenic phenotype several diet induced obesity (DIO) models also exist, that use different combinations of high fat food to generate the phenotype. Oka *et al.*<sup>[106]</sup> designed a DIO model in zebrafish by overfeeding adults for 8 wk with freshly hatched nauplii *Artemia* (brine shrimp), which are part of the normal food in zebrafish facilities. The DIO animals exhibited an increased BMI (calculated as the body weight divided by the square of the body length), increased plasma triglycerides and hepatosteatosis. These parameters were improved by a calorie restricted diet following overfeeding. A comparative transcriptome analysis between visceral adipose tissue of DIO zebrafish, DIO mice, DIO rats and obese humans revealed common pathophysiological pathways. Genes related to blood coagulation and lipid metabolism were significantly dysregulated in the four obese groups, including apolipoprotein H (*apoh*), *il6* and *il1 $\beta$*  as regulatory molecules appearing in the coagulation cascade, as well as *sreb1*, peroxisome proliferator activated receptor alpha (*ppar $\alpha$* ) and gamma (*ppar $\gamma$* ), nuclear receptor subfamily 1 group H member 3 (*nr1h3*) and leptin (*lep*), which are regulatory molecules involved in lipid metabolism in obese zebrafish, rats, mice and humans. These results indicates that immune molecules occur in obesity pathways in both zebrafish and mammals. Further research with this DIO model tested the anti-obesity effects of different vegetables in zebrafish, including regular and Campari tomatoes, pumpkins, eggplants, and others<sup>[107]</sup>. Campari tomatoes have significant lipid-lowering proprieties because they suppressed the increase of body weight and plasma triglycerides in DIO zebrafish, reduced lipid accumulation in the liver, and increased the genes involved in fatty acid oxidation such as proliferator-activated receptor gamma co-activator 1 $\alpha$  (*ppar $\gamma$ c1 $\alpha$* ) and peroxisome proliferator-activated receptor  $\alpha$ b (*ppar- $\alpha$ b*). Additionally, the same group tested the anti-adipogenesis proprieties of green tea extract (GTE) in the same DIO model<sup>[108]</sup>. GTE treatment decreased the volume of visceral but not subcutaneous WAT and increased the liver expression of acyl-coenzyme A oxidase 1, palmitoyl (*acox1*), acyl-coenzyme A dehydrogenase (*acadam*) and *ppar $\alpha$* , which are part of  $\beta$ -oxidation and lipid catabolism. Also decreased the expression of suppressor of cytokine signaling 3b (*socs3*) in visceral fat, which inhibits leptin signaling. Another approach using GTE as a treatment in adult zebrafish used a standard chow supplemented with gluten,  $\alpha$ -potato starch, corn oil and lard in order to create four diets for DIO models with different fat contents. The results indicated no differences in fat accumulation between the groups of high-fat (HF) or of low-fat (LF). As in the previous model, GTE decreased body weight and fat volume in animals on a HF diet, and increased the activity of the enzyme 3-hydroxyacyl-coenzyme A dehydrogenase in liver and skeletal muscle, which is part of the  $\beta$ -oxidation pathway, demonstrating the utility of this model to test different natural anti-obesogenic compounds.

Another recent DIO model based on overfeeding

adult zebrafish was generated by feeding them two times the standard fish chow than the controls<sup>[109]</sup>. DIO animals showed increased total weight, showed liver steatosis, as well as the overexpression of *tac4*, *col4a3*, *col4a5*, lysyl oxidases and genes involved in retinoid metabolism. A liver transcriptomic analysis after inoculation with LPS showed that immune system genes responded to LPS stimulation, including Toll-like receptors, ubiquitin mediated proteolysis, RIG-I-like receptor signaling pathway, MAPK and Jak-STAT signaling pathway in control lean animals. No alterations were observed in obese animals, and there was also no difference between obese animals and uninjected obese controls. Studying the differences between obese and non-obese zebrafish in other organs during LPS-stimulation or in other infection models could be of great interest, because obese animals are in a basal inflammatory state.

Though adult models of obesity seems to be more popular, larvae obesity models are also promising. The zebrafish obesogenic test<sup>[110]</sup> was created for the *in vivo* study of the effect of diet composition, chemical pollutants, and/or drugs on white adipocyte tissue. Larvae of a standard length of 7.5-9 mm were fed with a three-day protocol, in which the first day started with a high-fat diet (HFD) based on hard-boiled chicken egg-yolks *ad libitum* for the entire day, followed by starvation the next day, and by exposure to different obesogenic/non-obesogenic compounds the third day. After the feeding period, HFD animals showed an increase in NR staining in blood vessels, which were reduced after the fasting period. This phenomenon was not observed in control animals fed with a standard diet (SD). When studying the interaction of the initial diet with different compounds, the researchers found that exposure to rosiglitazone, a PPAR $\gamma$  agonist used in type II diabetes treatment, increased the lipids deposits in both SD and HFD animals, and this effect was inhibited by T0070907, a PPAR $\gamma$  antagonist. A similar result was observed with tributyltin, a renowned environmental obesogen that binds to PPAR $\gamma$  and retinoid X receptor, for which both SD and HDF larvae exhibited an increase in adipose deposits. However, additive effects between any of the two chemical and HFD were not observed. Although this model showed an increase in blood vessel lipids for a HFD in a short period of time, this result does not reflect obesity as a chronic disease, because differences in the accumulation of lipids in the visceral WAT between SD and HFD animals or increase in weight and size of HFD larvae were not observed. Perhaps a longer exposure period to the HFD would affect these parameters.

Progatzky *et al.*<sup>[111]</sup>, showed that the exposure to a HF diet or a high-cholesterol diet (HCD) in zebrafish larvae induced an inflammatory response in hours, with infiltration of myeloid cells in the intestine, dependent on inflammasome activation by IECs. They demonstrated that the inflammation was directly induced by cholesterol binding to the Niemann-Pick C1-like receptor, with the participation of the apoptosis-associated speck-like protein containing a CARD (ASC) and activation of

**Table 2 Zebrafish models of obesity**

| Model                          | Age        | Induction                                                                       | Characteristics                                                                                                                                                                                                                                                                  |
|--------------------------------|------------|---------------------------------------------------------------------------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Genetic models                 |            |                                                                                 |                                                                                                                                                                                                                                                                                  |
| AgRP overexpression            | All stages | AgRP expressed under the control of $\beta$ -actin promoter                     | Weight gain and linear growth; increased BMI; visceral adipose accumulation; increased triglycerides; larger visceral adipocytes <sup>[102]</sup>                                                                                                                                |
| Tg(krt4:Hsa.myrAkt1)cy18       | All stages | Expression of constitutively active human AKT1                                  | Weight gain; increased BMI; triglycerides accumulation; adipocyte hyperplasia; ectopic adipose tissue; increased expression of adiponectin, adiponectin receptors, leptin receptor; increased inflammatory molecules TNF $\alpha$ , IL1 $\beta$ , MMP2 and MMP9 <sup>[104]</sup> |
| DIO models                     |            |                                                                                 |                                                                                                                                                                                                                                                                                  |
| Artemia overfeeding            | Adult      | Overfeeding with nauplii artemia for 8 wk                                       | Increased BMI; high plasma triglycerides; hepatosteatosis <sup>[106]</sup>                                                                                                                                                                                                       |
| Chow overfeeding               | Adult      | Overfeeding with standard fish chow for 8 mo                                    | Weight gain; hepatosteatosis <sup>[109]</sup>                                                                                                                                                                                                                                    |
| Zebrafish obesogenic test (OZ) | Larva      | High-fat diet based in hard-boiled chicken egg-yolk ad libitum during one day   | Increase in blood vessel lipids in a short time <sup>[110]</sup>                                                                                                                                                                                                                 |
| HCD                            | Larva      | HCD, cholesterol mixed in fish standard dry food for 6 h. Extended HCD for 10 d | Infiltration of myeloid cells in intestine dependent of the inflammasome, microbiota and NF $\kappa$ B activation; extended feeding leads to visceral fat accumulation, liver steatosis, intestine inflammation, impaired peristalsis <sup>[111]</sup>                           |

HCD: High cholesterol diet; BMI: Body mass index; TNF: Tumor necrosis factor; IL: Interleukin; MMP: Matrix metalloprotease.

caspase-1, which is part of the inflammasome complex<sup>[112]</sup> that produces high levels of active IL-1 $\beta$ . Furthermore, this inflammation was dependent on the microbiota and NF $\kappa$ B activation. Finally, extended feeding with a HCD produced the accumulation of visceral fat, liver steatosis, sustained inflammation in the intestine, and impaired peristalsis. This study verified a direct link between inflammation and high-fat diets, specifically the activation of the inflammasome complex by cholesterol in the intestine, and opened a new window to the study of innate inflammation in the context of obesity and its influence in other chronic inflammatory diseases.

By last, a study analyzing two flame retardants, tetrabromobisphenol-A and tetrachlorobisphenol-A, as possible obesogens using zebrafish larvae<sup>[113]</sup> showed lipid accumulation in larval stage and late-onset weight gain in juvenile animals, which was most likely caused by the compounds' activity as a PPAR $\gamma$  agonist. This method could be interesting for the analysis of the inflammatory state under such conditions, using these substances as agonists of PPAR $\gamma$ .

The zebrafish models related to obesity maybe are not so well known as mice models, nonetheless, the examples presented here (Table 2) are evidence of the conserved signals that control lipids metabolism and the flexibility of the zebrafish as model of metabolic diseases.

## CONCLUSION

The models presented in this review exhibit the utility of zebrafish as a model of diseases and demonstrate that this animal as an intermediate between models involving simpler invertebrates and more complex higher mammals and can be used as an alternative or a complement to pre-clinical and drug screening studies that involve conserved metabolic and inflammatory pathways. Furthermore, the characteristics of zebrafish

such as physiological homology, rapid development and a low cost of production, make this animal a great option for research on new therapies for inflammatory diseases.

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## Hepatocellular carcinoma: Where are we?

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### Abstract

Hepatocellular carcinoma (HCC) is the second cause of death due to malignancy in the world, following lung cancer. The geographic distribution of this disease

accompanies its principal risk factors: Chronic hepatitis B virus and hepatitis C virus infection, alcoholism, aflatoxin B1 intoxication, liver cirrhosis, and some genetic attributes. Recently, type II diabetes has been shown to be a risk factor for HCC together with obesity and metabolic syndrome. Although the risk factors are quite well known and it is possible to diagnose HCC when the tumor is less than 1 cm diameter, it remains elusive at the beginning and treatment is often unsuccessful. Liver transplantation is thus far considered the best treatment for HCC as it cures HCC and the underlying liver disease. Using the Milan criteria, overall survival after liver transplantation for HCC is about 70% after 5 years. Many attempts have been made to go beyond the Milan Criteria and according to recent works reasonably good results have been achieved by using a histochemical marker such as cytokeratine 19 and the so-called "up to seven criteria" to divide patients into categories according to their risk of relapse. In addition to liver transplantation other therapies have been proposed such as resection, tumor ablation by different means, embolization and chemotherapy. An important step in the treatment of advanced HCC has been the introduction of sorafenib, the first oral, systemic drug that has provided significant improvement in survival. Treatment of HCC patients must be multidisciplinary and by using the different approaches discussed in this review it is possible to offer prolonged survival and quite good and sometimes even excellent quality of life to many patients.

**Key words:** Hepatocellular carcinoma; Treatment; Liver cancer; Epidemiology; Liver transplantation; Percutaneous ethanol injection; Chemoembolization; Chemotherapy; Radiofrequency ablation

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**Core tip:** This review summarizes on the current state of the art of treatment of hepatocellular carcinoma (HCC). After a brief chapter on epidemiology, risk factors and biology of HCC, the review presents all possible therapeutic approaches for HCC, from the most effective

such as liver transplantation to important but palliative treatments which can prolong patient survival such as different types of trans-artery-chemo-embolization and chemotherapy.

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## INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the ten most commonly occurring solid cancers worldwide and is the second cause of death from malignancy. The most recent data indicate that its incidence is still increasing in many countries whereas the most effective way of reducing mortality due to HCC is prevention<sup>[1]</sup>.

These observations appear to be in contrast with what we know about this lethal cancer. Most of its risk factors are known. It is possible to diagnose HCC when the tumor is 1 cm diameter or less and it can be treated either surgically or medically. So why we are losing the battle against an enemy we know so well?

It is clear that there are some other risk factors which are not yet known. We do not understand the precise mechanisms by which liver cells become neoplastic clones without the possibility of our immune system to controlling them. Most important of all, the natural history of HCC before it has been diagnosed is unknown. Sophisticated technology for searching for cancer cells in blood has revealed that even small tumors distribute thousands of cancer cells into the circulation that then may begin the process of metastasis much earlier than was formerly thought<sup>[2]</sup>. In other words, the concept of "early cancer" that has been applied successfully to other situations, such as gastric cancer, has not yet proven for HCC. Although it is believed that not all viable cancer cells are able to metastasize, but only those with stem cells properties, we must consider these aspects before saying we can cure cancer patients<sup>[2,3]</sup>. Relapse can occur years later, well after the standard 5 years that are considered. Breast cancer is the best example of this phenomenon.

Liver cells undergo replicative activity throughout life (thus the concept of the "streaming liver") but it has been not clarified whether this process helps to eliminate "initiated" cells or, on the contrary, helps them to arrive in the blood circulation. Moreover, since HCC occurs mostly in the cirrhotic or chronically inflamed liver, it is possible that chronic liver disease encourages the process of metastasis from tiny neoplastic nodules.

This review will focus on some of these aspects of HCC and the various current and future treatment options.

## EPIDEMIOLOGY

According to the most recent data, the global incidence of

HCC is still increasing, although it varies throughout the world. The highest rates occur in Eastern Asia whereas the positive effect of vaccination against hepatitis B virus (HBV) infection is showing the first results in China and Taiwan now. The lowest rates are in Northern Europe and North America. These data are mainly explained on the basis that two of the risk factors for HCC, HBV and hepatitis C virus (HCV) infections, are largely diffused in Eastern Asia. In Italy, for example, HBV-related HCC is now rapidly declining, in comparison to HCV-related HCC because of the introduction of mass HBV vaccination several years ago<sup>[4,5]</sup>. Moreover, aflatoxin-B1 intoxication, an important risk factor for HCC, is quite common in those geographic areas where the incidence of HCC is high due to inadequate cereals and food storage conditions. The known risk factors for HCC are reported in Table 1.

Some pathological aspects of the liver such as cirrhosis and fibrosis, conditions resulting from chronic inflammation due to chronic HBV and HCV infection or to alcohol intoxication, can lead to cancer through several mechanisms, some of which are linked to chronic inflammation such as hepatocyte necrosis, liver regeneration and fibrosis and others that are specific of each virus. For example, HBV causes about 70%-80% of HCC cases in East Asia, and possesses transformation activity due to some of its proteins, *i.e.*, protein X, or by integrating part of its DNA genome into that of humans and thus altering it<sup>[6,7]</sup>. HCV, which is an RNA virus without an inverse transcriptase, cannot integrate its genome with the human, but it has several proteins that have transforming properties. In addition, HCV infects the immune system and endothelial cells, leading to immune dysfunction and chronic deregulated angiogenesis. Both conditions are known risk factors for cancer<sup>[8,9]</sup>.

Alcoholism is an important risk factor for HCC because it causes fatty liver, necro-inflammation, fibrosis, liver cirrhosis and malnutrition, especially when it is associated with HCV infection. Alcohol abuse is the most important risk factor for HCC in North America and Northern Europe.

There are other risk factors for HCC, *i.e.*, hemochromatosis, fructosemia, tyrosinemia, galactosidemia, diabetes type II, genetic propensity, or clinical conditions such as obesity or hypothyroidism. Hemochromatosis, for instance, is the most common genetic defect (1/200 born in the West) and it leads to the accumulation of iron in the body, causing heart, joint, endocrine and liver diseases. Its prevention could be a key step to preventing HCC and other fatal diseases.

In recent years, other important risk factors have been described such as type II diabetes, metabolic syndrome and non-alcoholic steato-hepatitis. Type II diabetes is so common in western countries (about 10% of the adult population) that it constitutes an enormous societal health problem. Type II diabetes also increases the risk for other types of cancer, especially the most deadly pancreatic cancer. It is also the main risk factor for heart and vessel diseases. Since its occurrence is much facilitated by obesity and the lack of physical exercise,

**Table 1** Identified risk factors for hepatocellular carcinoma

|                                          |
|------------------------------------------|
| Age                                      |
| Ethnicity                                |
| Male Gender                              |
| Liver cirrhosis                          |
| Chronic HBV infection                    |
| Chronic HCV infection                    |
| Hemochromatosis                          |
| Alcoholism                               |
| Aflatoxin-B1 intoxication                |
| Tyrosinemia, galactosidemia, fructosemia |
| Alpha1 anti-trypsin deficiency           |
| Genetics predisposition                  |
| Anabolizing hormones                     |
| Estrogen contraceptives                  |
| Obesity                                  |
| Type II diabetes                         |
| Glucose overload                         |
| Metabolic syndrome                       |
| Hypothyroidism                           |
| Fatty liver                              |
| Non-alcoholic steato-hepatitis           |

HBV: Hepatitis B virus; HCV: Hepatitis C virus.

its prevention should become a target for all healthcare systems in developed countries where large portions of the population are becoming more and more overweight and sedentary.

As mentioned above, also fatty liver significantly increases the risk of HCC<sup>[10]</sup>. This condition per se does not seem a risk factor, but when it becomes the expression of underlying metabolic disorders or other more severe diseases it may become a predisposing lesion of HCC, as in the large use of anabolic hormones to increase body mass. On the other hand, it now seems possible to exclude the use of modern contraceptive pills from this category of risk factors<sup>[11]</sup>. Estrogen actually may reduce the risk of HCC as suggested by the observation that HCC occurs more frequently in men rather women with a ratio ranging from 2:1 to 4:1.

Ionizing radiation and smoking are also risk factors for HCC although there are no specific works on these. Finally, last but not least, we cannot forget that HCC is an age-related disease. Although it can occur at every age from infants to the elderly, nevertheless its frequency increases in individuals from 30 to 70 years of age, according to the population under consideration (in much younger individuals in Africa and East Asia where HBV is prevalent, and in older subjects in Europe or America).

The process that transforms a normal liver cell into neoplastic one is not known, although changes in normal liver histology are likely to play an important role together with genetic alterations due to viruses, oxygen derived free radicals, toxic compounds and changes in the host immune system.

Cancer is a genetic- and age-related disease and HCC is not an exception. All the risk factors that we have taken into consideration may result in changes in the hepatocyte DNA and, since the process of transformation

is quite long, the immune system is also involved in the development of clinically evident cancer. This means that the pathway to HCC must be long enough to cause an irreversible phenomenon. Chronic liver diseases and cirrhosis are very good examples of this old observation. There are thousands of patients who have had acute HBV or HCV infections many years earlier and then who developed HCC after a chronic disease that lasted decades in the literature. If either HBV or HCV are directly and rapidly oncogenic as experimental research shows, why does it take so long to develop HCC in humans? We know that HBV-related protein X or HCV NS3, NS5 or core proteins or others have the capacity to transform normal cells *in vitro*, but why does this not happen in all patients and, more importantly, why does it take 20-30 years for a neoplastic nodule to appear in man? Is it only due to our still rudimentary diagnostic tools which do not perceive tiny cancerous foci, or are some individual's immune systems able to combat the disease, or are there more complex pathways which we do not yet understand?

Consider the recent data published by two of the top scientists worldwide on colon cancer: The main risk factor according to their data is bad luck. After thousands of papers published on this issue, billions of dollars spent on research on it, the bitter conclusion is that the most important factor, but not the only one, in getting colon or lung cancer is bad luck<sup>[12]</sup>. What a terrible conclusion!

The liver is an example that fits quite well what Tomasetti and Vogelstein have shown<sup>[12]</sup>. Since liver stem cells have quite intense replicative activity and since the greater the replication, the higher the risk that one or more daughter cells have mutation in their DNA, it makes sense that the risk of acquiring HCC is higher in the presence of risk factors, such as HBV or HCV infection, alcoholism, aging, excessive iron, ionizing radiation, and smoking, however HCC also occurs in subjects who do not have any of the above risk factors.

Vaccination against HBV infection, prevention of HCV-infected transfusions, prevention of alcoholism or aflatoxin intoxication are crucial factors in the prevention of HCC. And we should also mention that besides the risk factors, there are also protective conditions that could help us to understand better how cancer develops. The most important protective factor for HCC is vaccination against HBV infection. Prevention of chronic HBV carrier status and chronic liver disease is crucial as shown recently by the decrease in HCC in Taiwan and China after the adoption of mass vaccination<sup>[4]</sup>. Reducing the HBV load in patients with liver cirrhosis and chronic hepatitis reduces also reduces the risk of HCC<sup>[13]</sup>. There are other factors that can help protect from HCC, such as regular consumption of vegetables and coffee drinking (3-4 cups/d). Vitamin D and calcium intake may also exert a protective role although results are controversial<sup>[14-17]</sup> (Table 2). It is also possible that other dietary factors act as protective factors, including consumption of phenols contained in red wine, green tea, olive oil, but these data

**Table 2 Protective or probably protective factors**

|                            |
|----------------------------|
| HBV vaccination            |
| Physical exercise          |
| Balanced diet              |
| High vegetable consumption |
| Calcium                    |
| Vitamin D                  |
| Coffee                     |

HBV: Hepatitis B virus.

require confirmation<sup>[18]</sup>.

## BIOLOGICAL FEATURES OF HCC

Despite recent advances in surveillance and management of HCC, its molecular biology is quite elusive. A complete definition of molecular events that lead to hepato-carcinogenesis is a major challenge in the clinical management of this disease. There is no doubt, however, that liver cirrhosis, chronic hepatitis, chronic HBV and HCV infections, and intoxication by alcohol, iron and aflatoxin-B1 all play a major role in causing HCC.

Hepato-carcinogenesis is a multi-step process that begins from an altered hepatic microenvironment, typically related to chronic liver disease, characterized by massive inflammation and fibrosis that are responsible for the deregulation of several signaling pathways and accumulation of genetic alterations in normal hepatocytes<sup>[19-21]</sup>. The latter are finally transformed into dysplastic lesions causing early carcinoma which finally progresses to HCC<sup>[22]</sup>. What seems particularly important in liver oncogenesis in humans is the lag time from the beginning of the inflammatory process or the exposure to risk factors and the occurrence of HCC. This review does not address specific aspects that could be involved in liver oncogenesis, but we do present some updated references that can provide more thorough information to the interested reader.

HCC is an extremely heterogeneous solid cancer<sup>[23]</sup> and factors such as platelet derived growth factor, transforming growth factors (TGFs), vascular endothelial growth factor (VEGF), hepatocyte growth factor, reactive oxygen species (ROS) and cellular factors such as hepatocytes, stem like, stellate and inflammatory cells are involved<sup>[24,25]</sup>. We believe that ROS and the oxidative stress that are associated with inflammation are of particular importance<sup>[26]</sup>. HCV infection, alcohol abuse or iron intoxication, for instance, all cause ROS and reactive nitrogen species (RNS) that can eventually overcome the liver's defense against oxidative stress, thus damaging DNA and cell proteins<sup>[27]</sup>. HBV-related inflammation also causes oxidative stress due to both the inflammation itself and protein X that stresses the endoplasmic reticulum *via* cAMP-responsive element-binding protein (CREBH)<sup>[26]</sup>. The presence of transforming proteins such as HBVx protein or HCV-related NS3, NS5, or core proteins may all lead to hepatocyte transformation. Intense angiogenesis in the liver during chronic HCV infection is also important and thus these patients are at

greater risk of developing HCC<sup>[28,29]</sup>.

Understanding the precise molecular processes occurring during these steps may offer a better therapeutic approach to HCC. Unfortunately, our understanding of how things occur *in vivo* is not sufficient to drive safe and effective therapy, which is the main reason why traditional chemo- and hormone therapies have produced discouraging results<sup>[30]</sup>. From a molecular point of view HCC is a heterogeneous disease that shows heterogeneity between different tumor nodules in the same patient and differences in the same nodule itself<sup>[31,32]</sup>. This may reflect the existence of distinct pools of cancer stem-like cells with different oncogenicity and independent genomic evolution. Thus, not only does each patient have his/her own HCC, but each nodule may be genetically unique<sup>[32-35]</sup>. With regard to this point, unfortunately the theory about the importance of cancer stem-like cells in HCC has produced little. There is a controversy regarding what they are and where they come from in HCC as well as in other cancers<sup>[36-39]</sup>. Like many, we think that they come from oval cells that, after prolonged proliferative activity, evolve towards a sort of erroneous differentiation. It is possible that oxidative stress causes DNA damage that results in initiation of the process. However, this is pure speculation, as we simply do not know! What we know very well is that without liver cirrhosis, chronic HBV and HCV infections or iron, ethanol or aflatoxin B1 intoxication, HCC is a rare disease.

## MOLECULAR ASPECTS OF HCC

Genome-wide surveys have been used to try to better understand HCC molecular mechanisms. Pathways commonly altered by genetic alterations (somatic mutations or homozygous deletions) include the Wnt/beta-catenin pathway, the p53 pathway, phosphatidylinositol 3-kinase (PI3K)/Ras signaling pathways, oxidative and endoplasmic reticulum stress modulators and processes responsible for chromatin remodeling<sup>[40]</sup>. Interestingly, different mutations segregated by the etiology of the underlying liver disease suggest that the carcinogenesis and development of mutations causing cancer may vary, depending on pre-existing liver disease. For example, inactivation of genes involved in chromatin remodeling is more common in patients with alcoholic cirrhosis, while p53 pathway deregulation is predominant in HBV-positive patients and Ras in those affected by HCV<sup>[40-43]</sup>.

Similar results have recently been obtained through transcriptome analysis of liver cancer that has shown a deregulation in some oncogenic signaling pathways such as those of TGF- $\beta$ 1, MYC, PI3K/Akt, Wnt/ $\beta$ -catenin, NOTCH and MET<sup>[41-43]</sup>.

Some of these HCC hallmarks have been analyzed in depth and their role as a potential target for HCC treatment is under investigation. VEGF and its receptors are known to be key mediators in HCC initiation, growth and diffusion<sup>[44,45]</sup>. Increased VEGF expression from low to high grade dysplastic nodules or advanced HCC and the correlation of VEGF levels with tumor stage and risk

of recurrence, support this hypothesis<sup>[44-46]</sup>. We have confirmed the importance of angiogenic phenotype in HCV-related HCC<sup>[47]</sup>. Fibroblast growth factor (FGF) and its family of receptors are recognized as a central player in augmenting HCC growth, invasion, and angiogenesis, making it an intriguing candidate for HCC therapy. FGF activation is involved in neovascularization<sup>[48]</sup>. Increased serum levels of FGF or activation of its receptors are associated with recurrence, treatment resistance, and poor prognosis<sup>[49]</sup> while synergistic interactions between FGF and VEGF have been shown to be a resistance mechanism to the antiangiogenic effects of VEGF-targeted agents<sup>[50]</sup>.

Several other pathways have been shown to be involved in HCC pathogenesis. In particular, promising results come from the PI3K/Akt/mTOR, EGFR, Ras/Raf/mitogen-activated protein kinase (MEK)/extracellular-signal-regulated kinase (ERK), and IGFR pathways<sup>[51,52]</sup>.

The PI3K/Akt/mTOR pathway is a major intracellular signaling cascade that is involved in the regulation of cell proliferation, growth and survival, through activation of tyrosine kinase receptors, such as VEGFR, EGFR, PDGFR, and IGFR. Nearly 50% of patients with HCC exhibit activation of the mTOR pathway, which may be partially attributable to activation signals from receptor tyrosine kinases such as IGFR and/or EGFR pathways. mTOR pathway activation is associated with aggressive HCC and decreased survival in these patients<sup>[53]</sup>. Despite promising results targeting mTOR, a substantial benefit in HCC patients has not been shown in clinical practice.

The Ras/Raf/MEK/ERK signaling cascade is another important intracellular pathway altered in HCC such as the Wnt pathway, however, while Raf inhibitors such as sorafenib have been developed in HCC therapy, Wnt inhibitors have not yet been developed.

cMET, a tyrosine kinase receptor with its ligand hepatocyte growth factor, has been implicated in HCC and probably in multidrug resistance<sup>[54]</sup> and represents a target that is undergoing intense investigation. Randomized phase II data of the cMET inhibitor, tivantinib, has demonstrated activity in patients with advanced HCC who progress on sorafenib with elevated expression of cMET by immunohistochemistry. Tivantinib is undergoing phase III investigation in this subgroup of patients<sup>[55,56]</sup>.

Given the molecular heterogeneity of HCC, the challenge is to identify in which patients any given alteration is critical. It is unlikely that any of these targeted agents will yield clinical success without selecting the patients whose tumors are most dependent on these pathways and are therefore most likely to benefit, and the identification of predictive markers of response is essential for the successful development of new targeted agents.

## SURVEILLANCE PROGRAMS AND DIAGNOSIS

If we exclude the so-called "incidental" occurrence of HCC that is found by chance in subjects undergoing ultrasound scan and/or computerized tomography (CT) for other

reasons, most HCC cases are suspected and diagnosed in a program of clinical controls or planned controls in patients known to be at risk for the disease. It is possible to diagnose HCC using different imaging techniques such as ultrasound, CT, nuclear magnetic resonance (NMR), but confirmation comes only with histology. There is a general agreement now that ultrasound should be the first tool for detecting HCC nodules in patients admitted to follow-up because of chronic liver disease or for the existence of an at-risk condition. Blood tests are much less useful, and they may help more in patient follow-up rather than in diagnosis. It is possible to find diagnostic algorithms made by experts after extensive discussion in the international literature<sup>[57,58]</sup>. However, we believe that confirmation of the presence of HCC by histology should be searched in all cases where image means (CT and/or NMR) are not conclusive or legal controversy may arise when such confirmation is lacking.

As mentioned above, HCC can first be observed by ultrasound scan, which is an inexpensive, easy, fast, and readily available technology in every part of the globe. Most scientific societies for the study of liver diseases agree that a 6-mo interval is the most convenient for checking up the development of HCC in liver patients<sup>[57,58]</sup> (Table 3).

Some authors suggest that surveillance becomes convenient when the ratio between cost and benefit achieves 3 extra mo of life at a cost of less than 50000 dollars<sup>[59]</sup>. The 6-mo interval comes from a large number of studies that have shown that the doubling time of HCC is about 6-mo, although very few of them have shown a clear gain in survival as compared to groups of patients who did not undergo programmed check-up<sup>[60]</sup>.

The effectiveness of ultrasound in detecting any form of HCC is quite good (94%), but its sensitivity drops to 63% when the target to be detected is small<sup>[61,62]</sup>. Because no ethical committee would permit a comparative study between controlled and uncontrolled cirrhotic patients, we think that the 6-mo interval is reasonable to follow-up patients. This interval becomes useless for assessing the response to treatment or to check for relapse in patients who have been treated for HCC. In addition, in patients where ultrasound has shown lesions of less than 1 cm diameter it should be repeated with a much shorter interval than 6-mo (we use 3-mo, normally). It is clear that once liver patients have been treated for HCC, they should be carefully followed-up with intervals inferior to 6-mo, either to follow how the treated nodule(s) is doing or to assess new lesions.

There is another exception that must be considered and it concerns those who are on the waiting list for liver transplantation. The occurrence of a small nodule of suspected HCC puts these patients into the priority line for transplantation according to the Milan criteria<sup>[61,62]</sup>.

Contrast-enhanced ultrasound does not increase the sensitivity for small HCC, but it helps in differentiating some lesions<sup>[63]</sup>. It is common that most of those with liver nodules undergo ultrasound-guided needle biopsy to assess the histology of the lesion. Expert pathologists

**Table 3** Patients where surveillance program is suggested by International Societies for the Study of the Liver (AASLD, EASL, EORTC, JSH, APASL)

| Society     | Target population                                   | Target population                                          |
|-------------|-----------------------------------------------------|------------------------------------------------------------|
| APASL, 2010 | Chronic hepatitis                                   | Cirrhosis                                                  |
|             | No recommendation                                   | HBV and HCV cirrhosis                                      |
| AASLD, 2011 | HBV-positive patients                               | HBV and/or HCV cirrhotics                                  |
|             | Male Asian HBV+ > 40 yr                             | Fourth stage PBC                                           |
|             | African > 20 yr; Familial predisposition + for HCC  | a1-antitrypsin deficit                                     |
|             |                                                     | Autoimmune hepatitis                                       |
|             |                                                     | NASH                                                       |
| JSH, 2011   | HBV and HCV chronic hepatitis                       | Non viral cirrhosis                                        |
|             |                                                     | HBV and HCV cirrhosis                                      |
| EASL, 2012  | HBV + active hepatitis                              | Child A and B cirrhosis                                    |
|             | HCV + hepatitis with advanced fibrosis F3 (Metavir) | Child C cirrhosis with indication to liver transplantation |

AASLD: American Association for the Study of Liver Diseases; JSH: Japan Society of Hepatology; EASL: European Association for the Study of the Liver; APASL: Asian Pacific Association for the Study of the Liver; NASH: Non-alcoholic steato-hepatitis; HBV: Hepatitis B virus; HCV: Hepatitis C virus; HCC: Hepatocellular carcinoma; PBC: Primary biliary cirrhosis; EORTC: European Organization for Research and Treatment of Cancer.

are needed to solve sometimes difficult diagnoses and the use of ultrasound scan, CT, or even NMR are very helpful in some cases. Before biopsy there are other techniques that help clinicians to reach the correct diagnosis.

Radiologists have studied the behavior of blood distribution inside the tumor, differentiating the artery phase from the portal phase (early and tardive). By using liver specific contrast it is possible to observe how the liver handles the contrast, suggesting the presence of HCC tissue or benign lesions. HCC is commonly a hyper-vascular lesion and contrasted CT can distinguish a rapid wash-in phase of the tumor (artery supply) from liver tissue that surrounds the HCC nodule. In patients with chronic liver disease the occurrence of a lesion having such characteristics strongly suggests the presence of HCC independently from other signs. Sometimes lesions are hypo-vascularized, especially those with a diameter of less than 2 cm. In these cases the most sensitive and specific technique is NMR with hepato-specific contrast that distinguishes the hypo-vascular lesion from other aspects of the liver, taking advantage of the absence of any metabolism of the contrast by neoplastic cells<sup>[64-68]</sup>.

In patients with chronic liver disease, especially with liver cirrhosis, it is relatively common to observe lesions of less than 1 cm diameter by ultrasound and CT scan. These lesions are difficult to diagnose, even when using ultrasound-guided biopsy because of the occurrence of false negatives. It is more prudent to wait and watch before doing anything, unless the patient is on the waiting list for liver transplantation. As mentioned before, this occurrence can move the patient into priority line for liver transplant. In patients with Child-Pugh stage A liver cirrhosis, repeated ultrasound check-ups must be done and if the lesion tends to grow, it may be considered for needle biopsy by expert hands. Once liver histology has been obtained, the pathologist should perform many specific tests such as CD 34, CK7, glypican 3, HSP-70 and others to assess the malignancy of the tissue according to guidelines of the European Association for

the Study of the Liver, or the European Organization for Research and Treatment of Cancer. There is controversy among different scientific societies regarding use of contrasted ultrasound in differentiating HCC, though CEUS can better identify a small lesion in a cirrhotic liver.

An issue that has been discussed intensely concerns whether liver biopsy of suspected nodules should be performed. From what was said above, we are in favor of liver biopsy in cases where not invasive techniques are not conclusive<sup>[69]</sup>. In addition, now that target therapy is becoming a reality, we think that good quality molecular study of each biopsy could add the knowledge that is required for administering the right target therapy. Circulating cancer cells could be the alternative in the future, but at present they do not give certainty about the presence and the site of HCC<sup>[70,71]</sup>.

As regards the use of biochemical blood parameters such as alpha-fetoprotein, or fucosylate alpha-pheto-protein or des-gamma-prothrombin, we agree with most western researchers that these parameters have limited utility in diagnosing HCC. They are especially misleading in screening programs because of poor sensitivity and specificity. On the contrary, they can be useful once HCC has been diagnosed to follow up the results of treatment. While this manuscript was in preparation a study concerning a possible new blood indicator for the presence of HCC in cirrhotic patients was published<sup>[72]</sup>. Only time will tell whether this new indicator for the presence of HCC will become of general use or will be disappointing, like many others.

## CLINICAL ASPECTS

As most solid cancers, HCC is asymptomatic for several months and sometimes for years. This means that HCC should be searched for in all known chronic liver disease patients with adequate diagnostic tools at regular intervals.

When HCC occurs in patients with an apparently healthy liver, the diagnosis is usually late and the patient

## HCC in liver cirrhosis, 2015

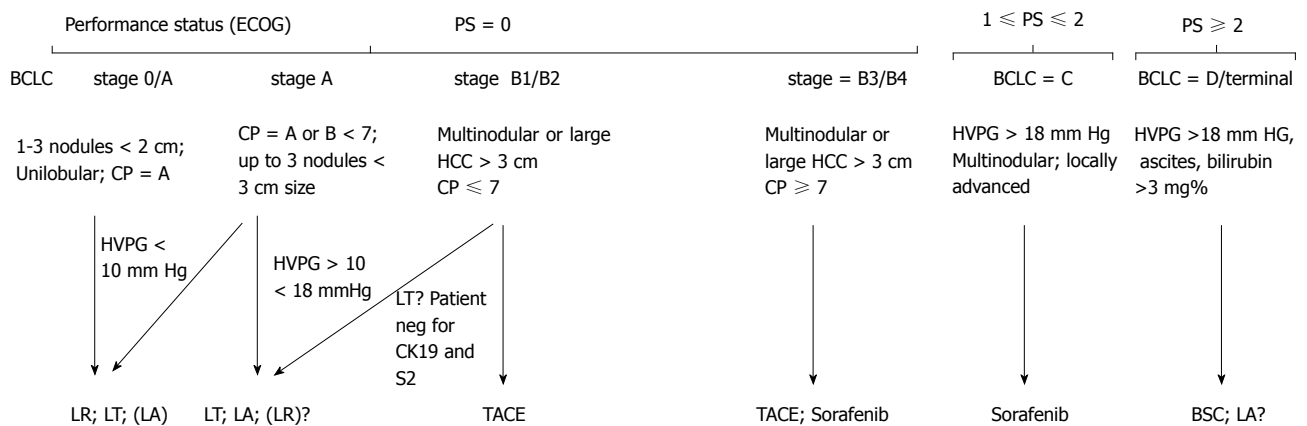


**Figure 1 Staging and possible strategy to treat patients with hcc and liver cirrhosis.** PS: Performance status according to ECOG; BCLC: Barcelona clinic liver cancer; HVP: Hepatic vein portal gradient; BSC: Best supportive care; CP: Child-Pugh score; CK19: Cytokeratin 19; S2: Patients beyond Milan Criteria (see text).  
References<sup>[73-75,79,123]</sup>

has advanced disease.

Symptoms, when present, are those of chronic hepatitis with the addition of pain in the right superior quarter of the abdomen, increased volume, and more fatigue. If a liver disease patient presents with a change in mood, edema in the legs, swollen abdomen due to ascites, rapid occurrence or deterioration of portal hypertension with esophageal or rectal varices, hemorrhoids or bleeding, the physician should consider that HCC may be developing. Decompensation of diabetes can also be a sign of the presence of HCC. Ultrasound scan without and with liver-specific contrast should be done rapidly and followed by a CT scan with contrast medium.

Early detection of liver nodules and diagnosis of HCC is crucial to ensuring that the patient have the greatest possibility of being cured or, at least, of prolonging survival and improving quality of life. It must be remembered that only 15%-35% of those who develop HCC can undergo surgery.

A patient's prognosis strongly depends upon the condition of his/her liver at diagnosis. Because of this, several authors have tried to make a "road map" to assist in selecting the proper therapy for the right patient.

## STAGING

As in all patients with cancer, staging is crucial before deciding any treatment. In addition, since more than 90% of HCC patients have an important underlying liver disease, careful staging of it must also be performed to avoid cancer treatment that damages the liver reserve. In the last 50 years several systems have been proposed to stage liver disease with or without HCC. With others we have developed in Italy a system to estimate the prognosis of patients affected by HCC and liver cirrhosis, the Cancer of the Liver Italian Program that, according to our own results and in the opinion

of many other researchers has been very precise in defining the prognosis of patients<sup>[73]</sup>. However, the Barcelona Clinic Liver Classification (BCLC) system with few modifications is more frequently used now because it also takes into consideration the choice of treatment. The BCLC staging system divides HCC associated with liver cirrhosis into: Very early stage (0 stage), early stage (stage A), intermediate stage (stage B), advanced stage (stage C) and terminal stage (stage D) and, compared to others, has the advantage of taking into account not only tumor burden, stage of liver cirrhosis, liver function, physical condition but also the efficacy of available treatment<sup>[74]</sup>. Patients have the best treatment opportunities at Centers with vast experience who utilize the BCLC system, which has recently been updated because of stage B heterogeneity, so B stage can now be divided into four subgroups, B1, B2, B3, B4 according to Child Pugh, Milan criteria, number and size of lesions, and ECOG performance status (Figure 1)<sup>[75,76]</sup>.

## TREATMENT

The treatment of HCC is difficult and requires a multi-disciplinary approach, whereby specialists in gastroenterology, hepatology, radiology, oncology, surgery and others need to bring their expertise to provide patients with the best and most updated therapies. It is possible to differentiate treatments into two main categories: Those that are potentially curative and those that are palliative.

## POTENTIALLY CURATIVE TREATMENTS

In this category we consider three types of approach: Liver transplantation, liver resection, and liver ablation. Each of these approaches has indications and contraindications, and need to be discussed carefully and tailored to each individual patient.

Liver transplantation is widely considered the only real potentially curative approach that provides treatment

of both HCC and the underlying liver cirrhosis. Its gold standard indication is early unresectable HCC in patients with compensated liver cirrhosis or HCC with no more than 3 nodules, Stage 0 or A according to the BCLC system. Recent reports show that 70% of transplanted patients for HCC are alive after 5 years, especially when transplantation was performed using the Milan criteria<sup>[77-79]</sup>. This percentage of success is extremely good when considering that most of these patients had liver cirrhosis and HCC. Unfortunately, only a minority of patients can undergo liver transplantation even when it is indicated. The number of donors is largely insufficient to cope with the worldwide need and the shortage of organs makes waiting lists longer each day in every country, so that many patients become unsuitable for transplantation or die while awaiting transplant. Many attempts have been made to go beyond the limits of the Milan criteria and results are under scrutiny now. One large review involving 770 consecutive transplanted patients for HCC shows that performing transplantation beyond the Milan criteria attains a 57% general rate of survival, after 5 years, with a rate of recurrence of 35%<sup>[80]</sup>. However, by using tissue cytokeratin 19 (CK 19) and the sum of the size of the largest tumor size plus the number of nodules up to 7, as for S2 patients, as prognostic markers, it was possible to divide the entire group of patients in two: (1) one group of individuals who were negative for CK 19 and were not included in the S2 group; and (2) another group resulting positive or to be in the S2 group. The results were quite different and impressive: The first group had a 64% survival rate and 19% relapse compared to the other group where survival dropped to 45% with a rate of recurrence of 53%<sup>[80]</sup>.

There are several reviews that address the issue of selection of patients to insert on the waiting list for transplantation<sup>[81,82]</sup>. In this general review it is sufficient to say that patients with early or very early unresectable HCC and liver cirrhosis with good liver reserve function (Child Pugh A) (stage 0 or A according to the BCLC system) are excellent candidates for liver transplant. Those who have poor reserve of liver function or too advanced HCC, stage C of the BCLC system, are not good candidates because of the surgery risk and/or frequent and/or rapid recurrence or appearance of metastases. Finally, there is a group of heterogeneous patients, included in the intermediate stage according to the BCLC system (stage B), where the decision on whether to submit patients to transplantation must be discussed among experts with different expertise. One possible pathway is indicated in Figure 1. Patients with Stage D disease are not candidates for transplantation nor for active treatment of HCC.

There are other problems with liver transplant: One is that many candidates for transplantation have chronic HBV or HCV infections that recur after transplantation and may quite rapidly reproduce liver cirrhosis. Effective treatments have been available for HBV infection for several years, including use of interferon, lamivudine and other antiviral drugs<sup>[83-85]</sup>. Treatment is usually

effective and eradicates the infection in most. Therapy should start before surgery and be continued for several months. It is more difficult to deal with HCV infection, especially that due to HCV genotype 1 that is particularly resistant to antiviral treatment with peg-interferon and ribavirin. Relapse of chronic hepatitis and liver cirrhosis occurs rapidly and, in addition, these patients show significantly worse prognosis than others<sup>[86]</sup>. Accordingly, active HCV infection has been a reason to exclude patients from transplantation in many centers. However, major progress has been made in the last 2 years in the treatment of chronic HCV infection including that due to genotype 1, through introduction of drugs such as sofosbuvir, ledipasvir, elbasvir and others that specifically target HCV proteins<sup>[87,88]</sup>. These drugs are able to clear the virus in most HCV patients though bitter discussion is in progress in several countries over the cost of these drugs and on their generalized use. It is too early to ascertain whether these new antiviral drugs have solved the problem. It must be kept in mind that HCV infects several sanctuaries of the body and since transplanted patients are chronically treated with immunosuppressive drugs, it is not possible to exclude later relapse of infection. Moreover, reactivation of HBV infection has been reported in patients on treatment with these new anti-HCV drugs and the issue is still open to possible surprises<sup>[89]</sup>.

To overcome the shortage of donors and to cut - waiting list time for recipients of a new liver it has been proposed to split the available deceased-donor livers (DDLT) or to take part of the liver from live donors (LDLT) (usually a relative of the patient). The results appear good according to what has been published especially in Asia, without any statistically significant increase in recurrence of HCC as compared to traditional cadaveric donors<sup>[90-93]</sup>. Complications of liver surgery of live donors make this option still debatable especially considering a mortality rate that ranges from 0.1% to 0.3% of the donor<sup>[93,94]</sup>.

What should be done to stop HCC progression while patients are on the transplant waiting list? The ideal approach would destroy the neoplastic tissue as much as possible without damaging the rest of the liver, without reducing functional reserve and, most importantly, without increasing the number of circulating liver cancer cells that cause metastases. Radiofrequency ablation (RFA) that causes almost full coagulative necrosis of the nodule in a single shot might be the best procedure, at least for small lesions. However, the use of trans-hepatic artery embolization (TAE) or trans-hepatic artery chemo-embolization (TACE) (see later) that theoretically deprive HCC of artery blood flow, necrotizing most of the tumor while allowing the presence of other neoplastic nodules to be assessed during the same procedure, are generally used. Many papers published on this issue show that TACE provides good results and increases overall survival compared to transplantation alone or TACE alone<sup>[95]</sup>. However, there are no controlled randomized studies comparing

TACE and RFA as neoadjuvant treatment prior to liver transplant. In addition, many centers are now using a different technique of TACE that uses beads eluting anticancer drugs or ionizing microspheres charged with Yttrium-99 to embolize the artery that feeds the tumor. Results are similar in terms of overall survival, although bead-TACE seems to produce fewer side effects than traditional TACE<sup>[95-98]</sup>. Bead-TACE also appears useful in downstaging HCC or in maintaining it at a steady stage while patients are awaiting transplant<sup>[99,100]</sup>.

### Liver resection

Liver resection is the other surgical option for treatment of HCC<sup>[101,102]</sup>. Liver surgery in patients with cirrhosis or chronic liver disease requires expert surgeons and teams trained to work together in these patients, who may develop liver failure after large resection. In addition, recurrence of HCC within 3 years is quite common (> 50%) even after the introduction of ultrasound during surgery. Other procedures have been proposed to improve the outcome of patients with HCC and liver cirrhosis who undergo liver resection. The adoption of the Milan criteria used for liver transplantation and NMR with gadoteric acid-enhanced MRI during surgery can improve detection of small lesions by 16% and makes surgery more precise and radical<sup>[103,104]</sup>.

Liver resection has some advantages over transplantation (organ saving, costs, expertise, etc.) and in addition leaves open the possibility of later transplantation, thus allowing many patients to gain time. Unfortunately, liver resection does not treat the underlying cirrhosis that is a pre-neoplastic lesion and it can be too risky for patients with advanced HCC and/or liver disease (stage C according to the BCLC). Even very selective resection can cause liver decompensation and/or gastrointestinal bleeding, especially in patients with high values (>10 mmHg) of portal hypertension and gastro-esophageal varices. This limitation has been disputed recently as it excludes too many patients (about one-fourth) who could benefit from surgery from effective treatment<sup>[105]</sup>.

Liver resection in patients with BCLC Stage B can be done quite safely by avoiding to operate those with serum bilirubin over 2 mg/dL and/or ascites. In selected patients, resection leads to an overall survival > 85% at 5 years, making this type of surgery the best option for treating early or very early HCC in well compensated liver cirrhosis (Child Pugh A).

### Liver ablation

Liver ablation performed using different techniques can be used in patients where surgery is not possible or too risky. Ablative techniques performed by experts in selected patients can allow survival comparable to transplantation even if more recent data have questioned this conclusion<sup>[106-112]</sup>. Of the ablative techniques, Percutaneous ethanol injection (PEI) was the first to be used in patients with small lesion(s) (< 3 cm diameter) HCC. Excellent results have been observed with recurrence rates at 5

years similar to resection. PEI consists in injecting ethanol inside the tumor mass to cause coagulative necrosis of all neoplastic nodules. PEI has been the most successful technique in treating small (less than 3 cm diameter) HCC nodules. It is easy, inexpensive, requires minimal equipment and expertise, and has been shown to prolong survival of these patients so much as to be comparable to surgery. PEI is so convenient and easy that it could be done at the patient's home even in poor countries where health systems are weak. Ethanol destroys cancer tissue and diffuses well in the cirrhotic liver, and the procedure can be repeated several times on the same nodule or on new ones. The side effects are minimal, no general anesthesia is required and the procedure can be performed in an ambulatory situation. The use of this simple and inexpensive procedure has shown that it is possible to prolong survival of many patients and allow them to maintain an excellent quality of life<sup>[106-112]</sup>. PEI can be used in patients with more than one nodule, usually no more than 3, and because it can easily be repeated on the already treated nodule, offers patients a simple and inexpensive treatment of their disease.

The ablative technique obtained by PEI has been improved by using RFA that can destroy cancer tissue in only one step. This procedure, more complex and expensive than PEI, has the advantage that it can treat cancer lesions with a larger diameter than PEI can (usually < 5 cm diameter) and many of them with one single application. Because of the pain involved in the procedure, RFA is more commonly performed with the patient under light anesthesia. Side effects occur more commonly than with PEI and but are rarely severe.

A comparison between PEI and RFA shows that when patients are carefully selected, the results in terms of overall survival are similar, though disease recurrence can be observed more frequently in patients treated by PEI<sup>[113,114]</sup>.

Ablation of the tumor can be obtained by other means, such as acetic acid or laser, but what is crucial is the expertise of the operator and good patient selection.

## PALLIATIVE TREATMENTS

As mentioned earlier, most HCC patients cannot undergo surgery and so, for them, especially those with locally advanced disease, the most effective and safe treatment is that which aims to destroy the most cancer tissue without affecting liver reserve function.

The procedures that can be used are PEI, RFA, TAE, TACE, in association or not with different forms of chemotherapy.

PEI and RFA are so easy and safe that they can be performed even in patients with advanced stage of liver cirrhosis (stage C according to the BCLC system). The main risks are infection and bleeding in patients with severe defects in blood coagulation (pro-thrombin time < 60% and platelet counts < 60000/mL<sup>3</sup>). The risk of diffusing cancer cells along the needle passage is real,

but in our experience is a rare event.

In several patients where surgery is not indicated, TAE and TACE can be employed to embolize the artery feeding the HCC. Patients for this procedure must be selected very carefully and the individual performing the procedure needs to be expert (usually a radiologist) in superselective embolization<sup>[114,115]</sup>. Necrosis of the tumor is rarely complete because of tumor angiogenesis that forms bunches of new small vessels that feed the cancer nodule. In addition, in many cases significant blood supply comes from portal blood and the liver cancer cells are ischemia resistant<sup>[116,117]</sup>. Nevertheless, the growth of the tumor may be impaired for weeks, making TACE or TAE the most commonly performed pre-transplantation procedure and the most used technique in HCC of large dimension and/or with too many nodules to be ablated.

Several authors have reported TACE prolonging survival also in patients with intermediate or advanced HCC. Considering that there are no other possible procedures in many patients, chemoembolization must be regarded as palliative but also active treatment<sup>[118,119]</sup>.

Recently, two retrospective works have compared liver resection and TACE in patients with BCLC stage B. In a Chinese study, resection appears as safe as TACE and provides better overall survival at 3 years (59% vs 29%) and 5 years (37% vs 14%) with a highly statistically significant difference ( $P < 0.001$ )<sup>[119]</sup>. In the other study, from Spain, the authors analyzed their results according to the revised BCLC classification system that divides stage B into 4 substages (B1, B2, B3 and B4) on the basis of Child Pugh score, inclusion in the Milan criteria and furthermore, size and number of lesions under or over 7, and Performance Status (P.S.) according to ECOG. They found that liver resection provides particularly good results in the B1 group (Child Pugh score 5-7, within Milan Criteria and  $< 7$ , and P.S. = 0) with an overall survival of 62.9% after 5 years and a recurrence rate of 25% as opposed to a recurrence rate of 60% in the TACE group ( $P < 0.018$ ). B3 (Child Pugh score = 7, beyond Milan criteria or  $> 7$  and P.S. = 0) or B4 (Child Pugh score 8 or 9, any Milan criteria or number, P.S.  $> 0$ ) patients had the worst prognosis after 5 years (15.4% as overall survival). These authors concluded that modern surgical techniques and good selection of patients with liver cirrhosis and HCC in BCLC stages B1 or B2 (Child Pugh 5 or 6, beyond Milan Criteria but number  $< 7$ , P.S. = 0) according to the new BCLC system can provide particularly good results with liver resection in terms of survival and recurrence rate compared to similar patients treated with TACE<sup>[120]</sup>.

In the last decade, the use of radio-labeled microspheres or embolizing beads that carry anticancer agents such as doxorubicin or cisplatin has been proposed to treat advanced HCC. The results have been encouraging and similar to those obtained by traditional TACE or TAE in terms of anticancer activity. Side effects appear less frequent and of minor gravity, making this new BEAD-TACE procedure one of the most favored in many

centers<sup>[121-124]</sup>.

Since all these treatments must be considered palliative and locally active, to improve their efficacy, it has been proposed to associate them with systemic chemotherapy.

## CHEMOTHERAPY

In intermediate or in selected advanced HCC patients who cannot be treated by ablative or embolizing techniques or where HCC is already extrahepatic, the only systemic chemotherapy that has been proved to be effective is sorafenib<sup>[125-127]</sup>. This drug (800 mg/d, orally) is a tyrosine kinase activity inhibitor that acts mainly as antiangiogenic to slow down the growth of the tumor. The response rate is not particularly high (about 4%) and toxicity may be relevant. Sorafenib has been described as decompensating liver cirrhosis, and then its dosage must be reduced to 600 mg/d or 400 mg/d. In a few cases therapy must be withdrawn and it is possible to reintroduce it at a lower dosage once liver function has recovered. Sorafenib can be too toxic in patients with advanced liver cirrhosis, provoking side effects that include mucositis, skin toxicity, fatigue, bleeding and liver failure<sup>[128,129]</sup>. It should be considered that drug resistance to sorafenib and other inhibitors of tyrosine-kinase activity has been reported<sup>[130]</sup>. It is also possible to try to reverse drug resistance by using other drugs that interfere with mechanisms of drug resistance<sup>[131]</sup>. Agents such as sunitinib, erlotinib, and many others have been proposed but toxicity or drug resistance have limited their use and they have been abandoned so far for the treatment of HCC.

Recently, as already mentioned when talking about the biology of HCC, and following experimental data showing the importance of the cMET pathway in HCC, a phase II randomized study was carried out using tivantinib, a specific inhibitor of the cMET, as second-line treatment after sorafenib. The results of this study show that especially in HCC showing MET expression tivantinib is active and significantly slows tumor growth. The authors conclude that a phase III study is warranted to see the real impact of this new drug in advanced HCC<sup>[55,56]</sup>.

Several authors have tried to improve the efficacy of these palliative treatments by associating sorafenib with TACE, TAE, ablation, or surgery<sup>[132-135]</sup>. Sorafenib has also been used as adjuvant chemotherapy in patients after complete resection of HCC to prevent relapse or as neoadjuvant chemotherapy before liver transplantation or resection. Though some encouraging preliminary results have emerged, none of these studies have shown clear advantages and must be regarded as experimental protocols which need to be repeated as controlled and much larger studies<sup>[136]</sup>.

Many years ago we performed a pilot study (phase II) by using traditional intrahepatic artery chemotherapy using 5-fluorouracil in patients who were not suitable for surgery or ablative techniques or

after relapse of HCC. This drug was well tolerated and our results, in terms of tumor response and survival, were positive compared with historic controls and in line with what others have recently shown, but the lack of randomized controls and small number of patients enrolled strongly limited the overall significance of this study<sup>[137]</sup>.

In conclusion, HCC is at present one of the most challenging cancers in clinical practice<sup>[138]</sup>. Though our understanding of its risk factors and how it develops have improved in the last decade, so much remains to be clarified. Currently the best way to significantly reduce the death rate remains prevention of HBV and HCV infection and alcoholism.

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## MicroRNA-regulated viral vectors for gene therapy

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### Abstract

Safe and effective gene therapy approaches require targeted tissue-specific transfer of a therapeutic transgene.

Besides traditional approaches, such as transcriptional and transductional targeting, microRNA-dependent post-transcriptional suppression of transgene expression has been emerging as powerful new technology to increase the specificity of vector-mediated transgene expression. MicroRNAs are small non-coding RNAs and often expressed in a tissue-, lineage-, activation- or differentiation-specific pattern. They typically regulate gene expression by binding to imperfectly complementary sequences in the 3' untranslated region (UTR) of the mRNA. To control exogenous transgene expression, tandem repeats of artificial microRNA target sites are usually incorporated into the 3' UTR of the transgene expression cassette, leading to subsequent degradation of transgene mRNA in cells expressing the corresponding microRNA. This targeting strategy, first shown for lentiviral vectors in antigen presenting cells, has now been used for tissue-specific expression of vector-encoded therapeutic transgenes, to reduce immune response against the transgene, to control virus tropism for oncolytic virotherapy, to increase safety of live attenuated virus vaccines and to identify and select cell subsets for pluripotent stem cell therapies, respectively. This review provides an introduction into the technical mechanism underlying microRNA-regulation, highlights new developments in this field and gives an overview of applications of microRNA-regulated viral vectors for cardiac, suicide gene cancer and hematopoietic stem cell therapy, as well as for treatment of neurological and eye diseases.

**Key words:** MicroRNA; MicroRNA regulation; MicroRNA target sites; Viral vectors; Adeno-associated virus; RNA interference; Gene therapy; Vector targeting

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**Core tip:** Post-transcriptional microRNA-induced suppression of gene expression is a simple new, highly efficient technology to restrict transgene expression to a specific tissue. It is based on the insertion of a target sequence for a cell-specifically expressed microRNA, typically into the 3' untranslated region of a transgene expression

cassette. MicroRNA-induced regulation can result in an up to 100-fold reduction of transgene expression in tissues where expression is not desired. This targeting strategy can be used in combination with other targeting strategies to further improve vector specificity for gene therapeutic approaches.

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## INTRODUCTION

Tissue-specific targeting of viral vectors is a key requirement for safe and efficient gene therapy. However, many viral vectors transduce a broad range of cell types. Improvement of specificity can be achieved by direct vector application into a defined location within the organ. But in general, transgene delivery by this means remains near the injection site and the application can lead to tissue injury, making this procedure unsuitable for many applications<sup>[1,2]</sup>. Other targeting strategies focus on improvement of target specificity after systemic vector application. In this regard, several approaches of transductional targeting have been successfully employed, including switching the virus serotype and capsid engineering *via* directed evolution, creation of vectors with chimeric or mosaic structures and insertion of antibodies or bi-specific fusion proteins containing the targeting ligands<sup>[3-5]</sup>. However, being based on the various natural entry mechanisms of infection of many viruses, this strategy has limitations. Transcriptional target-specific expression of the transgene using tissue-specific promoters represents another frequently used approach to improve vector specificity. Although this targeting strategy has been used successfully in many applications, it is limited by the small number of promoters whose activity is exclusively restricted to the target tissue and sufficiently strong to induce transgene expression at therapeutic levels<sup>[6,7]</sup>. Based on discovery of the RNA interference mechanism enabling post-transcriptional suppression of gene expression<sup>[8]</sup>, microRNA-dependent suppression of transgene expression has been emerging as a promising new approach to improve vector targeting. Since its initial application for reduction of off-target transgene expression in antigen presenting cells (APC)<sup>[9]</sup>, microRNA-dependent control of transgene expression has been used to restrict transgene expression in gene therapeutic approaches, to control oncolytic viruses, for live attenuated virus vaccine development and basic research. In this review we will provide an overview of this technology, its application in gene therapy and discuss perspectives for its further development.

## MICRORNAS - IMPORTANT PLAYERS IN CELLULAR GENE REGULATION

MicroRNAs are small endogenous non-coding RNA sequences of approximately 21 bp that post-transcriptionally regulate gene expression of about more than 60% of human protein-coding genes<sup>[10,11]</sup>. They are involved in most of cellular processes, including development, differentiation, proliferation and apoptosis<sup>[12-16]</sup>. MicroRNAs are highly conserved between species and expressed specifically and at certain levels dependent on tissue, lineage or differentiation state<sup>[17]</sup>. More than 2500 unique mature human microRNAs have been identified so far (<http://www.mirbase.org/>). Individual microRNA species can vary widely in copy number ranging from less than 10 to more than 30000 copies per cell<sup>[18-20]</sup>. Beside the tissue-specific expression profile, several microRNAs are dysregulated in cancer<sup>[21,22]</sup>, infectious diseases<sup>[23]</sup> or diseases of the heart<sup>[24]</sup> and liver<sup>[25]</sup>, which gives them potential as targets for new therapies.

MicroRNAs are usually processed from a precursor molecule (pri-microRNA) that folds into hairpin structures with imperfectly base-paired stems. Pri-microRNAs are further processed by nuclear and cytoplasmatic cleavage proteins, resulting in a short RNA duplex (see references for greater detail<sup>[10,26]</sup>). One strand of the duplex, the guide strand (microRNA), is selected based on the relative free energies of the microRNA duplex ends<sup>[27,28]</sup> and is loaded into a multi enzyme complex, the RNA-induced silencing complex (RISC). The less common product is defined as the passenger strand (microRNA\*), and is assumed to be degraded<sup>[26]</sup>. Alternatively both strands of the RNA duplex, namely the 5' strand (miR-5p) and the 3' strand (miR-3p) become mature functional microRNAs<sup>[29-33]</sup>. The mature microRNA is associated with an Argonaute (AGO) family protein, that constitutes the core of the RISC, and functions by base-paired binding to the corresponding target site located in the mRNA, resulting in repression of protein synthesis<sup>[10]</sup>. However, some microRNAs are able to activate mRNA translation<sup>[34-38]</sup>. Complete complementarity of microRNA and its target site leads to endonucleolytic central cleavage of the microRNA/mRNA-duplex by AGO2<sup>[26,39-43]</sup>, using a mechanism similar to RNA-interference mediated by siRNAs. In plants, this mechanism is the predominant one<sup>[44]</sup>, while the prevalent mechanism in animals involves binding with incomplete complementarity<sup>[12]</sup>, resulting in inhibition of translation and/or initiation of mRNA degradation<sup>[10,39,45-50]</sup>. In contrast to plants, whose microRNA target sites are mostly located in protein-coding regions, target sites in animals are often found as repeats in the 3' UTR of the mRNA<sup>[51-54]</sup>.

Some important rules relating to the interaction between microRNA and its target site were determined by experimental and bioinformatic analysis. The mRNA targeting specificity of a microRNA is determined by perfect match between the seed sequence, a conserved

sequence which is usually situated at positions 2-7 or 2-8 of the 5'-end of the microRNA, with a corresponding sequence in the mRNA<sup>[12,51-54]</sup>. An adenine opposite microRNA position 1 and an adenine or uracil opposite microRNA position 9 are not essential, but increase the efficacy of binding<sup>[54]</sup>. MicroRNAs exhibiting the same seed sequence belong to the same microRNA family and can regulate the same mRNA targets<sup>[51]</sup>. A single microRNA species can regulate the production of hundreds of proteins, most likely by recognizing the same seed-matched sequence in the mRNA<sup>[55]</sup>. A second common characteristic of endogenous microRNA-mRNA interaction comprises nucleotide mismatches in microRNA positions 9-12, thereby most likely preventing an AGO2-mediated cleavage of the target mRNA<sup>[10,56]</sup>. A third characteristic is that matches within the seed region alone are not always sufficient to induce gene repression; stabilization of microRNA binding may need additional complementarity in the 3' part of the microRNA. In particular if seed matching is suboptimal, nucleotides at microRNA positions 13-16 become important<sup>[51,57]</sup>. Thus the 3' portion can help to compensate for a single nucleotide mismatch in the seed region, as experimentally confirmed for let-7 sites in *Caenorhabditis elegans* lin-4<sup>[58]</sup> and for miR-196 site in mammalian Hoxb8<sup>[43]</sup> mRNA. Additional factors can influence binding stability and thereby the efficacy of microRNA-mediated gene regulation<sup>[54,57,59,60]</sup>. Thus a more effective repression can result from an AU-rich nucleotide composition near the target site<sup>[57]</sup>. Additionally, more than 15 nucleotides between miR-TS and the stop codon may reduce competition between proteins involved in translation and microRNA-mediated silencing, respectively<sup>[57]</sup>.

## DISCUSSION

### **Engineering, optimization and limits of artificial target sites for microRNA-mediated post-transcriptional transgene silencing**

Endogenously expressed microRNAs can also be used to specifically modulate the expression of an exogenously applied nucleic acid as a therapeutic cDNA. Therefore artificial microRNA target sites, referred to as miR-TS in this review, serve as targets for a specific microRNA, resulting in post-transcriptional silencing of the transcript<sup>[9]</sup> (Figure 1). In contrast to transcriptional targeting using tissue-specific promoters that positively regulate transgene expression, expression is negatively controlled by tissue-specifically expressed microRNAs. It was shown that insertion of miR-TS completely complementary to the microRNA in an arrangement of multiple tandem repeats of miR-TS results in strong repression of transgene expression in cells with corresponding microRNA expression<sup>[9,17,61]</sup>. In this case the transcript is endonucleolytically cleaved, similar to degradation by siRNAs, and the microRNA-RISC is rapidly recycled<sup>[39]</sup>. Usually the microRNA guide strand mediates transgene repression, thus a corresponding sequence representing

the miR-TS is inserted into the transgene expression cassette. However, recently Kim *et al.*<sup>[62]</sup> discovered that expression of a therapeutic transgene can also be regulated by the passenger strand of a microRNA (miR-122) linked to the transgene, thereby eliminating the risk of affecting expression of endogenous microRNA guide strand-regulated genes.

There are several factors influencing the efficacy of microRNA-regulated transgene expression systems. First the cell type- and tissue-specific expression of a chosen microRNA candidate is an important factor that must be taken into account for microRNA-regulated transgene expression systems. Many suitable microRNAs fulfilling this requirement have already been defined (please see reference<sup>[63,64]</sup>). Among them miR-122, a microRNA, almost exclusively expressed in liver tissue, represents the best candidate for a specific hepatocyte-de-targeting<sup>[20,65,66]</sup>. Indeed, successful miR-122-mediated suppression of transgene expression in liver was found in several studies<sup>[67-69]</sup>. However, specific microRNA expression may not be sufficient to achieve a stringent microRNA-dependent transgene repression.

Second, the expression level of a microRNA is crucial for microRNA-mediated regulation. High-throughput assays revealed that only the most abundant microRNAs within a cell mediate significant target suppression<sup>[70]</sup>. Consequently a fraction of microRNAs that is highly and specifically expressed in certain tissues, possibly involved in defining and maintaining tissue identity<sup>[71]</sup>, are the most potent to de-target transgene expression. In general more highly expressed microRNAs repress their targets to a greater degree<sup>[67]</sup>. However, the relationship between microRNA expression and its repression activity is nonlinear; in one case it was shown that a 10-fold increase of microRNA concentration results only in a 10% increase of repression<sup>[72]</sup>. In addition, microRNA expression level is not the only determinant of repressive activity and repression capacity can differ among microRNAs. Kozomara *et al.*<sup>[72]</sup> showed that although some microRNAs exhibit similar expression levels, they mediate very different repression levels. Conversely some microRNAs repress their targets to similar degrees despite of large difference in expression levels. Another key factor for microRNA-mediated repression of a miR-TS-containing transcript is the need for a certain minimal microRNA expression threshold, quantified as 100 copies/pg small RNA by Brown *et al.*<sup>[73]</sup>. The value is not only important because it defines the minimal amount which is necessary to suppress transgene repression but it also indicates that under a certain level, microRNAs are unable to induce repression. This consideration becomes important if the chosen microRNA is expressed in both the tissue where transgene expression is intended as well as in the tissue where it is not. Therefore microRNAs expressed under the minimal expression limit may not affect transgene expression in the targeted tissue.

Third, the configuration of the miR-TS is another important factor affecting repression efficacy. Corre-

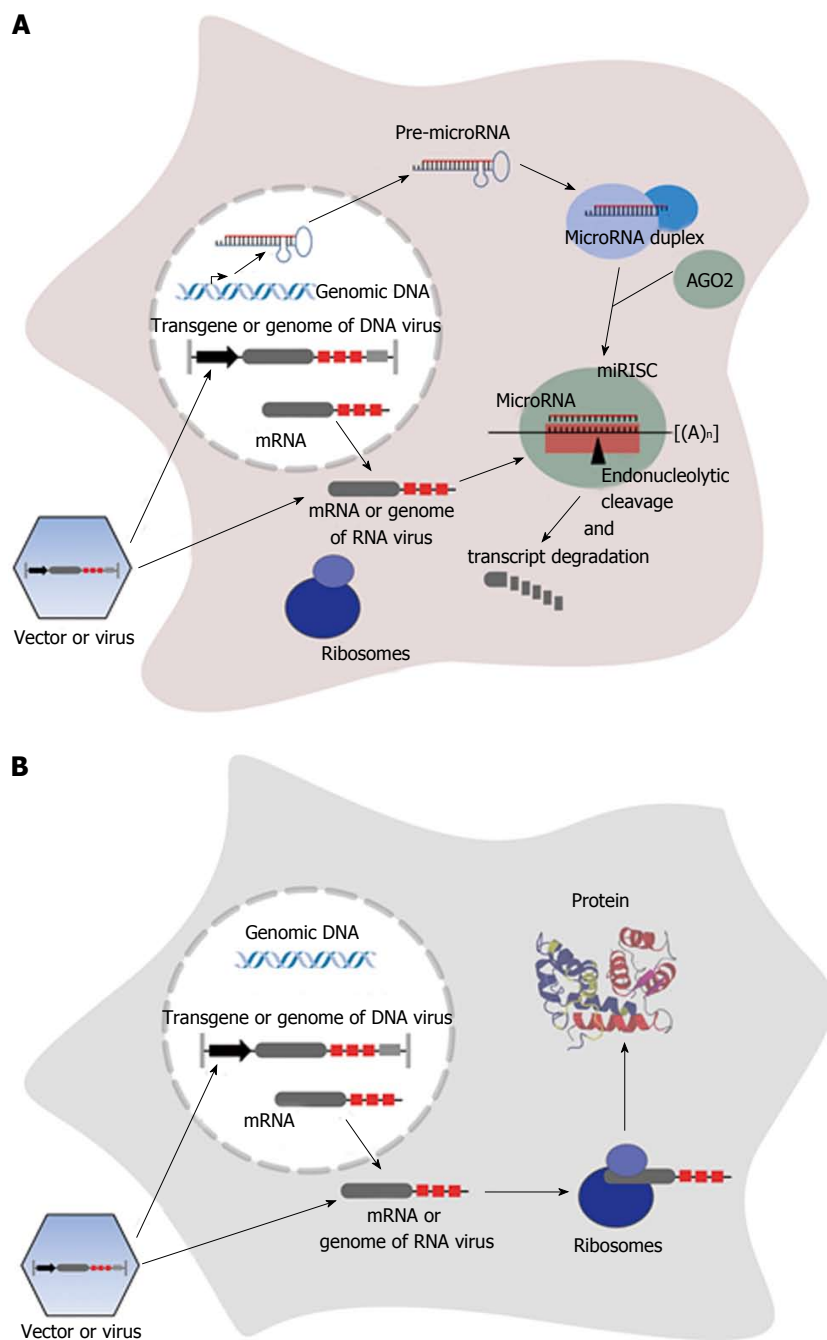


**Figure 1 Principle of microRNA-mediated suppression of transgene expression and viral replication.** After transduction/infection of a cell, vector DNA/viral DNA is transcribed in the nucleus (for lentiviral vectors integrated into host DNA). The transcript/viral RNA containing artificial miR-TS (red boxes) is transported into the cytoplasm. If corresponding microRNA is expressed (A), it binds to miR-TS and the target RNA is endonucleolytically cleaved and degraded. If certain microRNAs are not expressed (B), the RNA is translated into protein. The protein shown in the three-dimensional structure is dimeric S100A1<sup>[180]</sup>. AGO: Argonaute; miRISC: MicroRNA-induced silencing complex.

sponding to the different mechanism of microRNA-induced gene silencing, complete complementary miR-TS allow *per se* higher suppression of the transgene than imperfectly complementary sequences<sup>[33,61,73-75]</sup>. As perfectly complementary target sequences are endonucleolytically cleaved between microRNA positions 10/11, the microRNA is rapidly recycled<sup>[39,69,76-78]</sup>. Consequently complete complementarity reduces the risk of microRNA saturation and thus induction of undesirable side effects because the bioavailability of the cognate

microRNA to regulate its natural targets is maintained<sup>[79]</sup>.

In addition, the number of miR-TS repeats affects microRNA-mediated suppression of transgene expression. In general an increased number of miR-TS enhances microRNA-dependent transgene repression. But repression efficacy does not linearly correlate with an increasing number of miR-TS, and above a certain number of miR-TS, only a relatively modest increase of repression of transgene expression is observed<sup>[67,73,80]</sup>. Thus maximal suppression of transgene expression of

viral vectors, even under optimal conditions (highly and specifically expressed microRNA, use of tandem miR-TS repeats), rarely exceeds 100-fold<sup>[9,61,67,68,81]</sup>. The reason for this limitation is currently not fully understood, but different factors may be involved. It seems that the rate-limiting steps in microRNA activity are the level and dynamics of association of the target mRNA with the microRNA-loaded RISC, rather than level of target transgene mRNA<sup>[17,72,82]</sup>, which is directly dependent of vector and expression systems. We and others have shown that 3-4 identical repeats of miR-TS are sufficient for efficient transgene repression (Table 1). An increase to 6 or even 12 copies is possible, but may only have a marginal effect on repression efficacy<sup>[67,80,83]</sup>. Moreover, an increase of miR-TS repeats can induce saturation of the respective microRNA, as more target sites means more substrate for the microRNA to bind, resulting in potentially undesirable off-target effects<sup>[76]</sup>. As shown by several studies, different miR-TS can be inserted in series in order to de-target various cell types with different microRNA expression profiles<sup>[17,61,69,84]</sup>. On the other hand, a combination of miR-TS corresponding to different microRNAs for a given cell type or tissue can enhance microRNA-mediated transgene suppression, especially if the microRNAs are only expressed at moderate levels<sup>[85]</sup>. Moreover, employing cooperative microRNAs may reduce the risk saturating the function of one microRNA<sup>[67]</sup>. An enhancement of microRNA-mediated suppression of transgene expression can also be achieved by combination of microRNA regulation with other regulation systems. Thus, Bennett *et al.*<sup>[86]</sup> developed an adenoviral vector (AdV) system utilizing both microRNA-mediated regulation and the Cre recombinase (Cre)-loxP recombination system to further reduce hepatic transgene expression and to achieve relatively pure spleen-/dendritic cell-specific expression thereby improving AdV-based vaccination against transgene products. Although insertion of miR-122TS into the transgene expression cassette of AdV suppressed liver expression by about 100-fold, significant transgene expression levels were still found in the liver<sup>[86,87]</sup>. Therefore they engineered an AdV carrying the transgene flanked by loxP sites and containing miR-122TS, while another AdV expressed Cre equipped with miR-TS for miR-142-3p, which is highly expressed in the spleen. Intravenous co-administration of the AdVs resulted in an approximately 3800-fold reduction of hepatic transgene expression and a slightly reduced, but substantial, transgene expression in the marginal zone of the spleen, which was sufficient to exhibit therapeutic value<sup>[86]</sup>. In most studies, small 4 to 6 nucleotide long spacer sequences have been used to separate miR-TS repeats<sup>[9,61,67,68]</sup>. Introduction of spacers may, in general, reduce steric hindrance of enzyme complexes binding to microRNA/miR-TS duplexes and thus facilitate better repression. Nevertheless spacer-free multimeric miR-TS were also successfully applied for transgene repression<sup>[69]</sup>. Furthermore it is thought that, compared to longer spacers, insertion of shorter spacers might reduce the risk of formation of secondary structures

around the miR-TS that might disturb base-pairing of microRNA with the miR-TS<sup>[88,89]</sup>. Thus whether and to what degree spacer sequences affect efficacy of designed microRNA-regulated suppression systems remains to be elucidated. Besides the functional importance of applied miR-TS, copy number of miR-TS, as well as spacing between miR-TS, are relevant to viral vector construction, as it directly enlarges the transgene expression cassette inserted into the vector genome. As miR-TS are small in size (about 22 bp), insertion of tandem repeats of miR-TS, including spacer sequences, generally does not constitute a capacity problem for most viral vectors. However, for some viral vectors with low packaging capacity, such as self-complementary adeno-associated virus (AAV) vectors<sup>[90]</sup>, keeping down the total length of miR-TS could be an essential requirement. In this regard, our group investigated whether shortening of miR-TS corresponding to miR-122 affects the miR-122-mediated repression function. Indeed, we found that a deletion of up to 5 nucleotides from the 5' end of the miR-122TS was well tolerated and did not influence transgene suppression<sup>[67]</sup>. However, more studies are necessary to confirm our results for other miR-TS.

Although a systematic investigation defining common standards for insertion of miR-TS into a transgene expression system is currently not available, location of the miR-TS in the mRNA is obviously important for repression efficacy. Based on knowledge of microRNA target recognition and microRNA-induced cleavage of cellular targets, it becomes obvious that secondary structures of the mRNA itself or structures formed by miR-TS insertion can also affect transgene suppression as accessibility to the target mRNA is hindered<sup>[78,91]</sup>. Since the 3' UTR of mRNAs almost always lack secondary RNA structure<sup>[92]</sup>, miR-TS can be placed there, usually near the stop codon, but insertion within the 5' UTR or the open reading frame is also possible<sup>[93-96]</sup>. Other factors influencing repression capacity of a given microRNA include increased nuclear localization of a microRNA, its stability<sup>[97]</sup> and the stability of the microRNA/miR-TS duplex<sup>[70,72]</sup>. Concerning the latter, analysis of the free energy of multiple duplexes revealed a weak negative correlation of duplex stability and microRNA-mediated repression. Therefore lower stability seems to favor transient interaction with target mRNAs and efficient down-regulation of multiple targets<sup>[39,72]</sup>.

In summary, optimal microRNA-mediated repression of the vector-encoded transgene needs high expression of the microRNA, insertion of 3-4 copies of miR-TS with complete complementarity, uniqueness with regard to regulation by other microRNA candidates and insertion within a site with low secondary structure. All these aspects need to be pre-evaluated for optimal microRNA-mediated regulation of transgene expression of viral vectors.

Although this technology displays many advantages compared to other methods, it is not without concerns. Given that endogenous microRNAs are involved in target regulation, miR-TS containing transgene mRNAs

**Table 1** Application of microRNA-regulated vectors for gene therapy

| Target cell/tissue for therapy            | miR-TS for  | Number miR-TS | Vector             | MicroRNA-regulated transgene                                      | De-targeted cell/tissue                                     | Ref.            |
|-------------------------------------------|-------------|---------------|--------------------|-------------------------------------------------------------------|-------------------------------------------------------------|-----------------|
| Heart                                     |             |               |                    |                                                                   |                                                             |                 |
| Heart                                     | miR-122     | 3, 5          | AAV9               | EGFP, lacZ, luciferase                                            | Liver                                                       | [67,68]         |
| Heart                                     | miR-122     | 3             | AAV9               | Human S100A1                                                      | Liver, skeletal muscle                                      | [61]            |
|                                           | miR-206     | 3             |                    |                                                                   |                                                             |                 |
| Cancer                                    |             |               |                    |                                                                   |                                                             |                 |
| PyMT mouse breast cancer                  | miR-1       | 4             | AAV9-ESGLSQS       | Herpes simplex virus thymidinkinase (and ganciclovir), luciferase | Heart                                                       | [129]           |
| Metastatic hepatocellular carcinoma model | miR-122     | 4             | AAV8               | Herpes simplex virus thymidinkinase (and ganciclovir), luciferase | Hepatocytes/liver                                           | [128]           |
| Glioblastoma cells <i>in vitro</i>        | miR-128     | 2             | Lentiviral         | Herpes simplex virus thymidinkinase (and ganciclovir)             | Neuronal differentiated cells                               | [127]           |
| Subcutaneous fibrosarcoma xenografts      | miR-122     | 4             | Adenoviral         | Luciferase                                                        | Hepatocytes/liver                                           | [181]           |
| Intradermal melanoma tumor                | miR-122     | 4             | Adenoviral         | Herpes simplex virus thymidinkinase                               | Hepatocytes/liver                                           | [87]            |
| Human glioma xenografts                   | miR-31      | 3             | Baculoviral        | Herpes simplex virus thymidinkinase (and ganciclovir)             | Astrocytes/brain                                            | [85]            |
|                                           | miR-127     | 3             |                    |                                                                   |                                                             |                 |
|                                           | miR-143     | 3             |                    |                                                                   |                                                             |                 |
| Hepatocellular carcinoma model            | miR-181     | 2             | Adenoviral         | Human telomerase reverse transcriptase RNA-targeting ribozyme     | Hematopoietic stem cell- and progenitor-derived blood cells | [130]           |
|                                           | miR-122     | 3             |                    |                                                                   | Hepatocytes/liver                                           | [131]           |
| HSC and iPSC                              |             |               |                    |                                                                   |                                                             |                 |
| Tumor-infiltrating monocytes/macrophages  | miR-126     | 2             | Lentiviral         | Interferon- $\alpha$                                              | Hematopoietic stem and progenitor cells                     | [134]           |
|                                           | miR-130a    | 2             |                    |                                                                   |                                                             |                 |
| Differentiated myeloid cells              | miR-126     | 2             | Lentiviral         | Gp91 <sup>phox</sup>                                              | Hematopoietic stem and progenitor cells                     | [135]           |
|                                           |             |               |                    |                                                                   |                                                             |                 |
| Differentiated hematopoietic cells        | miR-126     | 4             | Lentiviral         | Galactocerebrosidase                                              | Hematopoietic stem and progenitor cells                     | [133]           |
|                                           | miR-130a    | 4             |                    |                                                                   |                                                             |                 |
| Lymphoid progenitors, myeloid cells       | miR-150     | 4             | Lentiviral         | GFP                                                               | Differentiated T and B lymphocytes                          | [132]           |
| Post-thymic T cells                       | miR-181a    | 4             | Lentiviral         | GFP, inhibition of negative thymic selection                      | Developing T cells                                          | [182]           |
|                                           |             |               |                    |                                                                   |                                                             |                 |
| Myeloid cells                             | miR-223     | 4             | Lentiviral         | EGFP, NGFR                                                        | Granulocytes and monocytes, mature dendritic cells          | [17]            |
| Immature dendritic cells                  | miR-155     | 4             |                    |                                                                   |                                                             |                 |
|                                           |             |               |                    |                                                                   |                                                             |                 |
| Human fetal fibroblasts                   | miR-302a, d | 4             | Lentiviral         | EGFP, mCherry                                                     | Human ES, neural progenitors                                | [137]           |
|                                           | miR-142-3p  | 4             |                    |                                                                   |                                                             |                 |
|                                           | miR-155     | 4             |                    |                                                                   |                                                             |                 |
|                                           | miR-223     | 4             |                    |                                                                   |                                                             |                 |
| ES, iPSC                                  | miR-292-3p  | 4             | Lentiviral         | GFP, differentiation of ES to neural progenitors                  | Pluripotent cells                                           | [138]           |
|                                           |             |               |                    |                                                                   |                                                             |                 |
| ES, iPSC                                  | let-7a      | 4             | Lentiviral         | GFP                                                               | Non pluripotent cells                                       | [136]           |
| CNS and eye                               |             |               |                    |                                                                   |                                                             |                 |
| CNS                                       | miR-1       | 3             | AAV9               | lacZ, EGFP                                                        | Heart, liver                                                | [69]            |
|                                           | miR-122     | 3             |                    |                                                                   |                                                             |                 |
| CNS/astrocytes                            | miR-124     | 4             | Lentiviral         | lacZ, glial glutamate transporter                                 | Neurons                                                     | [141]           |
| CNS/cortical excitatory neurons           | miR-128     | 4, 8, 12      | Lentiviral         | EGFP                                                              | Cortical inhibitory neurons                                 | [83]            |
|                                           | miR-221     | 4, 8          |                    |                                                                   |                                                             |                 |
| Retina/eye                                | miR-124     | 4             | AAV5               | EGFP                                                              | Photoreceptors/retinal pigment epithelium                   | [143]           |
|                                           | miR-204     | 4             | AAV8               |                                                                   |                                                             |                 |
| Photoreceptor/eye                         | miR-181c    | 4             | AAV2 (quadY-F+T-V) | GFP                                                               | Ganglion cells and inner retina                             | [144]           |
|                                           |             |               |                    |                                                                   |                                                             |                 |
| Immune system and other applications      |             |               |                    |                                                                   |                                                             |                 |
| Skeletal muscle                           | miR-142-3p  | 2, 4, 8       | AAV1               | Ovalbumin human sarcoglycan                                       | Antigen presenting cells                                    | [152,153]       |
| Hepatocytes/liver                         | miR-142-3p  | 4             | Lentiviral         | Factor IX, VIII, induction immunologic tolerance                  | Antigen presenting cells                                    | [9,145,147,183] |
|                                           |             |               |                    |                                                                   |                                                             |                 |
| Dendritic cells/spleen                    | miR-142-3p  | 4             | Adenoviral         | Luciferase, lacZ, combination with Cre-loxP system                | Hepatocytes/liver                                           | [86]            |
|                                           | miR-122     | 4             |                    |                                                                   |                                                             |                 |
| Neural stem cells                         | miR-124a    | 4             | Lentiviral         | GFP, identification of neurons                                    | Neurons                                                     | [184]           |
| Adipose tissue                            | miR-122     | 3, 8          | AAV8               | Human leptin                                                      | Hepatocytes/liver                                           | [185]           |
| Intramuscular delivery                    | miR-122     | 6             | AAV8               | 201Ig immunoadhesin, luciferase                                   | Liver, skeletal muscle                                      | [186]           |
|                                           | miR-206     | 6             |                    |                                                                   |                                                             |                 |

|                        |          |   |                                   |                                                                     |                       |       |
|------------------------|----------|---|-----------------------------------|---------------------------------------------------------------------|-----------------------|-------|
| Human fibroblasts HFL1 | miR-124  | 4 | Lentiviral, replication deficient | Neural conversion genes <i>Ascl1</i> , <i>Brn2</i> and <i>Myt1L</i> | Human induced neurons | [179] |
| Skeletal muscle        | miR-208a | 2 | AAV9                              | Human calpain3                                                      | Heart                 | [81]  |

miR-TS: Artificial microRNA target sites; HSC: Hematopoietic stem cell; CNS: Central nervous system; AAV: Adeno-associated virus; GFP: Green fluorescent protein; EGFP: Enhanced green fluorescent protein; iPSC: Induced pluripotent stem cells; NGFR: Nerve growth factor receptor; ES: Embryonic stem cells; HFL1: Human fetal lung fibroblast 1.

are in competition with cellular target mRNAs. By the use of complete complementary miR-TS, enabling rapid microRNA recycling, and defining an optimal target site number, the risk of microRNA saturation can be minimized. We and others showed that endogenous microRNA profile or microRNA-regulated genes were not altered after administration of optimized miR-TS-bearing vectors<sup>[61,67,69,81]</sup>.

### Application of microRNA-regulated transgene expression systems

Several promising results using microRNA-dependent regulation have been obtained for different disease models and vector systems (Table 1).

### Application to cardiac gene therapy

AAV vectors have been established as the leading vector type for myocardial gene transfer in preclinical and clinical applications<sup>[98]</sup>. Their main advantage is their ability to transduce cardiomyocytes *in vivo* at high efficiency. In addition, AAV vectors allow long lasting expression of encoded transgenes and do not trigger a strong immune response or inflammation<sup>[99-102]</sup>. Importantly AAV vector-driven expression is performed without integration of the vector genome into the host genome<sup>[103]</sup> which, in consequence, reduces the risk of long term, irreversible side effects<sup>[104-106]</sup>. As conventional AAV vectors containing capsid proteins of serotype 2 (AAV2) revealed a natural cardiac tropism, their transduction efficiency was limited. Therefore discovery of naturally occurring AAV serotypes carrying variations in the amino acid sequence of the capsid protein<sup>[107,108]</sup> enabled the development of pseudotyped AAV vectors and led to substantial improvement of cardiac gene transfer. AAV9 vectors are the most cardiotropic serotype upon systemic vector administration for transgene delivery into the heart of rodents<sup>[109-115]</sup>, whereas AAV6 vectors seems to be superior to other serotypes in large animals<sup>[116]</sup>. In humans, a clinical phase 2b trial showed promising results upon intracoronary administration of an AAV1 vector expressing the calcium regulatory protein SERCA2a in patients with advanced heart failure<sup>[117]</sup>. However, improved cardiac transduction by serotype switch did not abrogate the intrinsic property of AAV resulting in sustained transduction of a broad range of tissues, in particular liver and skeletal muscle.

Transcriptional targeting represents another approach to increase cardiac specificity of AAV vectors. To restrict AAV vector-mediated transgene expression to the heart upon intravenous injection, a variety of cardiac-specific

promoters have been investigated. Promoters of the heavy chain (MHC)<sup>[118-120]</sup> and the light chain (MLC)<sup>[121,122]</sup> of cardiac protein myosin are most potent to transcriptionally mediate heart-specific transgene expression. However, endogenous cardiac-specific promoters are too large to be packaged into AAV vectors and their core elements alone provide only weak transgene expression. Smaller hybrid promoters consisting of a cardiac core element and another strong enhancer element increased cardiac expression<sup>[109,122]</sup> but transgene expression was also detected in other tissues, especially in the liver, skeletal muscle and pancreas<sup>[61,122]</sup>. At this point it seems unlikely that promoter-only approaches will sufficiently target the vectors, therefore microRNA-dependent regulation of AAV vector-mediated transgene expression has shifted into focus to further improve cardiac specificity of AAV vectors (Figure 2). Two studies carried out by Qiao *et al.*<sup>[68]</sup> and Geisler *et al.*<sup>[67]</sup> addressed microRNA-mediated suppression of transgene expression in liver after systemic AAV9 vector application. Among three microRNA candidates (miR-122, miR-192 and miR-148a) that have been described to be selectively expressed in liver<sup>[65,123]</sup>, we found that miR-122 was most abundantly expressed in murine liver, whereas expression in murine heart was approximately 5 orders of magnitude lower. Systemic application of AAV9 vectors containing miR-122TS in the 3' UTR of an EGFP reporter confirmed effectiveness of miR-122-mediated suppression in the liver, whereas cardiac expression remained unaffected. Interestingly, microRNA-mediated transgene suppression in the liver was by far more efficient than transcriptional control by the en-CMV-MLC0.26 cardiac hybrid promoter<sup>[67]</sup>. The other study carried out by Qiao *et al.*<sup>[68]</sup> confirmed improved specificity of miR-122-regulated AAV9 vectors. Similar to our study, they reported that microRNA-mediated increase of cardiac specific expression was even more efficient than transcriptional control of transgene expression by an en-MHC-TNT hybrid promoter.

Abundant transgene expression upon systemic AAV9 vector application can also be found in the skeletal muscle. Therefore transgene suppression in this tissue is necessary to further enhance the cardio-specificity of AAV vector-mediated gene transfer. According to the guidelines for microRNA selection (see above), only miR-206 fulfills the major requirements of microRNA-mediated repression: It is highly expressed in the skeletal muscle and absent in the heart. However, we found that a transgene containing miR-206TS was strongly suppressed in both the skeletal muscle and heart of mice

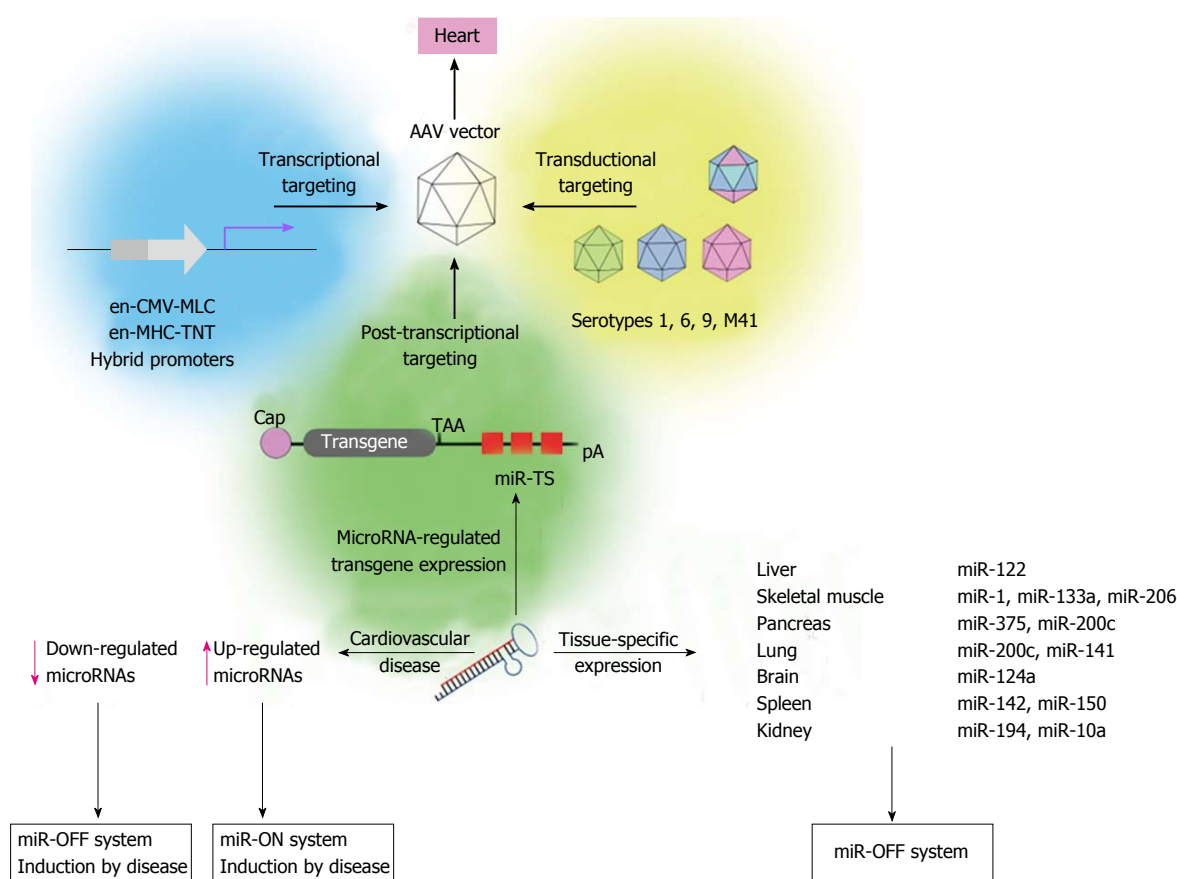


**Figure 2 Adeno-associated virus vector-based microRNA targeting strategies to improve cardiac-specific gene therapy.** Cardiac-specific gene transfer of AAV vectors can be obtained by different targeting strategies, including transcriptional targeting using cardiac hybrid promoters<sup>[68,122]</sup>, transductional targeting<sup>[109-113,187,188]</sup> or microRNA-regulated post-transcriptional transgene suppression. miR-OFF system (right site): Expression of miR-TS-bearing transgenes can be selectively suppressed in non-cardiac tissue by microRNAs, which are expressed in these tissues, but not in the heart. For the miR-OFF system (left site): If microRNAs are selectively down-regulated in cardiovascular diseases, transgene expression may be restricted to these cells by use of miR-TS corresponding to down-regulated microRNAs. If microRNAs are up-regulated in cardiovascular diseases, corresponding miR-TS can be used in the miR-ON system<sup>[178]</sup> for regulation of a repressor protein to restrict transgene expression to respective cells. Shown microRNAs are specifically expressed in mouse tissues (unpublished). AAV: Adeno-associated virus; pA: Polyadenylation site; CMV: Cytomegalovirus; MLC: Myosin light chain; MHC: Myosin heavy chain; TNT: Troponin T.

following systemic application of AAV9 vector. The reason for this is binding of the miR-1, which shows high sequence homology to miR-206 and is highly expressed in heart tissue. By introducing single nucleotide substitutions into the seed region of the microRNA/miR-206TS duplex, we generated mutated miR-206TS that became resistant to miR-1 regulation but remained fully sensitive to miR-206<sup>[61]</sup> (Figure 3). This was a result of compensatory effects induced by perfect complementarity of the 3' portion of miR-206 to mutated miR-206TS. *In vivo* transgene expression of AAV9 vectors bearing mutated miR-206TS and miR-122TS was strongly suppressed in both skeletal muscle and the liver, whereas expression in heart remained unaffected<sup>[61]</sup>. Interestingly, contrary to the generally applicable concept of miR-TS recognition and repression by microRNAs, we observed that insertion of single nucleotide mutations within miR-206TS can even enhance miR-206-mediated transgene repression. Although the underlying mechanism remains to be elucidated, we observed similar results for miR-122TS repression but not for other modified miR-TS (data not published).

### Application to suicide gene cancer therapy

Regulation of transgene expression by endogenous microRNAs has been exploited for vector-mediated suicide gene therapy with Herpes Simplex Virus thymidine kinase (HSV-tk). Expression of the HSV-tk mediates phosphorylation of the prodrug ganciclovir (GCV), thus inhibiting DNA replication in rapidly dividing cancer cells. As phosphorylated GCV is also toxic in normal cells<sup>[124]</sup>, tumor cell-specific expression of HSV-tk is required. AdVs are widely used in cancer gene therapy. However, they exhibit strong hepatotropism after systemic application and can induce severe hepatotoxicity after HSV-tk delivery<sup>[125,126]</sup>. Repression of HSV-tk containing miR-122TS induced by miR-122 distinctly reduced hepatotoxicity upon local delivery by Adv, without altering the antitumor effects, as miR-122 is weakly expressed in transduced melanoma tumor cells but highly expressed in hepatocytes<sup>[87]</sup>. In another approach, a lentiviral vector (LV) expressing HSV-tk in combination with ganciclovir showed improved tumor specificity *in vitro* by miR-128-mediated transgene regulation, a microRNA that is differentially expressed

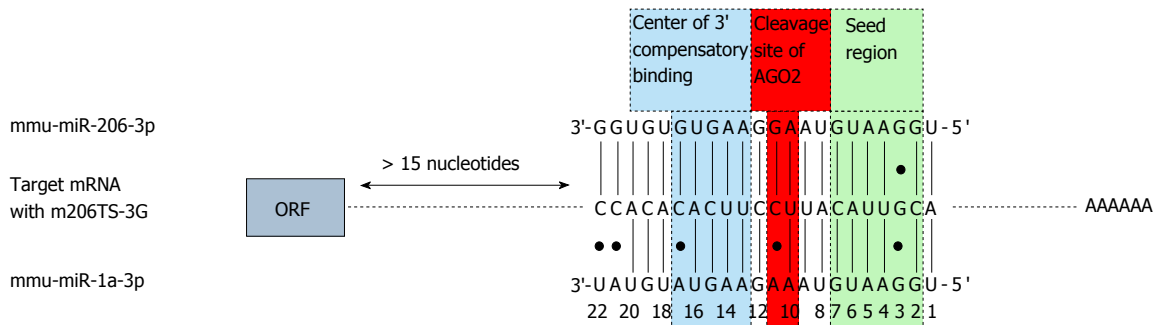


**Figure 3** Sequence alignment of murine miR-206, miR-1 and mutated miR-206TS, referred to as m206TS-3G. The last of these sequences contains a C to G transition compared to miR-206TS at microRNA nucleotide position 3. Vertical dashes, Watson-Crick pairing; dots, mismatches; green boxed capital letters, seed region. Potential cleavage site of AGO2 between nucleotide 10 and 11 is boxed in red. The center of the potential 3' compensatory microRNA binding is boxed in blue. Nucleotide positions are numbered according to microRNA sequence. ORF: Open reading frame; AGO: Argonaute.

between glioblastoma cells and normal brain tissue<sup>[127]</sup>. Other studies confirmed the reduction of side effects by microRNA-dependent suppression of suicide genes in cancer gene therapy approaches. Wu *et al.*<sup>[85]</sup> found that in addition to transcriptional control by a glioma-specific promoter, insertion of miR-TS for the miR-31, miR-127 and miR-143 into baculoviral vectors led to repression of HSV-tk in normal neural cells. As these microRNAs are more highly expressed in these cells than in glioma cells, this approach led to reduced HSV-tk/GCV induced cytotoxicity. More recently, Della Peruta *et al.*<sup>[128]</sup> improved tumor specificity of systemically applied AAV8 vectors expressing HSV-tk linked with miR-122TS in combination with a liver-specific promoter in a syngeneic metastatic murine hepatocellular carcinoma (HCC) model. Administration of the vector and GCV treatment resulted in a reduction of cancer growth and number of metastases without liver toxicity. Moreover Trepel *et al.*<sup>[129]</sup> used miR-TS of the miR-1 to inhibit cytotoxicity in heart after HSV-tk delivery with an AAV9 vector capsid variant. Animals treated with the vector were protected from cardiac HSV-tk expression and drug-induced development of dilative cardiomyopathy, whereas delivery of the suicide gene to tumors significantly inhibited tumor growth after GCV treatment. Another study recently carried out by Won *et al.*<sup>[130]</sup> improved specificity of cancer gene therapy by developing microRNA-regulated trans-splicing ribozyme that targets human telomerase reverse transcriptase (hTERT) RNA in cancer cells. The group I intron-based ribozyme targets and cleaves its substrate RNA and trans-splices an exon attached at its 3' end (e.g., a therapeutic RNA) onto the cleaved target RNA, resulting in expression of the therapeutic RNA and repression of substrate RNA. As the ribozyme specifically targets hTERT RNA positive cancer cells, but also hematopoietic stem cell-derived blood cells, the ribozyme was modified by inserting target sites for the blood cell-specific miR-181a downstream of its 3' exon. Analysis of AdV-mediated expression of the hTERT-targeting trans-splicing ribozyme harboring miR-181aTS with HSV-tk gene as a 3' exon under control of a liver-specific promoter and GCV treatment resulted in specific anticancer effects. Moreover systemic vector

application in an orthotopic multifocal HCC mouse model demonstrated a regression of liver tumor nodules and tumor volume, with minimal hepatotoxicity<sup>[130]</sup>. In a similar approach, AdV-mediated administration of the hTERT-targeting trans-splicing ribozyme containing miR-122TS resulted in efficient anti-cancer effects and reduced hepatotoxicity<sup>[131]</sup>.

#### Application to hematopoietic and pluripotent stem cell therapy

For hematopoietic stem cell (HSC) therapy, lineage- and differentiation stage-specific microRNA expression can be used to specifically express a transgene in a discrete subset of progeny. Although integrating vectors, such as LVs, mediate long-term expression in progenitor cells, expression needs to be often restricted to their differentiated progeny. Therefore Brown *et al.*<sup>[17]</sup> showed that miR-223 de-targets transgene expression in the myeloid progeny of HSC (granulocytes and monocytes), thus restricting expression to the lymphoid progeny. Similarly, transgenes were equipped with miR-TS for miR-150 and miR-155, thus repressing their expression in mature T and B cells and mature dendritic cells, but not in lymphoid progenitors or immature dendritic cells, respectively<sup>[17,132]</sup>. On the other hand, microRNA-regulation technology also worked if the expression of a toxic transgene needs to be avoided in hematopoietic pluripotent stem/progenitors cells (HSPC). In this regard it has been shown that miR-126 and miR-130a, both expressed in the HSPC compartment, but not in mature blood cells, suppressed LV-mediated transgene expression of galactocerebrosidase containing the corresponding miR-TS in HSC, allowing long-term hematopoiesis after vector transduction and a stable bone marrow graft in mice. Moreover a robust expression of galactocerebrosidase in mature hematopoietic cells enabled successful treatment of globoid cell leukodystrophy in a mouse model<sup>[133]</sup>. In a similar approach Escobar *et al.*<sup>[134]</sup> targeted interferon- $\alpha$  expression to tumor-infiltrating monocytes/macrophages using a combination of transcriptional and miR-126-/miR-130a-mediated control during post-transplant hematopoiesis to limit HSC exposure to the transgene thereby inhi-

biting breast cancer progression. Furthermore in an approach using therapeutic LV encoding gp91<sup>phox</sup> linked with miR-126TS under the control of a myeloid-specific promoter, high levels of myeloid-specific transgene expression was achieved for X-linked chronic granulomatous disease therapy, entirely sparing the miR-126 positive CD34<sup>+</sup> HSC compartment. As ectopic gp91<sup>phox</sup> expression in HSC could cause production of reactive oxygen species that may damage DNA, alter cell growth or induce apoptosis, the transcriptionally and post-transcriptionally regulated LV reduced off-target expression and effectively corrected the X-phenotype in gp91<sup>phox</sup>-deficient mice<sup>[135]</sup>.

MicroRNA-regulated reporter expression systems can also be used to track the differentiation of stem cells and have been widely disseminated in the field of pluripotent stem cell technology (induced pluripotent stem cells, embryonic stem cells)<sup>[17,136-138]</sup>. A big challenge in this field is the *in vitro* generation of a homogenous cell population of a pre-defined lineage which can be successfully transplanted. Therefore cell type- and differentiation-specific microRNAs are helpful to identify and select cells with desired differentiation fates. Upon differentiation, cells can be selected either by combining miR-TS with a positive or negative regulator such as neomycin or thymidine kinase<sup>[17,136]</sup> or by combining with a reporter, such as GFP, and sorting by fluorescent activated cell sorting (FACS)<sup>[138]</sup>, respectively.

#### **Application to central nervous system and eye**

Gene therapy holds promise for treating of genetic as well as metabolic disorders of the central nervous system (CNS) and in particular AAVs have received growing interest as viral vector systems for gene delivery in the last few years. Besides the well-known generally favorable properties of AAV vectors, several AAV serotypes are able to cross the blood-brain barrier and thus effect an efficient transduction of cells of the CNS with systemic vector application<sup>[139]</sup>. Based on the broad range of tissue targeting by AAV9 vectors, Xie *et al.*<sup>[69]</sup> exploited miR-TS technology to restrict AAV9 vectors to the CNS. Therefore they constructed AAV9 vectors with miR-TS completely complementary to hepatocyte-specific miR-122 and muscle-specific miR-1 in order to repress reporter gene expression in liver, heart and the skeletal muscle. *In vivo* comparison of vectors, each bearing one or three miR-122TS or/and miR-1TS, found that the number of miR-TS repeats inversely correlated with reporter expression levels in tissues where the corresponding microRNA is expressed. Moreover systemic application of the microRNA-regulated AAV9 vector resulted in distinct suppression of the transgene in the liver and muscle, whereas it was not altered in the brain. Most vector systems transducing the CNS have a strong neurotropism. However, for several neurodegenerative disorders, certain cell types of the CNS, such as astrocytes, need to be specifically targeted, as they exhibit important physiological functions and may display altered functions. Among CNS-specific

microRNAs, miR-124 is specifically expressed in neurons but absent in astrocytes<sup>[140]</sup>. Accordingly, Colin *et al.*<sup>[141]</sup> demonstrated that insertion of miR-124TS into a LV is sufficient to repress transgene expression in neuronal cells *in vitro* and *in vivo*, whereas expression remained unaffected in astrocytes. Moreover, exploiting microRNA regulation enables more than targeting transgene expression to neurons or supporting cells. Thus Sayeg *et al.*<sup>[83]</sup> showed that a combinatory regulation of a locally applied LV by miR-128 and miR-221 targeted transgene expression between neuronal subtypes in the murine neocortex as expression was selectively inhibited in excitatory neurons but not in inhibitory cells.

Inherent retinal degeneration of the eye results from impaired function of the retinal pigment epithelium (RPE) or photoreceptor (PR) cells and leads to severe visual deficits and blindness<sup>[142]</sup>. In an approach to restrict AAV-vector-mediated EGFP reporter gene expression to either RPE or PRs, Karali *et al.*<sup>[143]</sup> subretinally applied miR-TS-bearing AAV5 vectors into the eye of mice and pigs. AAV vectors containing miR-204TS were selectively repressed in RPE, which was in agreement with the miR-204 expression in RPE. In contrast the EGFP reporter was repressed in PRs but not in RPE if the vector was equipped with miR-TS for miR-124, a microRNA that is expressed in PRs but absent in RPE. For advanced retinal gene therapy, Kay *et al.*<sup>[144]</sup> recently developed an AAV2 vector capsid mutant with a strongly increased transduction efficacy for PRs upon intravitreal vector delivery. Furthermore, transgene expression was restricted to PRs only by use of a combinatorial approach of a PR-specific promoter and incorporation of multiple target sites for miR-181c, a microRNA which is expressed in retinal ganglion cells and the inner retina.

#### **Application to reduce transgene-directed immunogenicity**

The concept of microRNA-mediated negative regulation of transgene expression was first shown by Brown *et al.*<sup>[9]</sup> in order to reduce off-target transgene expression in APC upon systemic injection of LV. By combining a hepatocytic-specific promoter and target sites for the hematopoietic-lineage specific miR-142-3p, a reduced transgene expression in APCs allowed a long-term expression of clotting factor F.IX for stable correction of hemophilia B mice upon systemic LV administration<sup>[145,146]</sup>. Furthermore it was shown that this combined approach induced an immunological tolerance for the transgene<sup>[147-149]</sup> resulting from hepatocyte-directed expression<sup>[147,150]</sup>. Further studies confirmed these results for other vector systems. Intramuscular application of AAV vectors leads to appearance of immune responses against the transgene and rapid decrease of transgene expression, possibly as a result of vector transduction of APC<sup>[151]</sup>. Thus Majowicz *et al.*<sup>[152]</sup> and Boisgerault *et al.*<sup>[153]</sup> inserted target sites for miR-142-3p into the transgene expression cassette of AAV1 vectors. Upon intramuscular application they observed prolonged expression of the transgene in the skeletal muscle, which correlated with

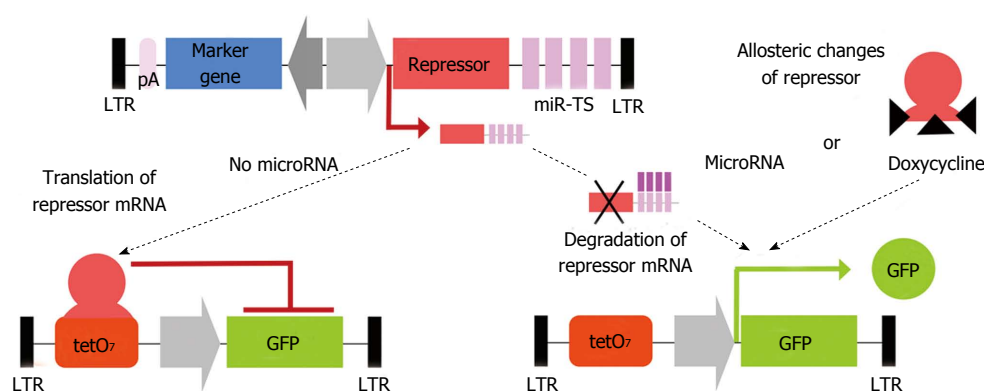


**Figure 4 Schematic of the miR-ON system using lentiviral vectors in the indicated conditions.** Two independent lentiviral vectors containing a repressor protein and a GFP reporter with operator sequences for the repressor protein, respectively. The repressor protein, either tetracycline repressor (TR) or tTR-Kruppel-associated box, constitutively binds to the tetO sequence, thereby suppressing reporter gene expression. If the repressor mRNA containing miR-TS is not translated into native protein due to microRNA-mediated degradation of the repressor mRNA or is unable to bind tetO sequence because of doxycycline-induced allosteric changes of the repressor, GFP expression is switched on. GFP expression is inhibited if the repressor is translated due to lack of an inhibiting microRNA, thereby allowing the repressor to bind to the operator sequence. Arrows indicate orientation of transcription. Modified according to [178]. LTR: Long terminal repeats; miR-TS: Artificial microRNA target sites; pA: Polyadenylation site; tetO7: Seven tandemly arranged tetracycline operator sequences; GFP: Green fluorescent protein.

reduced immunogenicity to the transgene.

### Oncovirotherapy and vaccines

Besides the development of microRNA-regulated viral vectors in order to de-target transgene expression in certain tissues, lineages or differentiation states or to identify cell subsets, modification of tropism of replication competent oncolytic viruses (OV) has gained growing interest. Although OVs preferentially replicate in tumor cells, application to cancer gene therapy is limited by their replication and corresponding induction of toxicity in normal tissues. By insertion of miR-TS for tissue-specific microRNAs into the viral genome, microRNAs have been used to reduce replication in normal cells, while maintaining their oncolytic potential in tumor cells. This was initially shown by Kelly *et al.* [154], who engineered a coxsackie A21 OV with miR-TS for the skeletal muscle-specific miR-206 and muscle-specific miR-133a, thus reducing myotoxicity while maintaining oncolytic potential. MicroRNA-regulation of OVs to increase tumor specificity has been successfully applied for several RNA [84,155-158] and DNA [80,93,159-167] viruses in the meantime. For detailed information the reader is referred to Ruiz *et al.* [168].

Another aspect that needs to be addressed is the enhancement of safety of live attenuated virus vaccines by microRNA-regulation [169]. Insertion of miR-TS into the viral genome leads to reduced primary replication in the viral target organ, whereas weaker replication in other tissues induces protective immunity of the host. This was successfully shown for poliovirus [170], influenza virus [171], flaviviruses [172-175] and alphavirus [176].

### Other applications

Activity of a given microRNA can be easily identified by coupling corresponding miR-TS to a reporter gene such as lacZ, GFP or luciferase and analysis of reporter gene expression. This allows dynamic measurement of microRNA

expression over time on a single cell basis. Because reporter gene expression is observed as microRNA-mediated shut down of expression, the absence of signal might be not distinguishable from false negative results of failed transduction or transgene silencing. By LV-mediated co-expression of the microRNA-regulated sensor reporter and a second non-microRNA-regulated reporter, both under the control of a bi-directional promoter, microRNA activity can be measured independently from vector dose, transduction level and promoter activity [9,17,133]. This principle has been applied to construct a microRNA reporter library for analysis of microRNA activity in FACS-sorted cells. This technique has many advantages, as it allows the measurement of microRNA bioactivity in heterogeneous cell populations such as HSC [133] or neural stem cells [177]. However, for microRNAs with expression levels close to the detection threshold of the reporter system, its reliability is limited and other methods are preferred. Thus recently a miR-ON system (Figure 4) was generated to identify small subpopulations of microRNA-expressing cells and to select them directly. This system includes transduction of a repressor containing miR-TS, together with a reporter under its regulation. Reporter activity is observed only if the upstream repressor mRNA is degraded and cells expressing corresponding microRNA become selectable by reporter gene expression [178].

Furthermore microRNA-mediated regulation of transgene expression can help to improve direct cell conversion as shown for conversion of human fibroblasts into functional neurons (human induced neurons) by a self-regulating vector. Insertion of miR-TS for the neuron-specific miR-124 linked to the neural reprogramming genes *Ascl1*, *Bmi1* and *Myt1L* of an integration deficient LV facilitated a down-regulation of conversion gene expression once the cell has reached a stable neuronal fate, thereby allowing for a more complete functional maturation of the cells in culture [179].

For additional applications in further fields, the reader is referred to Table 1.

## CONCLUSION

In this review we have described the features of microRNA-regulated transgene expression, including its technical mechanisms and its application to transgene delivery by viral vectors. The variety of examples showed how microRNA regulation can be diversely exploited in different contexts as vectors, routes of application or fields of research in general. Compared to other targeting strategies, including transcriptional and transductional targeting, microRNA-regulation displays many advantages. Insertion of approximately 100 bp sequence of miR-TS can be easily accomplished by conventional cloning techniques. In general the small size of miR-TS prevents interference with the packaging capacity for therapeutic genes or foreign genes, thus making it applicable for both viral vectors and viruses. This system has now become a major technique for specific application of therapeutic transgenes, for increased tumor specificity of oncolytic viruses and enhanced safety of recombinant live attenuated virus vaccines. Nevertheless each miR-TS needs to be carefully evaluated with regard to its optimal effectiveness. Moreover, as many viral vectors have a broad tissue tropism, which in general requires the use of several target sites to restrict a transgene to a specific tissue or cell type, target site optimization is required. The miR-ON system may be an alternative to overcome this disadvantage. Importantly, it may also be a platform to develop microRNA-regulated shRNA and artificial microRNA expression systems, which are currently not available.

Based on the results of the overall studies investigating potential side effects induced by microRNA-mediated transgene regulation, no major side effects have been observed to date, suggesting the mechanism is safe for therapeutic use. But there is a need to consider specific guidelines concerning the complementary between target sites and its corresponding microRNA, the number of target sites and the strength of transgene expression. In summary, microRNA regulation has been emerged as a powerful new technology to improve vector-mediated transgene expression *in vitro* and *in vivo*. It has the potential to be translated into clinical applications, thereby improving efficacy and safety of gene- and virotherapeutic approaches to the degree that their use can become more common and be applied to additional indications.

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## Comments on human eurytremiasis in Brazil

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### Abstract

Eurytremiasis is an important parasitic disease of cattle that was recently suggested to be a neglected and emerging human disease in Brazil. Based on a misinterpretation of the life cycle of the parasite, it was suggested that a great number of people could be infected with this fluke in the country. In the present letter, aspects of the life cycle of

*Eurytrema* spp. are revisited and clarified. The mechanism of transmission previously reported for the few accidental human cases involved the ingestion of raw or undercooked insects (grasshoppers and crickets) harboring the infective metacercariae. In reality, the zoonotic potential of *Eurytrema* species is extremely low, and human eurytremiasis is not, and probably never will be, a zoonotic disease in countries where entomophagy is not a common food habit.

**Key words:** *Eurytrema*; Parasite transmission; Tropical medicine; Metacercariae; Zoonosis

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**Core tip:** In the present letter, the life cycles of species of *Eurytrema*, pancreatic flukes of ruminants, are revised. Given the transmission of the parasite by the ingestion of infected insects, we suggest that human eurytremiasis is not a zoonotic disease in countries where entomophagy is not a food habit. Therefore, eurytremiasis should not be considered a neglected or emerging disease in Brazil.

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### TO THE EDITOR

Eurytremiasis is a helminthic disease caused by dicrocoeliid trematodes belonging to the genus *Eurytrema*, which includes flukes that parasitize the pancreas of ruminants in South America and Asia. In Brazil, this pancreatic disease is endemic in localities from central-western, southern and southeastern regions, where the infection prevalences of up to 75% have been reported in cattle<sup>[1-3]</sup>. Parasitism by *Eurytrema* spp. is generally subclinical; however, pathological alterations, such as

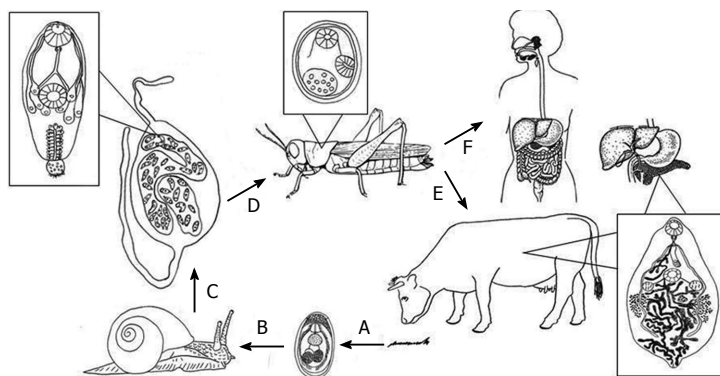


**Figure 1** The life cycle of the pancreatic fluke *Eurytrema coelomaticum*. A: Eggs containing formed miracidia are passed through the feces of definitive vertebrate hosts (ruminants) and ingested by the first intermediate host, the land snail *Bradybaena similis*; B: Miracidia hatch into the digestive tract of the snails and develop successive generations of sporocysts (mothers and daughters) that produce microcercous cercariae; C: Daughter sporocysts containing cercariae are expelled from the snail in the terrestrial environment and ingested by the second intermediate host, insects from the order Orthoptera (grasshoppers and crickets); D: Encysted metacercariae are formed inside the body cavity of the insects; E: Insects containing metacercariae are ingested by ruminants. The metacercariae are excysted in the intestine and actively migrate to the pancreas, where they grow, mature sexually and produce eggs, which are passed in the feces; F: The accidental infection of humans occurs exclusively through the ingestion of insects, which is unlikely in Brazil.

chronic interstitial pancreatitis, have been documented. Moreover, in cases of high worm burdens, chronic wasting and death of animals are verified and can be related to damage to cattle breeding<sup>[4]</sup>. The infection of humans with this pancreatic trematode, although possible, is extremely rare. In fact, only about a dozen cases of eurytrema, diagnosed by coproscopy or necropsy, were reported in China and Japan several decades ago, as reviewed by Ishii *et al.*<sup>[5]</sup>.

Recently, important aspects related to the epidemiology, diagnosis, treatment and control of eurytrema in Brazil were discussed by Schwartz *et al.*<sup>[3]</sup>. However, the authors unfortunately made some mistakes regarding the life cycle and therefore the transmission mechanisms of *Eurytrema*. Perhaps based on this incorrect information, they suggested that it is possible that a great number of people could be infected with this fluke in the country. The authors suggested, without scientific support, that the absence of reports and the probable occurrence of human eurytrema in Brazil are justified by (1) false negative results in coproparasitological tests; (2) the subclinical condition of the infection; (3) the use of serological tests in parasitological diagnosis; and (4) the existence of feeding habits (ingestion of wild watercress) in areas with high prevalence of the parasite as constituting an important risk factor for human infections<sup>[3]</sup>. Although animal eurytrema is already a well-known and admittedly important disease in the country, some corrections are necessary to demonstrate that the lack of documented human cases in Brazil is not related to the factors enumerated above but to intrinsic factors related to the life cycle of the parasite. Thus, human eurytrema is not an emerging or neglected zoonotic disease in Brazil, as discussed below.

The life cycle of species of the genus *Eurytrema* (Figure 1) take place in the terrestrial environment and involves a land snail, the Asian tramp snail (*Bradybaena similis*), as the first intermediate host. Insects, mainly grasshoppers (*Conocephalus* spp.) and crickets (*Oecanthus* spp.), are

the second intermediate hosts<sup>[6,7]</sup>. The transmission of the parasite to the definitive host occurs exclusively by the eating these insects containing infective metacercariae. In the case of ruminants, this event occurs accidentally during the act of grazing. Unlike what was presented and discussed by Schwartz *et al.*<sup>[3]</sup>, the formation of metacercariae in the external environment, including in edible plants, is not observed in the case of *Eurytrema* spp. Strangely, the transmission pattern of *Eurytrema* species discussed by Schwartz *et al.*<sup>[3]</sup> is similar to that of *Fasciola hepatica* (*F. hepatica*), a zoonotic liver fluke from ruminants, which is transmitted by the ingestion of the metacercariae present in plants or water. In fact, the figure legend describing the life cycle of *Eurytrema* presented by Schwartz *et al.*<sup>[3]</sup> cited references on the life cycle of *F. hepatica*, suggesting that the authors accidentally combined the life cycles of *Eurytrema* spp. and *F. hepatica*. However, it is already widely known that the life cycle of *Eurytrema* spp. and all other species belonging of the family Dicrocoeliidae obligatorily require second intermediate hosts (arthropods). The ingestion of wild watercress is therefore not a risk factor for human eurytrema.

Regarding the few human cases of eurytrema already diagnosed in Asia, there is no conclusive evidence on the mechanism of transmission of the parasite, although it had been suggested that transmission likely occurred through the ingestion of grasshoppers harboring metacercariae of the parasite<sup>[5]</sup>. In Brazil, there are some reports of snails and insects naturally infected with these flukes in areas associated with the occurrence of parasitism in cattle<sup>[8,9]</sup>; however, no case of human eurytrema has been reported so far. It should be noted that the use of insects, including orthopterans, as a food source is a common practice in some Asian countries<sup>[10]</sup>. Thus, the ingestion of raw insects containing the metacercariae of *Eurytrema* may be involved in the few human cases previously diagnosed in Asia. However, entomophagy is not a food habit in Brazil;

therefore, the transmission of *Eurytrema coelomaticum* is an event that is both extremely rare and unlikely. Certainly this difference in the eating habits explains the lack of human cases in the country. The possibility of the accidental ingestion of infected insects, perhaps by children or mentally ill individuals from endemic areas for cattle eurytremiasis, cannot be completely excluded. Given the remote chance for transmission of this fluke to humans, there are no reasons for the eurytremiasis to be considered an emerging or neglected disease in the context of tropical medicine and public health. However, the importance of the parasite in veterinary medicine should be emphasized, and the new initiatives presented by Schwartz *et al.*<sup>[3]</sup> for experimental studies on antiparasitic drugs, immunological aspects and pathological alteration related to eurytremiasis in the context of veterinary medicine should be encouraged.

Although the zoonotic potential of several helminths of veterinary importance have recently gained worldwide attention<sup>[11,12]</sup>, it is important to keep in mind that there are species of helminths whose zoonotic potential is extremely low due to myriad of biological factors, including impediments related to the mechanism of transmission. The possibility of future occurrences of accidental cases of humans require constant surveillance, however it cannot be indicative of a new disease among so many that already require our attention in the area of tropical medicine.

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## Selected suitable seed cell, scaffold and growth factor could maximize the repair effect using tissue engineering method in spinal cord injury

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**Author contributions:** Ji WC designed the study and wrote the manuscript; Zhang XW guided the experiment; Qiu YS modified the grammar and guided the thesis writing.

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### Abstract

Spinal cord injury usually leads to permanent disability, which could cause a huge financial problem to the

patient. Up to now there is no effective method to treat this disease. The key of the treatment is to enable the damage zone axonal regeneration and luckily it could go through the damage zone; last a connection can be established with the target neurons. This study attempts to combine stem cell, material science and genetic modification technology together, by preparing two genes modified adipose-derived stem cells and inducing them into neuron direction; then by compositing them on the silk fibroin/chitosan scaffold and implanting them into the spinal cord injury model, seed cells can have features of neuron cells. At the same time, it could stably express the brain-derived neurotrophic factor and neurotrophin-3, both of which could produce synergistic effects, which have a positive effect on the recovery of spinal cord. The spinal cord scaffold bridges the broken end of the spinal cord and isolates with the surrounding environment, which could avoid a scar effect on the nerve regeneration and provide three-dimensional space for the seed cell growth, and at last we hope to provide a new treatment for spinal cord injury with the tissue engineering technique.

**Key words:** Tissue engineering; Seed cell; Scaffold; Growth factor; Spinal cord injury

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**Core tip:** Spinal cord injury is a thorny disease to doctors. Tissue engineering including three core contents-seed cells, scaffolds and growth factors - is a promising method to cure this disease. Selecting suitable factors and maximizing the repairing effect is very important. This article is a hypothesis to choose the suitable factor which hopes to build up a promising method to treat spinal cord injury.

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## INTRODUCTION

Spinal cord injury (SCI) is a thorny issue to clinic doctors and scientists, the incidence of which increases year by year<sup>[1]</sup>. In China, SCI incidence is nearly 60 people per million and in the United States there are 12000 new patients each year, with the estimated totally number of Americans living with a SCI nearly 259000<sup>[2,3]</sup>. This disease not only causes great harm to the patient mentally and physically, but also increases the economic burden on the society, so it is very important to develop novel strategies to treat SCI.

In the basic research, scientists focus their attention on the following methods<sup>[4-6]</sup>: (1) using drugs; (2) taking advantage of stem cells; (3) making use of growth factors; (4) inhibiting inflammatory responses and hypertrophic scars; (5) promoting the axon growth; and (6) using tissue engineering technology. Among these, the most important method is tissue engineering technology, including three core factors-seed cells, scaffolds and growth factors (Figure 1). Thus how to select suitable seed cells, scaffolds and growth factors is very important, which could maximize the repair effect.

## HYPOTHESIS

Our hypothesis is to use the tissue engineering to repair SCI in rats. Adipose-derived stem cells (ADSCs) have the characteristic of multipotential differentiation, and under certain conditions, they could differentiate into neuron cells. Constructing lentiviral vectors carried brain-derived neurotrophic factor (BDNF) and neurotrophin-3 (NT-3) gene independently; we used it to transfect neural cells induced by the ADSCs, and thus seed cells have the properties of neurons; and at the same time, it could stably express BDNF and NT-3. Making the silk fibroin/chitosan scaffolds (SFCS), seeded transfect cells on scaffolds, implanting the composite into a 2 mm spinal cord transection rat model and taking advantage the characteristic of double gene modified neurons, they could continuously express BDNF and NT-3, and the trait of tissue engineering is shown. We are looking forward to providing a new treatment for SCI which could reduce the disability rate and mortality of SCI, and offering a solid theoretical basis for the clinical treatment of SCI (Figure 2).

## EVALUATION OF THE HYPOTHESIS

### Seed cell

Seed cell is the first core factor in tissue engineering which has also restricted the development of tissue

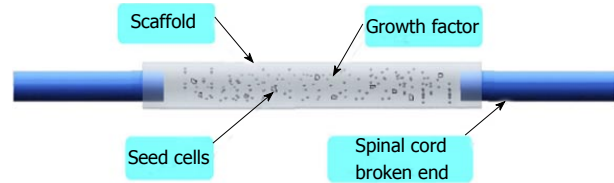


Figure 1 Scheme of using tissue engineering method to repair spinal cord injury.

engineering as a bottleneck<sup>[7]</sup>. Seed cell could be divided into non-stem cells and stem cells<sup>[8]</sup>. The former includes: Schwann cells, olfactory ensheathing cells, fibroblast, and endothelial cells; the latter is divided into two types: (1) adult stem cell: ADSCs and muscle derived stem cells (MPSCs); and (2) non-adult stem cell: Embryonic stem cell (ES), neural stem cells and bone mesenchymal stem cells (BMSCs). In the above seed cells, ES and neural stem cells have critically important usage in the basic research in the early period, but there exist some problems, such as ethical problem, reject reaction and limitation of source which could not be used widely in clinics<sup>[9]</sup>; BMSCs are widely used in basic research, but in clinical application, we need to extract the bone marrow from patients, which will cause trauma<sup>[10]</sup>. Thus more and more scientists focus their attention on the adult stem cells, which have all the above advantages and at the mean time avoid their shortcoming<sup>[11]</sup>. Although MPSCs are adult stem cells normally used in the tissue engineering, most of the experiments use them to cure heart diseases and urological diseases; and just a few studies use them to repair nerve injury; the reason is that the ability of neurogenic differentiation for MDSCs is lower than ADSCs<sup>[12-14]</sup>. So we think if we want to select a suitable adult stem cell to cure the SCI, ADSCs are better, which are first separated by Zuk *et al.*<sup>[15]</sup>. Its morphology, biological characteristics and immune phenotype is similar to BMSC, and the most advanced of this stem cell is minimally invasive for the patient<sup>[16]</sup>. Thus stem cells are better than non-stem cells, and in the stem cell, adult stem cells are better than non-adult stem cells, ADSCs are the best.

### Scaffold

Except for the seed cell, the scaffold is also very important, which could be divided into different types according to various standards. For example, according to the source of materials it could be divided into natural scaffold and synthetic scaffold; according to the ingredient, it could be divided into single ingredient scaffold and composite ingredient scaffold; according to different states, it could be divided into solid scaffold and liquid scaffold<sup>[17]</sup>. Compared to the single ingredient scaffold, the composite ingredient scaffold which includes several components is perhaps better, because it could avoid the disadvantage existing in the single ingredient scaffold and maximize the scaffold function<sup>[18]</sup>. In addition, compared to the synthetic scaffolds, the natural ingredients scaffold may be better, and the reason is that the immune reaction



**Figure 2 Technology road mapping.** SFCS: Silk fibroin/chitosan scaffolds; ADSC: Adipose-derived stem cells; NT-3-RFP: Neurotrophin-3-red fluorescent protein; BDNF: Brain-derived neurotrophic factor; LV-GFP: Lentivirus Green fluorescent protein.

and inflammation reaction is lighter after being implanted in the body<sup>[19]</sup>.

Silk fibroin was obtained from the silk degumming and a kind of natural structural albumen was used as a scaffold in tissue engineering in many experiments. For example, Parry in their research uses silk fibroin to cure spinal fusion and finds this scaffold has a good

effect<sup>[20]</sup>. But the shortcoming of this scaffold is that it is very brittle in a dry state and difficult to handle with<sup>[21]</sup>. Chitosan is another scaffold which has been researched on its biocompatibility, biodegradability, and biotoxicity for a long time, although rapid degradation and high swelling property are its problem<sup>[22]</sup>. Thus the blending of both materials to make SFCS was suggested, and the reason

is that SFCS could avoid the exclusive limitation of pure silk fibroin and chitosan, and at the mean time it has excellent mechanical properties<sup>[23]</sup>. Therefore, we think using SFCS as a scaffold to repair SCI may be a bright future direction.

### Growth factor

From the above we know that seed cells and scaffolds are very important if we want to use tissue engineering technology to cure SCI, but besides them, growth factors could not be ignored, including nerve growth factors, BDNF, NT-3, glial cell line - derived neurotrophic factors and others, among which the most important are NT-3 and BDNF<sup>[24]</sup>. BDNF was extracted from porcine brain in 1982 and has a widespread effect on central neurons, which through the activation and binding of TrkB to expresses its biological effects, it could prevent neurons death and improve the pathological state of neurons after SCI<sup>[25]</sup>. NT-3 is found in the cloning of NGF gene, which is a multifunctional gene and has the effect on maintaining the neuron and dopaminergic neuron differentiation<sup>[26]</sup>. Up to now, NT-3 is considered to be the only gene which has a clear effect that could promote the corticospinal tract growth after SCI by binding and activating the TrkC to produce its biological effect<sup>[27]</sup>. Thus we want to know whether we could use BDNF and NT-3 at the same time, which may have a synergistic effect. If we want to implement this idea, we need to resolve the new challenge-how to use them, because injecting the growth factor directly may cause it to be lost and fail to take a long effect. Using gene modified technology could solve this problem. Morizono *et al.*<sup>[28]</sup> did a comparison of efficiency among three kinds of viruses to transfect ADSCs and found that the highest transfection efficiency is in lentivirus. Thus lentivirus may be better, and another reason why to choose this virus carrier is that it has a main characteristic which could insert nearly 8 KB exogenous fragments and provide a possibility that connects two genes in the same carrier in the future research, and if using this transfect stem cell, gene expression may be higher than using two single genes to transfect at the same time.

## CONCLUSION

If our hypothesis is confirmed, we can significantly improve the repair effect on the SCI, but we clearly know that SCI will still trouble doctors and researchers for a long time in future, and this hypothesis just provides a possibility for people in future research. If we want to successfully cure the SCI and let patients recover normally after injury, there is a long way to go. All in all, with the deepening research about tissue engineering, we think this method could be used in the clinics to cure SCI at last, which promises bright future for the patients.

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*WJEM* covers topics concerning clinical laboratory medicine (applied and basic research in hematology, body fluid examination, cytomorphology, genetic diagnosis of hematological disorders, thrombosis and hemostasis, and blood typing and transfusion), biochemical examination (applied and basic research in laboratory automation and information system, biochemical methodology, and biochemical diagnostics), clinical microbiology (microbiological laboratory quality control and management; microbiological specimen collection and its influencing factors; conventional, automatic or molecular detection of clinical microorganisms; monitoring of bacterial and fungal drug resistance, drug resistance mechanisms, and rational application of antibiotics; monitoring and control of nosocomial infections), immunodiagnostics (laboratory diagnosis of infectious diseases, tumor markers and their application, laboratory diagnosis of autoimmune diseases, and immunotechnology), and clinical laboratory management (laboratory quality control and management, traceability and calibration, information management system and laboratory automation, and laboratory biosafety management).

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## Basic Study

## Lipofuscins prepared by modification of photoreceptor cells *via* glycation or lipid peroxidation show the similar phototoxicity

Alexander Dontsov, Anna Koromyslova, Mikhail Ostrovsky, Natalia Sakina

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### Abstract

#### AIM

To investigate the effect of two ways of lipofuscin production (lipid peroxidation and glycation) on lipofuscin fluorescence characteristics and phototoxicity and to compare them with the properties of natural lipofuscin.

#### METHODS

Model lipofuscins were prepared on the basis of bovine photoreceptor outer segments (POS) with bisretinoid A2E addition. One set of samples was prepared from POS modified by lipid peroxidation, while another set from POS modified by glycation with fructose. Fluorescent properties and kinetics of photoinduced superoxide generation of model lipofuscins and human retinal pigment epithelium (RPE) lipofuscin were compared. The fluorescence spectra of samples were measured at 365 nm excitation wavelength and 380-650 emission wavelength.

#### RESULTS

The fluorescence spectra of model lipofuscins are almost the same as the spectrum of natural lipofuscin. Visible light irradiation of both model lipofuscins and natural lipofuscin isolated from RPE cells leads to decrease of a fluorescence maximum at 550 nm and to appearance of a distinct, new maximum at 445-460 nm. The rate of photogeneration of reactive oxygen forms by both model lipofuscins was almost the same and approximately two times less than that of RPE lipofuscin granules.

## CONCLUSION

These data suggest that fluorescent characteristics and phototoxicity of lipofuscin granules depend only to an insignificant degree on the oxidative modification of POS proteins and lipids, and generally are defined by the bisretinoid fluorophores contained in them.

**Key words:** Model lipofuscins; Retinal pigment epithelium; Photoreceptor outer segments; Bisretinoids; Glycation; Lipid peroxidation; Superoxide

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**Core tip:** The aim of this work is to investigate the influence of different ways of protein-lipid modification of photoreceptor outer segments (POS) on the spectral characteristics and toxicity of lipofuscin. Therefore, model lipofuscins were prepared by protein-lipid modification of POS with products of lipid peroxidation or glycation reaction and the subsequent addition of the fluorophore A2E. The type of photoreceptor modification has no effect on model lipofuscins phototoxicity.

Dontsov A, Koromyslova A, Ostrovsky M, Sakina N. Lipofuscins prepared by modification of photoreceptor cells *via* glycation or lipid peroxidation show the similar phototoxicity. *World J Exp Med* 2016; 6(4): 63-71 Available from: URL: <http://www.wjgnet.com/2220-315X/full/v6/i4/63.htm> DOI: <http://dx.doi.org/10.5493/wjem.v6.i4.63>

## INTRODUCTION

Fluorescent (aging pigment) lipofuscin accumulates during lifetime and is one of the most important factors limiting the life of a cell. Lipofuscin is found in tissues of various organs such as brain, heart, liver, kidneys and skin. Especially a lot of lipofuscin accumulates in aging postmitotic tissues such as nerve and muscle. Lipofuscin is insoluble and cannot to be utilized by neither lysosomal enzymes nor proteasomal system of a cell<sup>[1]</sup>.

In the eye lipofuscin is largely accumulated in the retinal pigment epithelium cells (RPE)<sup>[2]</sup>, being one of the key biomarkers of aging and oxidative stress. It also plays an important role in the development of retinal pathologies<sup>[3]</sup>.

RPE lipofuscin granules are composed of a complex mixture of fluorophores, which determine fluorescent characteristics of a granule<sup>[4]</sup>. The most studied fluorophore of lipofuscin granules is pyridinium bisretinoid - A2E<sup>[5]</sup>.

Both lipofuscin granules and fluorophore A2E are toxic to cells. Upon irradiation with visible light, they are able to generate reactive oxygen species<sup>[6-8]</sup>, enforce damage of membrane structures<sup>[9-11]</sup>, inhibit lysosomal and proteasome degradation of proteins<sup>[12-14]</sup> and lipids<sup>[15]</sup>, as

well as to induce apoptosis of RPE cells<sup>[16,17]</sup>.

It is generally accepted that lipofuscin granules are formed as a result of incomplete digestion during phagocytosis of exhaust disks of photoreceptor outer segments (POS) in RPE cells. Inability of proteins and lipids in lipofuscin granules to be utilized is associated with the processes of oxidation and formation of intra- and inter-molecular covalent cross-linking. This modification of molecules in granules is considered to be mainly connected to reactions of lipid peroxidation that can actively pass in the retina and RPE tissues<sup>[18]</sup>. The reactive electrophilic aldehydes produced by process of lipid peroxidation, especially malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE), react readily with amino acid residues of lysine, histidine, cysteine and arginine, as well as with amino groups of phospholipids. This results in damage and disruption of their structure and catabolism<sup>[19]</sup>. Indeed, most of the proteins identified in lipofuscin from human RPE are modified in reactions with MDA and 4-HNE<sup>[19]</sup>. Proteins modified by lipid peroxidation reactions are called advanced lipoxidation end products (ALE).

Reduced susceptibility of phagolysosome proteins to proteolysis after their modification by malondialdehyde and 4-hydroxynonenal is supposed to be an important factor in lipofuscinogenesis<sup>[20]</sup>.

However, it is well known that covalently crosslinked proteins and lipids are also produced under conditions of hyperglycaemia resulting from the Maillard reaction<sup>[21]</sup>. These so-called advanced glycation end products (AGE) are believed to play an important role in the development of diabetic complications<sup>[22]</sup> and various eye pathologies<sup>[23]</sup>. AGE products were found to accumulate with age in cadaver eyes within the RPE in Bruch's membrane and drusen<sup>[24,25]</sup>. AGE accumulation leads to the reduced activity of lysosomal enzymes and, consequently, to the increased accumulation of lipofuscin<sup>[25]</sup>.

AGE products were also found directly in lipofuscin granules. Horie *et al.*<sup>[26]</sup> has first shown that lipofuscin from brain tissue of elderly people is formed not only in reactions of lipid peroxidation, but also in glycation reactions. Apparently, this can also occur in the lipofuscin granules of the RPE. For instance, AGE-modified proteins were also found in lipofuscin granules of human RPE<sup>[19]</sup>.

All in all, that suggests that during lipofuscinogenesis modification of proteins and lipids in phagolysosome of RPE cells can occur either as a result of lipid peroxidation and ALE products formation, or by glycation reactions and formation of AGE products. The later can happen if there is an increased content of reducing sugars in the cell. There is currently no information about the properties of lipofuscin granules formed under the conditions of hyperglycemia. The aim of this work is to investigate the influence of different ways of protein-lipid modification on the spectral characteristics and toxicity of lipofuscin. It is assumed that the fluorescence of RPE lipofuscin determines the fundus autofluorescence (FAF) *in vivo*<sup>[27,28]</sup>. Monitoring FAF is noninvasive diagnostic method used in detection retinal degenerative diseases such as recessive

Stargardt disease, Best macular dystrophy, age-related macular degeneration (AMD) and Diabetic Retinopathy<sup>[3]</sup>. So the model systems of lipofuscin study may be important for FAF interpretation. Therefore, model lipofuscin was prepared on the basis of POS and the fluorophore A2E. Model lipofuscin preparing was accomplished by incorporating modified POS and the fluorophore A2E. Modification of POS was carried out either by the reaction with products of lipid peroxidation (LPO-lipofuscin model), or by glycation reaction in the presence of fructose (LFF-lipofuscin model). Properties of the model lipofuscins were compared with natural lipofuscin from human eye RPE.

## MATERIALS AND METHODS

### *Isolation of lipofuscin granules from human RPE*

Cadaver eyes from 40-75 years old donors without any ophthalmic diseases were kindly provided by the Eye Tissue Bank of the Sv. Fyodorov Eye Microsurgery Institute, Moscow. Experiments on tissue isolated from human-cadaver eyes were performed in compliance with officially accepted procedures, in particular Russian Federation law N 4180-I dated 22.12.1992, "on human organs or tissue transplantation" (with modifications and additions dated 20.06.2000, 16.10.2006, 09.02.2007, and 29.11.2007); section II "removal of organs or tissue from dead bodies"; clause 8 "presumption of consent for removal of organs or tissue"; clause 10 "permission to remove organs or tissue from dead bodies". According to section II (clause 8) of this law, consent from the donor or the next of kin to use organs or tissue is not required<sup>[29]</sup>. On the basis of the Russian Federal Service on Surveillance in Healthcare (Roszdravnadzor) licenses No. 99-01-005317 dated 30.04.2008 and No. FS-99-01-008251 dated 18.02.2013, the Eye Tissue Bank located in the Sv. Fyodorov Eye Microsurgery Complex (Beskudnikovskiy bld. 59a, Moscow, Russia, 127486, [http://www.mntk.ru/mntk-moscow/Scientific-units/biology/glaznoy\\_bank/](http://www.mntk.ru/mntk-moscow/Scientific-units/biology/glaznoy_bank/)) obtains human cadaver eyes from the mortuary departments of the Moscow Forensic Medical Examination Bureau. These licenses permit the use of tissue isolated from human-cadaver eyes for transplantation and scientific research. Permission was obtained from the chief medical officer of the Sv. Fyodorov Eye Microsurgery Complex, under a scientific collaboration agreement between the Complex and the Emanuel Institute of Biochemical Physics dated 11.01.2011, to perform scientific research in the Laboratory of Physical and Chemical Bases of Vision at the Institute with RPE from cadaver eyes. Cadaver eyes, after removal of corneas for transplantation, were delivered by the Eye Tissue Bank to the Laboratory by road in a box for human organs. Cadaver eyes after isolation of RPE were returned to the Eye Tissue Bank for use, in compliance with officially accepted procedures.

Lipofuscin granules were isolated from RPE cells by a modified technique described in<sup>[30]</sup>. The obtained granules were dissolved in phosphate buffer and stored at -70 °C.

### *Isolation of POS*

POS were isolated from bovine retinas by a modified method of McDowell<sup>[31]</sup>. Obtained POS were dissolved in 0.1 mol/L potassium phosphate buffer (pH 7.6) and stored at -20 °C.

### *A2E chemical synthesis*

A2E samples were synthesized and purified by the method described in<sup>[32]</sup>. Briefly, a mixture of all-trans-retinal and ethanolamine (Sigma) in a ratio of 2.3:1 in absolute ethanol was stirred in the presence of acetic acid (1 eq.) at room temperature in the dark for 2.5 d. The mixture was then evaporated under vacuum; the pellet was dissolved in chloroform (Himmed, Russia) and chromatographed on a silica gel-chloroform column (200-400 mesh, 60 Å, Sigma). Purified A2E was dissolved in methanol (Sigma). Purity of final A2E was monitored by HPLC on a chromatograph Knauer (Germany), fitted with a Diasfer-110 C18 reverse phase analytical column, and with the mobile phase consisting of acetonitrile/water (84% of acetonitrile in mobile phase) + 0.1% trifluoroacetic acid (TFA)<sup>[33]</sup>.

### *Preparation of model lipofuscins*

The model lipofuscins were prepared from POS<sup>[34]</sup>.

**Model of lipofuscin from oxidized POS:** POS from bovine eyes were subjected to the process of autooxidation. A suspension of POS containing 10-12 mg protein per 1 mL K-phosphate buffer was incubated at 4 °C in the dark for 30-45 d. The concentration of TBA-reactive substances in the autooxidised POS was  $12.0 \pm 1.0$  nmol/mg protein. Autooxidised POS were dissolved in 0.1 mol/L K-phosphate buffer, pH 7.6 (final concentration 1.3-1.5 mg protein/mL) and incubated at 37 °C in the dark with constant stirring for 72 h. After incubation, modified POS were dialyzed against phosphate buffer to remove unreacted low molecular weight molecules. For dialysis, Float-A-Lyser (SPECTRUM Labs) cellulose-ester membranes with a Molecular Weight Cut-Off of approximately 3.5 kDa were used. Dialysis was carried out for 1.5 d at 6 °C. Solution of the A2E in methanol was added to the purified modified POS to a final concentration of 60-70 nmol/mL. The prepared complex was centrifuged at 10000 g in a Beckman Allegra 64R centrifuge for 20 min. The pellet was resuspended in 0.1 mol/L K-phosphate buffer (LFO-lipofuscin).

**Model lipofuscin from weakly oxidized POS:** Freshly prepared POS from bovine eyes (concentration of TBA-reactive substances did not exceed 0.5-0.7 nmol/mg protein) were dissolved in 0.1 mol/L K-phosphate buffer containing 50 mmol/L fructose (Sigma), to a final concentration of 1.3-1.5 mg protein/mL and incubated with constant stirring at 37 °C for 72 h in the dark. No fructose was added to the control samples. After incubation, modified samples were treated in the same way as oxidized POS. The final pellet was resuspended in 0.1 mol/L K-phosphate buffer (LFF-lipofuscin).

### Determination of wet weight of lipofuscin

To determine the wet weight of natural and model lipofuscin aliquots of samples were subjected to centrifugation using Amicon-Ultra-0.5 filters at 10000 g for 30 min. The concentration of samples was calculated in mg wet weight per 1 mL. For our samples it was the following: LFO-lipofuscin approximately  $110 \pm 15$  mg/mL, LFF-lipofuscin approximately  $80 \pm 12$  mg/mL and for RPE lipofuscin approximately  $100 \pm 20$  mg/mL. A2E content in model lipofuscins was approximately 0.8-1.0 nmol/mg of wet weight. A2E concentration was calculated by using  $\varepsilon = 3.1 \times 10^4$ /mol/L per centimeter at 430 nm wavelength.

### The fluorescence spectra

The fluorescence spectra of samples were measured on a spectrofluorometer (Shimadzu RF-5301) at 365 nm excitation wavelength and 380-650 emission wavelength. All experiments were made using pure solvent as a reference.

### Irradiation of samples

For irradiation of samples an incandescent lamp KGM 24-150, 150 watt, equipped with a focusing system and heat filter was used. Irradiation energy was 80 mW/cm<sup>2</sup>. Illumination was carried out under constant stirring at room temperature, monitoring the initial sample volume. The spectral range of irradiation was set to 390-700 nm.

### Concentration of peroxidation products

The concentration of peroxidation products was determined by the accumulation of the reaction products with thiobarbituric acid (Sigma) (TBA-reactive substances - TBARS), which was measured by absorption at a wavelength of 532 nm<sup>[35]</sup>.

### The concentration of superoxide radicals

The superoxide concentration was measured by cytochrome c reduction (Fe<sup>3+</sup>), inhibited by superoxide dismutase by a modified method from<sup>[36]</sup>. The reaction mixture contained Hanks buffer supplemented with 20 mmol/L sodium bicarbonate, pH 7.6, 100 mmol/L cytochrome c, 50 µg/mL catalase and 0.05% cetyltrimethylammonium bromide. The concentration of model lipofuscins and lipofuscin from human RPE in the samples was 3-5 mg/mL. All reagents used for this method were obtained from Sigma. The mixture was irradiated with visible light (irradiation energy 80 mW/cm<sup>2</sup>) under constant stirring. Superoxide concentration was measured in a spectrophotometer (Shimadzu UV-1700) by the increase in maximum absorbance at 550 nm (molar absorption 21/mol/L per centimeter)<sup>[37]</sup>.

### Statistical analysis

The data were expressed as the mean  $\pm$  SD. For the statistics, Student's *t*-test was used. *P* < 0.05 was considered as statistically significant.

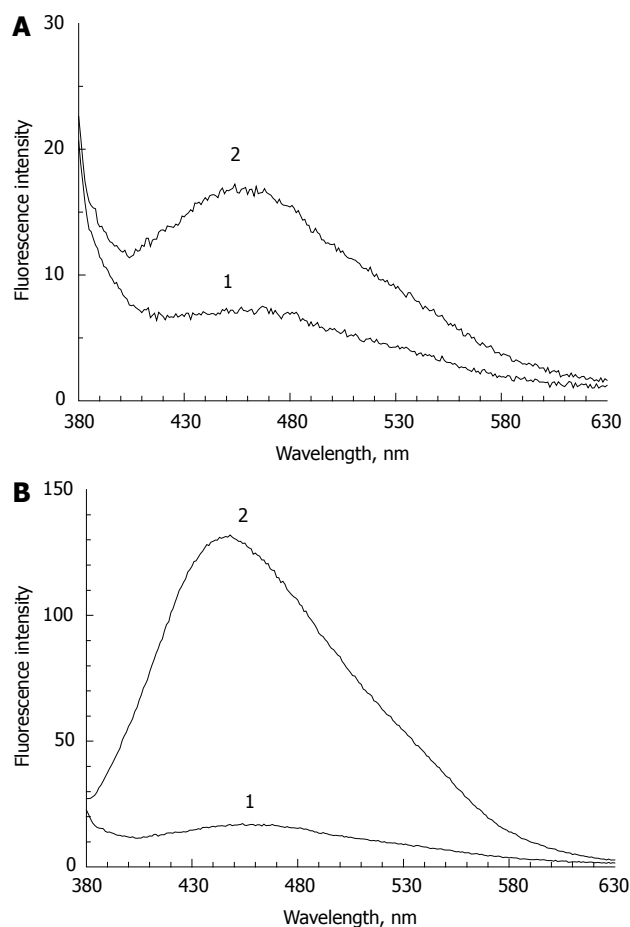


**Figure 1** Fluorescent products accumulation in weakly oxidised bovine photoreceptor outer segments after 72 h incubation at 37 °C. A: POS fluorescence incubated in the absence of fructose: curve 1 - prior to incubation; curve 2 - after incubation; B: POS fluorescence incubated in the presence of fructose: curve 1 - prior to incubation; curve 2 - after incubation. Fluorescence was measured in 0.1 mol/L K-phosphate buffer, pH 7.6, excitation wavelength - 365 nm.

## RESULTS

### Model lipofuscin fluorescence spectra are not altered by the ways of POS modifications

The preparation of model lipofuscin samples included two stages. First stage - modification of proteins and lipids from POS during incubation at 37 °C in the presence of either lipid autooxidation products (oxidation way) or fructose (glycation way).

Figure 1 shows the fluorescence spectrum of POS samples containing a small concentration of peroxidation products and incubated in the absence (Figure 1A) or presence (Figure 1B) of fructose. In the absence of fructose, a 72-h incubation causes an approximately two-fold increase in the fluorescence maximum. In the presence of fructose, the intensity of POS fluorescence at 445-450 nm increased about 8 times. At the same time, an increase of absorption at a wavelength of 320 nm was observed (data not shown). It appears that optical absorption and fluorescence increases due to the glycation reaction and formation of fluorescent Schiff

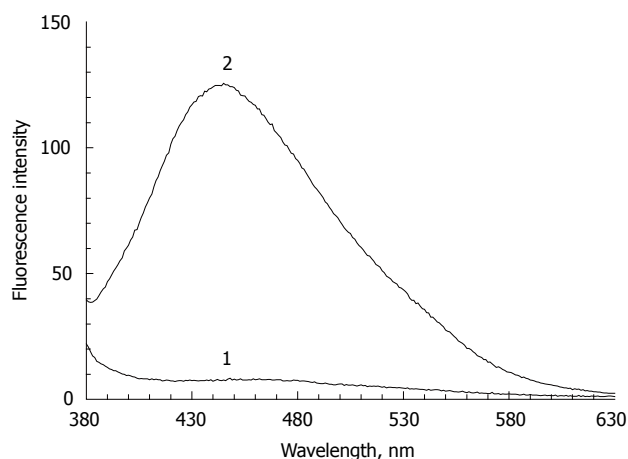


**Figure 2** Fluorescent products accumulation in the pre-oxidized photoreceptor outer segments at 72 h incubation at 37 °C. Curve 1 - prior to incubation; curve 2 - after incubation. Measurement conditions as in the legend under Figure 1.

bases in proteins and lipids. In the absence of sugar, this process is very slow and could be explained by residual products of lipid peroxidation.

In addition, following the incubation of POS samples with a high concentration of lipid peroxidation products, a significant increase in fluorescence intensity occurs (almost 15 times) irrespective of the fructose presence (Figure 2). In this case, the emission wavelength maximum practically coincides with the emission wavelength maximum of POS samples incubated with fructose.

The second stage of model lipofuscin preparation involves mixing modified and dialyzed POS with A2E followed by precipitation of the complex and solubilization of the precipitate in phosphate buffer, as described in Materials and Methods. This procedure leads to a significant change in spectral fluorescent characteristics of the modified POS. There was a significant decrease in fluorescence intensity at a wavelength of 445-450 nm (the major fluorescence peak observed before the addition of A2E, Figure 3A, curve 1) and an appearance of the emission maximum at 550 nm (Figure 3A, curve 2). The fluorescence maximum at 550 nm is typical for A2E and could be explained by the presence of these molecules in the model lipofuscin samples.

The reason for the sharp decrease of the fluorescence maximum at 445-450 nm is not fully understood. It could be due to fluorescence quenching of Schiff bases by A2E molecules or by their destruction.

Our model lipofuscins and lipofuscin from human RPE have very similar fluorescence spectra. Figure 3B shows the fluorescence spectra of LFO lipofuscin (curve 1) and natural lipofuscin (dashed curve 2) at an excitation wavelength of 365 nm. The spectra are almost completely identical. It should be appreciated, however, that the fluorescence emission spectra of lipofuscin from human RPE can considerably vary at excitation 360-365 nm, producing emission maxima between 460 and 630 nm<sup>[38]</sup>.

This dispersion appears to depend on many factors,

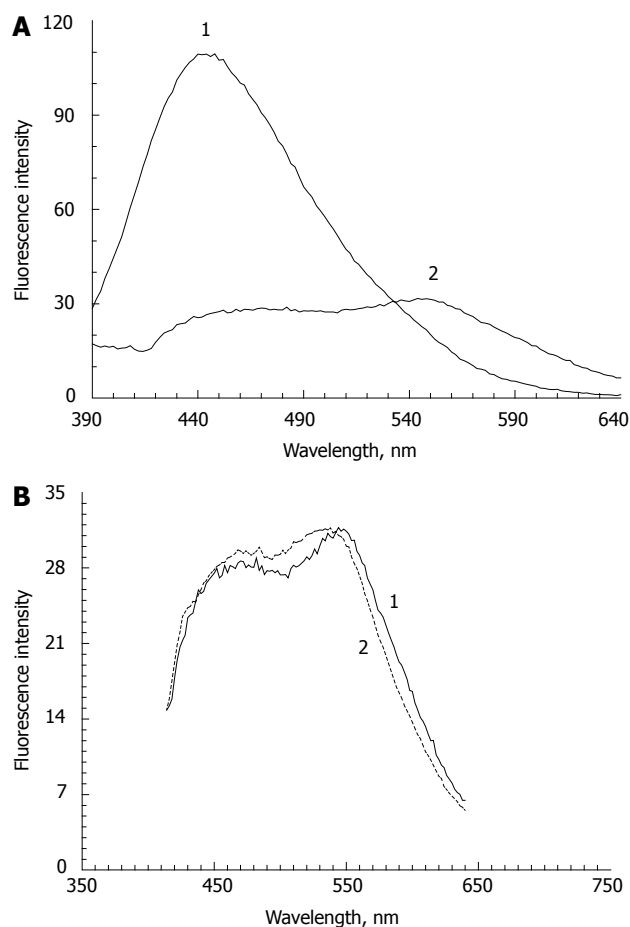


**Figure 3** Production of model lipofuscin in the reaction between modified photoreceptor outer segments and bisretinoid A2E. A: A2E binding by modified photoreceptor outer segments leads to a change in their fluorescence characteristics: curve 1 - POS modified oxidized prior to incubation with A2E; curve 2 - modified POS containing A2E (LFO - lipofuscin); B: Comparison of fluorescence characteristics of the model lipofuscin (LFO) and natural lipofuscin granules from human RPE: curve 1 (solid line) - LFO; curve 2 (dotted line) - lipofuscin granules from human RPE. Model lipofuscin preparation is described in materials and methods, excitation wavelength - 365 nm. POS: Photoreceptor outer segments; RPE: Retinal pigment epithelium.

including age and diet quality<sup>[39]</sup>. The fluorescence spectra of the LFO and LFF lipofuscins in these conditions were almost identical. These results show that the fluorescence spectrum of the lipofuscin model is largely determined by its fluorophores content; in this case, the fluorophore A2E.

### ***The photobleaching processes of both model and RPE lipofuscins demonstrate very similar kinetics***

Figure 4 shows the fluorescence spectra of LFF lipofuscin (Figure 4A) and lipofuscin from RPE (Figure 4B) upon irradiation with visible light with different exposures. It can be seen that in both cases the 550 nm peak disappears while the fluorescence amplitude significantly increases in the short wavelength region. Emission maximum after a 3-h exposure is shifted to 445-450 nm for LFF and to 460 nm for RPE lipofuscin. Apparently, the reason for this shift is associated with

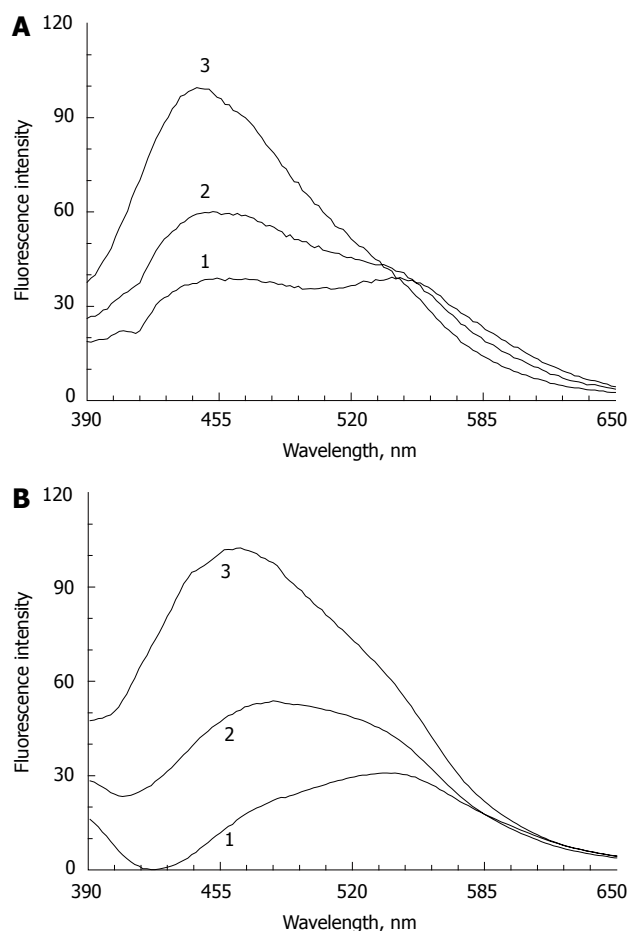


**Figure 4** Effect of visible light irradiation on the fluorescence characteristics of lipofuscin. A: Change of fluorescence spectra of the model lipofuscin (LFF): 1 - prior to irradiation, 2 and 3 - after irradiation for 1 and 3 h, respectively; B: Change of fluorescence spectra of human RPE lipofuscin granules: 1 - prior to irradiation, 2 and 3 - after irradiation for 1 and 3 h, respectively. RPE: Retinal pigment epithelium.

photodestruction of fluorophore A2E, leading to the formation of its oxidized derivatives<sup>[4]</sup>. The fluorescence spectra of these oxidized compounds are shifted to a shorter wavelength range.

It is also possible that A2E destruction reveals the underlying fluorescence of modified POS proteins and lipids. Because the model lipofuscin contains only fluorophore A2E, but RPE lipofuscin contains a mixture of different fluorophores, we can assume that, in natural RPE lipofuscin fluorophore A2E plays an important role in determining the total fluorescence.

#### **Irradiation of model lipofuscins show almost the same rate of superoxide photogeneration**

Lipofuscin phototoxicity is known to be primarily determined by the ability of lipofuscin to generate reactive oxygen species. We have previously shown that phototoxicity of RPE lipofuscin is higher than toxicity of just its A2E content<sup>[11]</sup>. This is probably due to the presence of other more active fluorophores in lipofuscin granules.

Model lipofuscins, prepared in this study, contain only one fluorophore A2E. Figure 5 shows the comparative

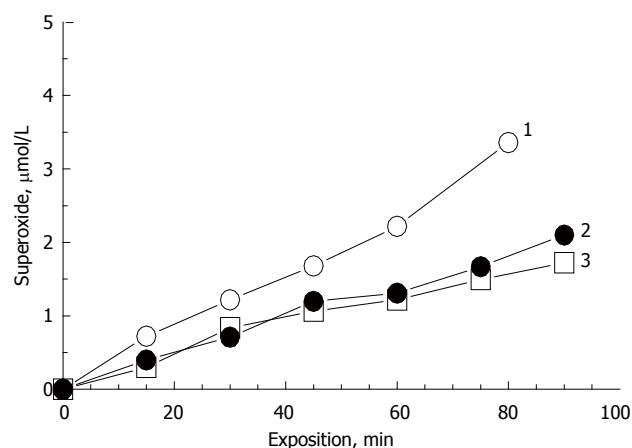


**Figure 5** Comparative kinetics of superoxide generation by irradiation of natural lipofuscin (curve 1) and model lipofuscin (curve 2 - LFF, curve 3 - LPO). The reaction conditions - see methods. Lipofuscin concentrations - 2.5 mg/mL.

kinetics of photogenerated superoxide upon irradiation of model and RPE lipofuscins. Model lipofuscins contained about 0.8-1.0 nmol A2E per 1 mg wet weight of sample.

Irradiation of LFO and LFF lipofuscin samples exhibited almost the same rate of superoxide generation (curves 2 and 3). A2E content in RPE lipofuscin can be roughly estimated according to the data from<sup>[40]</sup>. In this study quantitative analysis of bisretinoids in human RPE lipofuscin showed that the average content of A2E and iso-A2E is 380 pmol per  $5.5 \times 10^7$  lipofuscin granules, which corresponds to  $6.4 \times 10^{-18}$  mol/granule. The weight of one lipofuscin granule is  $1.3 \pm 0.2$  pg<sup>[41]</sup>, which gives the value of about 5 nmol/mg of A2E content in human RPE lipofuscin granules. This roughly corresponds to the A2E content in our model lipofuscin, assuming that the dry weight of the model pellets is about 20% of wet weight.

However, the rate of superoxide photogeneration catalyzed by RPE lipofuscin (curve 1, Figure 5) was significantly higher compared to that of model lipofuscin (curves 2 and 3, Figure 5). Average rates of superoxide photogeneration in all experiments are presented in the Table 1. The higher rate of superoxide photogeneration by RPE lipofuscin compared to A2E-containing model lipofuscins could be explained by the presence of other photoactive fluorophores<sup>[11,42-44]</sup> because the concentration of A2E in RPE lipofuscin does not exceed the concentration of the fluorophore in the model lipofuscins.

## **DISCUSSION**

As noted earlier, lipid peroxidation products are undoubtedly important for the formation of lipofuscin. Although the process of AGE products accumulation during normal aging has been well studied, much less is known about the role of advanced glycation end products in the formation of lipofuscin, which accumulates with age in RPE cells<sup>[45]</sup>.

An important role in this process belongs to fructose.

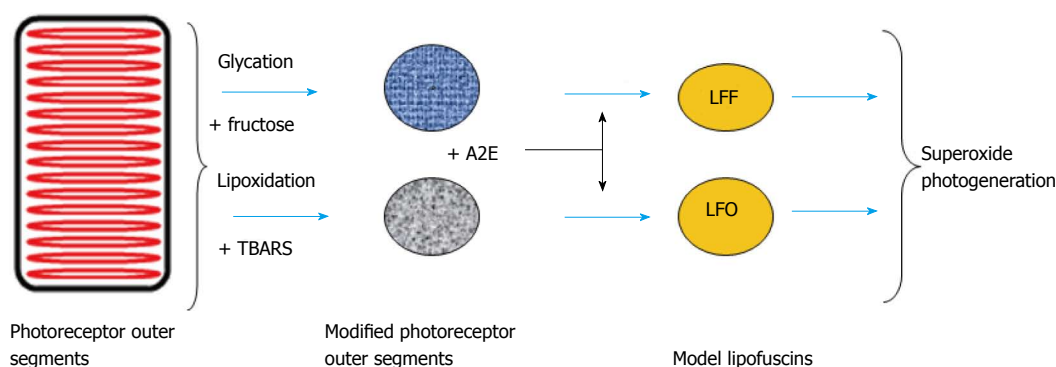


**Figure 6** Schema of processes generation of retinal pigment epithelium lipofuscin from modified photoreceptor outer segments and fluorophore A2E. POS: Photoreceptor outer segments; TBARS: TBA-reactive substances.

**Table 1** Superoxide generation rate in model and natural lipofuscins

| Sample name                                                                                        | Superoxide generation rate<br>nmole/min mg wet weight |
|----------------------------------------------------------------------------------------------------|-------------------------------------------------------|
| Model lipofuscin from oxidized POS<br>(LPO-lipofuscin)                                             | 8.5 ± 0.6                                             |
| Model lipofuscin obtained from not<br>oxidized POS in the presence of fructose<br>(LFF-lipofuscin) | 9.2 ± 0.4                                             |
| Natural lipofuscin from human eye                                                                  | 15.7 ± 0.8                                            |

Data represents mean values of 5 measurements on each type of lipofuscin with standard deviation,  $P < 0.05$ . POS: Photoreceptor outer segments.

Although its concentration in the blood is significantly lower than the glucose concentration (approximately 35  $\mu\text{mol/L}$ , while the concentration of glucose is approximately 5 mmol/L), the cellular concentration of fructose can be much higher. This increase could be induced by hyperglycaemic conditions when fructose formation through the polyol pathway is activated<sup>[46]</sup>. An elevated concentration of glucose is known to activate the cellular enzyme aldose reductase, which catalyzes the transformation of glucose into sorbitol. Sorbitol is then oxidized to fructose in a reaction catalyzed by the polyol dehydrogenase enzyme. It has been established that the retina and RPE are characterized by high expression of aldose reductase under hyperglycaemia<sup>[47-49]</sup>.

Fructose is a much more effective glycation agent in Maillard reactions than glucose. This is probably due to the fact that the open form of glucose molecules, which is directly involved in the Maillard reaction, is present at only 0.0002% of the content of the inactive cyclic form, whereas the fructose open form reaches 0.7% of the cyclized form<sup>[50]</sup>. A significant difference between glucose and fructose in the induction of carbonylation of target molecules may be explained by the formation of glyceraldehyde during fructose metabolism, while during glucose metabolism glyceraldehyde-3-phosphate is formed, which is much less active in Maillard reactions<sup>[51]</sup>.

Hyperglycaemia and polyol path activation greatly increase retinal sensitivity to oxidative stress<sup>[52]</sup>. All these facts suggest that aging and diabetes create conditions

in the retina and in RPE cells that promote development of advanced glycation reactions. Therefore, in lipofuscin, cross-links in proteins and lipids may form, not only by their interaction with reactive carbonyls produced by lipid peroxidation, but also by reactions with products of glycation. Lipofuscin thus should be considered as fluorescent pigment generated by modification of lipids and proteins through the action of lipid and carbohydrate reaction pathways. Schema of such processes generation of RPE lipofuscin from modified POS and fluorophore A2E is shown in Figure 6.

Our experiments indicate that fluorescent characteristics of the modified POS are essentially independent of the method of their modification. And various modifications of POS seem to have no or only a small influence on the spectral properties of obtaining lipofuscin.

Processes of fructozylation (glycation) and lipid peroxidation in POS cause fluorescence with the same maximum emission (445-450 nm) at an excitation wavelength of 365 nm. However, binding of the A2E fluorophore by modified POS led to a significant decrease of the original short wavelength emission maximum of modified POS. That also led to the appearance of a long-wave fluorescent peak, characteristic of the fluorescence profile of A2E. It also suggests that the fluorescence of lipofuscin is only slightly dependent on the nature of the fluorescent compounds arising from the modification of proteins and lipids. The fluorescence profile is mainly determined by the bisretinoid fluorophores present in lipofuscin granules.

Model lipofuscins prepared by fructozylation and lipid peroxidation contained approximately equal concentrations of fluorophore A2E and showed the same rate of superoxide photogeneration. Consequently, we hypothesize that the lipofuscin toxicity is independent from paths of protein and lipid modification. Instead, it mainly depends on the bisretinoid content.

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## COMMENTS

### Background

Lipofuscinogenesis in the retinal pigment epithelium (RPE) cells is connected in many respects with formation of the modified proteins and the lipids incapable of degradation by lysosomal enzymes. Modification of proteins and lipids is carried out by their interaction with the active carbonyl compounds accumulated in reactions of lipid peroxidation and glycation. These two different ways of modification are used for preparing of model lipofuscin systems in this study.

### Research frontiers

The authors examined the effect of different ways of protein-lipid modification of photoreceptor outer segments (POS) on the fluorescence characteristics and toxicity of model lipofuscins and compared them with properties of natural lipofuscin.

### Innovations and breakthroughs

The results of this study are proposed that fluorescent characteristics and phototoxicity of lipofuscin granules depend only to an insignificant degree on the oxidative modification of proteins and lipids and generally are defined by bisretinoid fluorophores containing in them.

### Applications

It is supposed that the fluorescence of RPE lipofuscin determines the fundus autofluorescence (FAF) *in vivo*. Monitoring FAF is noninvasive diagnostic method used in detection some retinal degenerative diseases such as recessive Stargardt disease, best macular dystrophy, and age-related macular degeneration. Preparation of model lipofuscins with predetermined fluorescent characteristics, defining by the relative content of the modified proteins and lipids, as well as the ratio of oxidized and reduced forms of fluorophores, is important for understanding the character of fluorescence ocular fundus and diagnostics associated pathologies. So the study of lipofuscin model systems may be an important factor for FAF interpretation.

### Terminology

Lipofuscin is fluorescent pigment that accumulates in aging postmitotic tissues. In the eye lipofuscin is mainly accumulated in the RPE, being one of the key biomarkers of aging and oxidative stress. Lipofuscin also plays significant role in the development of retinal pathologies.

### Peer-review

This is an interesting manuscript.

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## Basic Study

# Morphogenesis of human embryonic stem cells into mature neurons under *in vitro* culture conditions

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## Abstract

### AIM

To describe the morphogenesis of different neuronal cells from the human embryonic stem cell (hESC) line, SCT-N, under *in vitro* culture conditions.

### METHODS

The directed neuronal cell line was produced from a single, spare, pre-implantation stage fertilized ovum that was obtained during a natural *in vitro* fertilization process. The hESCs were cultured and maintained as per our proprietary in-house technology in a Good Manufacturing Practice, Good Laboratory Practice and Good Tissue Practice compliant laboratory. The cell line was derived and incubated in aerobic conditions. The cells were examined daily under a phase contrast microscope for their growth and differentiation.

### RESULTS

Different neural progenitor cells (NPCs) and differentiating neurons were observed under the culture conditions. Multipotent NPCs differentiated into all three types of nervous system cells, *i.e.*, neurons, oligodendrocytes and astrocytes. Small projections resembling neurites or dendrites, and protrusion coming out of the cells, were observed. Differentiating cells were observed at day 18 to 20. The differentiating neurons, neuronal bodies, axons, and neuronal tissue were observed on day 21 and day 30 of the culture. On day 25 and day 30, prominent neurons, axons and neuronal tissue were observed under phase contrast microscopy. 4', 6-diamidino-2-phenylindole staining also indicated the pattern of differentiating neurons, axonal structure and neuronal tissue.

### CONCLUSION

This study describes the generation of different ne-

neuronal cells from an hESC line derived from biopsy of blastomeres at the two-cell cleavage stage from a discarded embryo.

**Key words:** Human embryonic stem cells; Multipotency; Neural differentiation; Neural progenitor cells; *In-vitro* fertilization

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**Core tip:** Human embryonic stem cells (hESCs) have the capability to regenerate and differentiate into a wide variety of cells. In the present study, we described the morphogenesis of different neuronal cells from an hESC line under *in vitro* culture conditions. The blastomeres were at the two-cell cleavage stage and were taken from a discarded embryo during an *in vitro* fertilization process. We showed that neuronal axons and tissues were generated by the joining of multiple cells that communicate and transfer signals to each other, thereby forming neuronal cells and tissue.

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## INTRODUCTION

Human embryonic stem cells (hESCs) offer an unlimited source of human cells<sup>[1]</sup>. hESCs have the capability to renew and differentiate into all cell types. Differentiation may occur at all development stages upon exposure to the appropriate signals. These signals act in a hierarchical manner that regulates the development of the embryo, which induces the hESCs to differentiate into specific cell types of the three germ layers<sup>[2,3]</sup>.

The pluripotent characteristic of hESCs is considered as a "double-edged sword". The determination of how to direct hESCs to a specific progenitor cell type is a major challenge<sup>[1]</sup>. hESCs are a renewable source of neural cells<sup>[4]</sup>. Different protocols are used to direct the neural differentiation of hESCs; however, across all culture systems, common signals and stages of differentiation are observed. The three main stages of hESCs' neural differentiation are neural induction, neural stem/progenitor expansion, and neuronal and glial differentiation. These stages may further be subdivided into multiple events to progress a stem cell through its different progenitor stages<sup>[1]</sup>.

The human neuronal system is ectodermal in origin and contains multipotent stem cells, *i.e.*, neuronal stem cells or neural progenitor cells (NPCs), which give rise to the different cells of the nervous system. Multipotency, the ability to differentiate into different types of neuronal

cells, *viz.* neurons, astrocytes and oligodendrocytes, is the key characteristics of NPCs<sup>[5,6]</sup>. hESCs can be differentiated into NPCs that exhibit a broad cellular developmental and lineage spectrum<sup>[7,8]</sup>. According to Wu *et al*<sup>[9]</sup>, *in vitro* differentiation of hESCs and *in vivo* embryonic formation of the neuroectoderm share some similarities.

The present study describes the generation of different neuronal cells from an hESC line (SCT-N) produced from biopsied blastomeres at the two-celled cleavage stage from a discarded embryo during an *in vitro* fertilization (IVF) process. These cells use defined animal free conditions during derivation and long-term culture, which make them suitable for clinical cell therapy. We showed that neuronal axons and tissues were not generated from single cell differentiation, but were produced by the joining of multiple cells that communicate and transfer signals to each other, thereby forming neuron tissue.

## MATERIALS AND METHODS

### Ethics statement

The study protocol was approved by an independent Institutional Ethics Committee (IEC) of Nutech Mediworld. The institutional committee for stem cell research and therapy of Nutech Mediworld, New Delhi, India reported the study to the National Apex Committee for Stem Cell Research and Therapy (NAC-SCRT). The study was conducted according to the Declaration of Helsinki<sup>[3]</sup>.

### Origin of the cell line

The directed cell line (neuronal) was obtained from a single, spare, pre-implantation stage fertilized ovum that was obtained during a natural IVF process. The donor gave her consent.

The hESCs were cultured and maintained according to our proprietary in-house technology (United States Granted Patent No US 8592, 208, 52) in a Good Manufacturing Practice (GMP), Good Laboratory Practice (GLP) and Good Tissue Practice (GTP) compliant laboratory. The cell lines were chromosomally stable and free from any animal product. The detailed cell culture and differentiation techniques were described in our previous paper<sup>[10]</sup>.

### Cell culture and derivation

The embryo was suspended in Roswell Park Memorial Institute (RPMI) medium and broken mechanically. Human beta-chorionic gonadotropin ( $\beta$ -hCG) and progesterone were added and the cells were incubated in a CO<sub>2</sub> water-jacketed incubator for 24 h in aerobic conditions. The cell suspension was then re-incubated in the same incubator after adding Dulbecco's Modified Eagle's Medium (DMEM, Himedia Labs, Mumbai, India) under anaerobic conditions. The details of cell culture and derivation are explained in our previous paper<sup>[10]</sup>.

For this experiment, the above cell line was taken and re-incubated in aerobic conditions after the addition of DMEM. The cells were examined daily for the growth

and differentiation of the neural tissue.

### **Sample collection and characterization**

Syringes of frozen cells containing 2.5 to 3.5 million cells per milliliter were thawed and raised to body temperature. After this slow thawing process, the cells were characterized for their expression of different markers<sup>[11]</sup> using reverse transcription polymerase chain reaction RT-PCR on a Bio-Rad T100 (Bio-Rad Laboratories Inc., Hercules, CA, United States), immunofluorescence using a Nikon Ellipse E200 (Nikon corporation, Minato-ku, Tokyo, Japan) and fluorescence-activated cell sorting (FACS).

### **Sterility tests**

A quality check for the integrity, viability and microbial contamination was performed for the stored cell batches and the cultured cells obtained from the GLP laboratory.

### **Phase contrast/confocal microscopy**

To investigate the differentiation of hESCs into different NPCs and neuronal tissue, hESCs were cultured for one month (day 0 of inoculation to day 30 of the culture). The cells were observed for differentiation at different days under a phase contrast microscope (Nikon Ellipse E200), which was used as the major instrument to determine neuronal differentiation.

### **4', 6-diamidino-2-phenylindole (DAPI) staining of cultured cells**

To determine the presence of nuclei and chromatin materials, hESCs were stained with DAPI. The cells were incubated in a DAPI solution at a concentration of 0.1-1 µg/mL for 5 min. DAPI stained cells were mounted in ProLong® Gold Antifade (Life technologies, OR, United States) and were evaluated under a fluorescence microscope.

### **Neuronal Nuclei (NeuN) staining**

The expression of the *NeuN* gene was examined in cells cultured for different days (0-30 d). The cultured cells were reacted with an anti-NeuN antibody. The staining was performed at day 4, 8, 16, 22 and 30 of culture.

### **Immunofluorescence**

Cells ( $1 \times 10^6$ ) were adhered on a cover slide coated with poly-L-lysine, followed by washing in PBS and fixing for 10-20 min in 4% paraformaldehyde (PFA) at room temperature. The cells were permeabilized in Triton-X (0.25%) for 20 min. BSA (2%) and donkey (5%) serum were used to block the PBS washed cells for 2 h.

The treated cells were incubated at 37 °C with a primary antibody against NeuN (1:100, Neuromics) and then with the secondary antibody Alexa fluor® 488 donkey (SC2044) anti-mouse IgG (Invitrogen, Life technology, 557782) for 1 h and 30 min respectively. The cells were counterstained with 0.1-1 µg/mL DAPI for 5 min, mounted in ProLong® Gold Antifade (Life technologies, OR, United States) and evaluated under a

fluorescence microscope (Nikon Micro E200).

## **RESULTS**

### **Sterility tests**

All the cell batches and the cultured cells were found to be viable and sterile. No microbial contamination was observed.

### **Differentiation of neuronal progenitor cells into neurons, oligodendrocytes and astrocytes**

Different NPCs and differentiating neurons were observed in the hESCs culture. Multipotent NPCs differentiated into all three types of cells of the nervous system, *i.e.*, neurons, oligodendrocytes and astrocytes, as observed under a phase contrast microscope (Figure 1).

### **Determination of morphological differentiation of hESCs into neurons and neuronal tissue by phase contrast microscopy**

The hESCs were cultured for 30 d and observed at regular interval under a phase contrast microscope. Low cell numbers were observed just after inoculation; however, after 24-48 h of culture, the cell number increased and the cells started to clump together. Further culture led to an increase in cell size and the generation of NPCs (Figure 2). From 6-8 d of culture, we observed increased numbers of NPCs, while some of the cells showed the first signs of differentiation.

We observed small projections, such as those on neurites or dendrites, and protrusions coming out of the cells. Significant differentiation of the cells was observed at 18-20 d of culture.

Differentiating neurons, neuronal bodies, axons and neuronal tissue were observed on day 21 and day 30 of culture. On day 25 and day 30, prominent neurons, axons and neuronal tissue were observed under phase contrast microscopy.

### **DAPI staining of differentiating neuronal cells**

Further validation of the cells' morphology, differentiation pattern, presence of chromatin material and presence of tissue structure in culture were examined by DAPI (nuclear staining). The DAPI staining confirmed the presence of nuclei inside the cells. DAPI staining was more prominent in smaller cells compared with larger cells, probably because the larger cells were less permeable to DAPI compared with smaller cells (Figure 3).

DAPI staining also indicated the pattern of differentiating neurons, axonal structure and neuronal tissue.

### **Expression profile of NeuN, an intracellular neuronal marker, in hESCs in culture, as determined by immunofluorescence and confocal microscopy**

*NeuN* staining of cells was observed on day 4, 8, 16, 22 and 30 of culture. We observed the staining of the cells from day 4 of culture, which continued on day 8, 16, 22 and 30 of culture. Tissue staining was observed on day

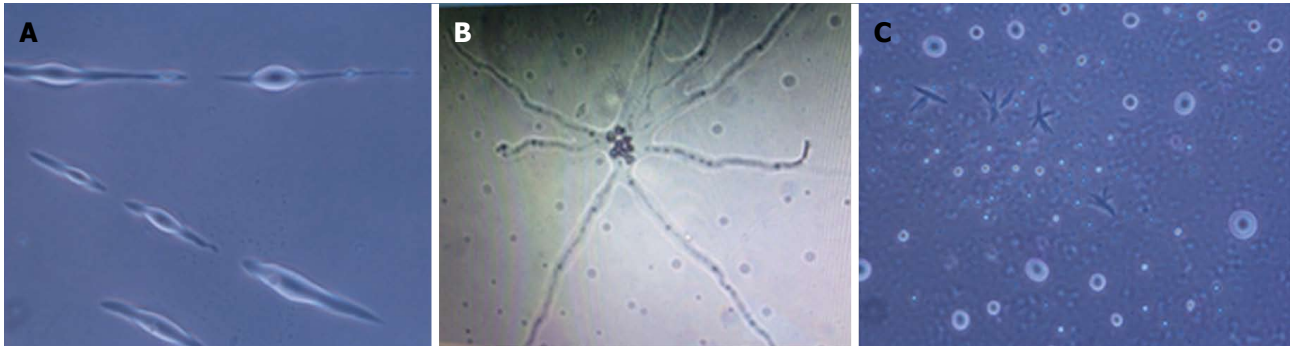


**Figure 1** Differentiation of neuronal progenitor cells into neuronal cells, oligodendrocytes and astrocytes under *in vitro* culture conditions. A: Neuron; B: Oligodendrocytes; C: Astrocytes.

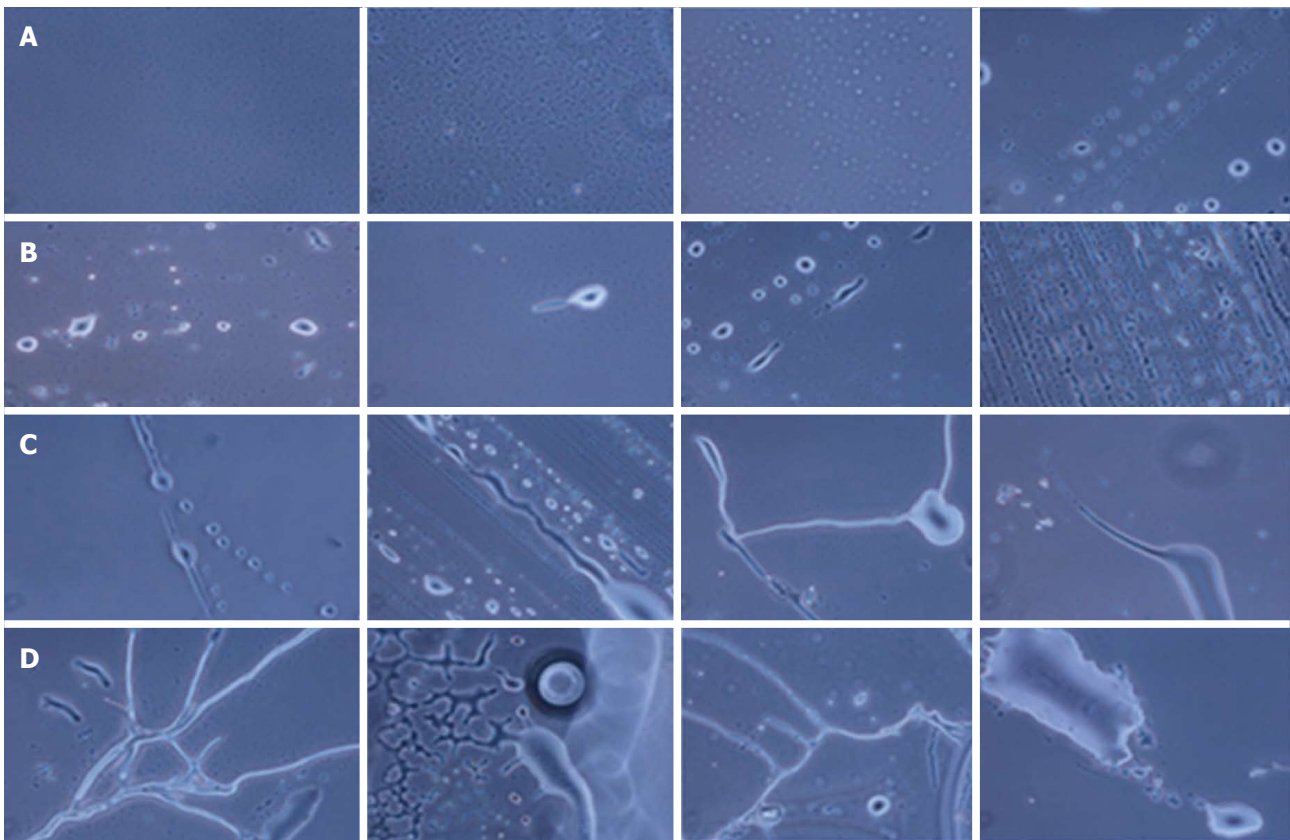


**Figure 2** Phase contrast images of neuronal cells and tissues at different days of culture. A: Day 0: Small stem cells; B: Day 5: Differentiating neurons; C: Day 16-18: Small protrusions, neuritis structure and end-joining of neurons; D: Day 30: Complete neurons and neuronal tissue.

16 onwards (Figure 4).

#### **Determination of the mechanism of neuronal axons and tissue formation under *in vitro* culture conditions**

During the formation of neurons, neuronal axons and neuronal tissue, the NPCs were first arranged in a line and then differentiated into neuronal cells with single or multiple projections. Furthermore, these projections joined the progenitor cells and increased in size, giving rise to small single protrusions. This protrusion increased in length and then joined to another cell's projection. The joining of one cell projection to another might either be direct or *via* joining to other progenitor cells. In this way,

these cells formed a chain and their cytoplasmic materials fused together to form neuronal axons (Figure 5).

Likewise, for the formation of neuronal tissue, the cells were first arranged in a line and then their projections joined together to form a network-like structure. Further growth and development of these joined cells in a network led to the development of the neuronal tissue. We also observed branching of neurons by the joining of single cell progenitor cells.

Under our culture condition, we observed that during differentiation of NPCs, all the cells do not commit towards differentiation at the same time. We hypothesized that among these cells, if one cell commits to differentiation,

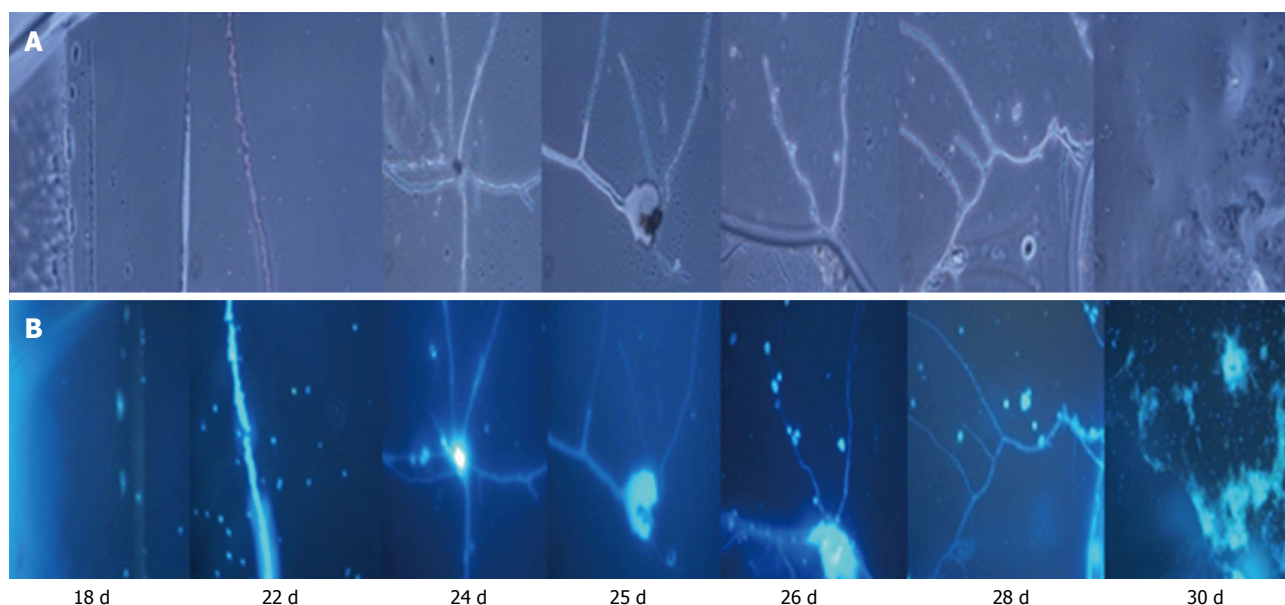


**Figure 3** 4', 6-diamidino-2-phenylindole staining of neuronal specific cells and tissues under *in vitro* culture condition. A: Cells viewed under a phase contrast microscope; B: 4', 6-diamidino-2-phenylindole stained cells.

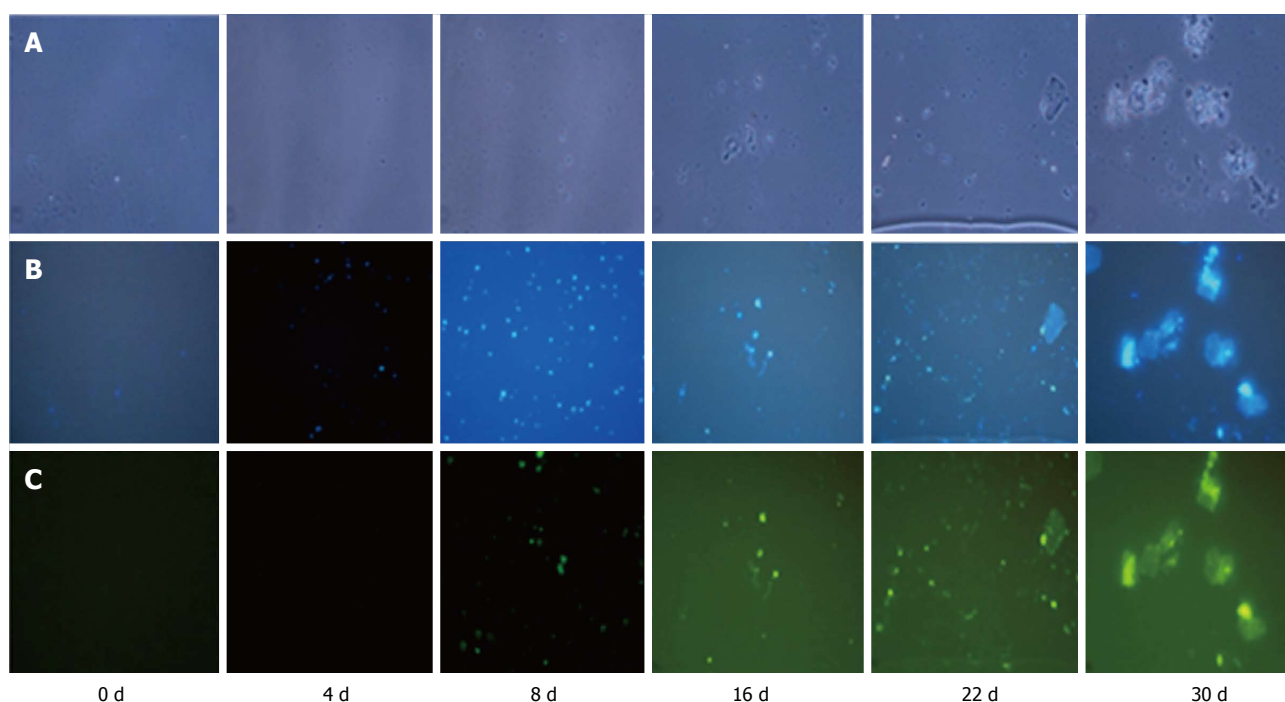


**Figure 4** Differentiation of human embryonic stem cells into neuronal specific cells and tissues under *in vitro* culture conditions. A: Cells viewed under a phase contrast microscope; B: 4', 6-diamidino-2-phenylindole stained cells; C: Green (NeuN) stained cells.

other surrounding cells will assist these progenitor cells for their growth, development and differentiation.

In this *in vitro* culture, we could observe single neuronal cell differentiation and the spread of a tissue-like structure. By joining together, these cells communicate and transfer signals to each other.

## DISCUSSION

In the present study, we determined the ability of hESCs

to differentiate into several neural cells and tissue. We showed the mechanism of division and differentiation of single NPCs into neurons, axons and neuronal tissue. Our results indicated that differentiation of hESCs towards lineage specific cells or tissue involves the generation of lineage-specific progenitors from hESCs that then undergo differentiation.

The pluripotent nature of hESCs allow them to generate the neuronal and glial cells found in neural tissue<sup>[5]</sup>. To direct and understand the differentiation process of hESCs into

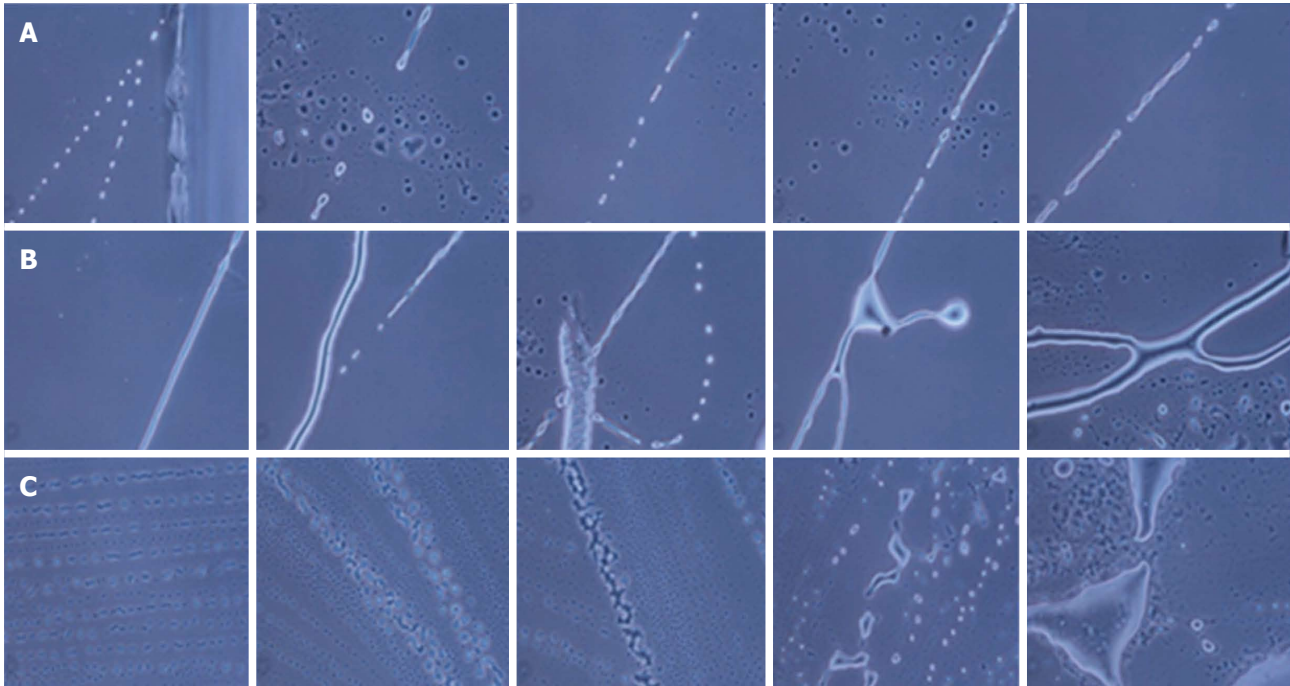


**Figure 5 Mechanism of differentiation of human embryonic stem cells into neurons and axons formation under *in vitro* culture conditions.** A: Day 5: Neural progenitor cells increased in size, arranged in a line and then joined together through small protrusions; B: Day 16-18: Cytoplasmic material mixed together to form long axonal structure; C: Day 30: Formation of neuronal tissue.

neuronal lineages, we cultured the cells in normal culture media, which was free from feeder layers and animal product. A unique feature of this culture media was that it did not contain any supplements. Under these culture conditions, the differentiation pattern of the stem cells was similar to the accepted model of the adult human and mouse nervous system differentiation process<sup>[12,13]</sup>. Ying *et al*<sup>[14]</sup> cultured embryonic stem cells (ESCs) in defined serum-free and feeder-free conditions, in the absence of bone morphogenetic proteins (BMP) signals, which resulted in efficient neural commitment and differentiation. Our results indicated that our unique culture conditions allow the lineage specific differentiation of neuronal cells without adding supplements.

In our study, hESCs generated NPCs that were capable of differentiating into neural cells and tissue. Microscopy revealed three types of nervous system cells, *i.e.*, neurons, oligodendrocytes and astrocytes. A previous study by Rubinoff *et al*<sup>[4]</sup> reported similar results: The proliferating progenitors generated from hESCs were able to differentiate into astrocytes, oligodendrocytes and neurons. Similarly, Zhang *et al*<sup>[15]</sup> demonstrated that hESCs-derived neural precursors generated all three types of neuronal cells *in vitro*.

After establishing the differentiation of hESCs in our culture conditions, we studied how hESCs were differentiated into neurons and neuronal tissue and the time course of differentiation. The differentiation stages of hESCs were followed at different days (0 day to 30<sup>th</sup> day). The cells started growing and were arranged in a line. Arranging of the cells in a line was identified as a characteristic of neuronal differentiation in our

culture conditions. After culturing hESCs for a week, small projections started to come out of the cells. The multipotent NPCs underwent asymmetrical cell division, which gave rise to smaller, undifferentiated multipotent stem cells and differentiated neuron cells. Hence, our results confirm the potential of hESCs to differentiate into neural cells. These results are consistent with previous studies<sup>[16]</sup>. In a study by Zhang *et al*<sup>[15]</sup>, hESCs differentiated to form neural tube-like rosette structures by day 7 of cell culture in a defined medium. Rubinoff *et al*<sup>[4]</sup> cultured hESCs for 3 to 4 wk to induce differentiation and derivation of NPCs. Early differentiation started after a week of culture, as indicated by changes in the cell morphology observed at the center of the colonies under phase contrast microscopy. Differentiation accelerated during next two weeks. Abranches *et al*<sup>[12]</sup> cultured ESCs for several days and observed that the cells developed very long cellular projections similar to those of radial glia, which then differentiated into all three neural lineages.

Differentiation of hESCs towards neuronal lineages leads to expression of neuronal-specific markers. Therefore, after establishing the differentiation of neurons and neuronal tissue in our culture conditions, we also confirmed the expression of neuronal system specific markers by immunofluorescence microscopy. *NeuN* is a specific marker that is expressed in neuronal differentiated cells and tissues<sup>[17]</sup>. Expression of this neuron-specific nuclear protein is a reliable marker of proliferative capacity that might indicate the physiological status of a post mitotic neuron<sup>[18]</sup>. In our previous study, we observed the expression of neuronal lineage-specific markers like nestin (neuronal progenital cells, NPCs) and *NeuN* (neuronal

marker of differentiated cells) in hESCs at the mRNA level<sup>[10]</sup>. On the 8<sup>th</sup> day of culture, we observed staining of smaller cells with the anti-NeuN antibody, which indicated that the cells might be entering differentiating mode. On day 30 of culture, we observed the staining of differentiated cells and differentiated tissue, which indicated that the later stage of the culture contained both differentiating cells and tissues. The spontaneous expression of neural markers may be considered as an evidence of the hESCs' differentiation towards neural lineages.

We also determined the mechanism of neuronal axons and tissue formation under *in vitro* culture conditions. Most of the studies on neurons and neuronal tissues differentiation have been done with either stem cells isolated from the pluripotent blastocyst stage or multipotent adult stem cells. These cells were found to account for the morphology and antigenic properties of neurons and astrocytes after their division and differentiation into neurons and astrocytes, respectively<sup>[19,20]</sup>. Reynolds *et al*<sup>[20]</sup> demonstrated the induced *in vitro* proliferation of striatum-isolated cells *via* epidermal growth factor. As far as we know, this is the first study to explain the mechanism of neuron network formation, axon formation and neuronal tissue differentiation of hESCs. Based on the principle that a brain-like structure can emerge as a self-organizing cytoarchitecture from stem cells *in vitro*<sup>[19]</sup>, we were able to show the mechanism of differentiation, extension of axons, cell joining and tissue differentiation from totipotent hESCs during *in vitro* culture. In our culture conditions, the pattern of aligning in a row of hESCs was found to be a characteristic feature of neuronal differentiating cells. We also observed that all the cells do not differentiate simultaneously, rather multiple single cells first align together and then join together through small protrusions, thereby forming neuronal cells, axon and tissue.

This study provided a system for generation of NPCs from hESCs. Moreover, the resulting NPCs from hESCs could serve as an expandable source for neuron production, which could be applied for many purposes, such as the treatment of neurodegenerative diseases. Recently, clinical trials using hESC derivatives to treat several neurodegenerative diseases have been approved, which led to the derivation of specific cell types from hESCs<sup>[6]</sup>. The development of neuronal tissue differentiation protocols will expand the application of hESCs-derived NPCs to neurological diseases. The stem cells used in our study have been injected into patients suffering from various neurodegenerative diseases such as cerebral palsy, spinal cord injury and Friedreich's ataxia and have shown successful results<sup>[11,21-23]</sup>.

To understand how to manipulate stem cells, and to restore and repair neural circuitry, it is important to identify the signals and mechanisms involved at each of these stages<sup>[1]</sup>. In view of the importance of understanding self-renewal and differentiation of hESCs at the transcriptional level, further studies are needed. This study will have a significant impact on the emerging field

of regenerative medicine.

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## COMMENTS

### Background

Human embryonic stem cells (hESCs) offer an unlimited source of cells. They have the capability to renew and differentiate into all cell types. Differentiation may occur at all development stages upon exposure to appropriate signals. These signals act in a hierarchical manner that regulates the development of the embryo, which induces the hESCs to differentiate into specific cell types of three germ layers.

### Research frontiers

The study describes the generation of different neuronal cells from the cell line SCT-N produced using hESCs from biopsy of blastomeres at the two-celled cleavage stage from a discarded embryo during an *in vitro* fertilization process.

### Innovations and breakthroughs

The hESC line uses defined animal free conditions during its derivation and long-term culture, which makes it suitable for clinical cell therapy. The author showed that neuronal axons and tissues were not generated from single cell differentiation but by the joining of multiple cells that communicate and transfer signals to each other, thereby forming neuronal tissue.

### Applications

The study provided a system to generate neural progenitor cells (NPCs) from hESCs. Moreover, the resulting NPCs could serve as an expandable source for neurons production, which could be applied for many purposes, such as the treatment of neurodegenerative diseases.

### Terminology

NPCs are multipotent, self-renewable cells that are responsible for forming the main phenotype of the nervous system.

### Peer-review

The paper "Morphogenesis of human embryonic stem cells into mature neurons under *in vitro* culture conditions" by Shroff, describes the differentiation of hESCs into neurons. It is a rapidly growing field of translational research that combines high expectations and safety concerns. Maintaining hESCs in defined conditions under strict GMP regulations is a very important part of the research, together with detailed step-by-step descriptions of differentiating cells and their characterization.

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