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MSC-derived exosomes: a novel possible treatment for liver fibrosis

Essay

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Abstract

Liver fibrosis, the precursor of cirrhosis, is a progressive disease leading to the impairment of hepatic function. It is characterized by excessive myofibroblast proliferation and extracellular matrix (ECM) deposition. In this essay, the cause and pathology of liver fibrosis are discussed. In the search for treatment, mesenchymal stem cells (MSCs) have come forward as an excellent candidate for cell therapy. Preclinical evidence has shown the antifibrotic nature of MSCs in liver fibrosis, having effect on many different aspects of the pathology. The effect of MSCs on ECM, myofibroblasts and hepatocytes in liver fibrosis are discussed, as well as their immunomodulatory and differentiating capacities. It has become clear that MSCs repair liver tissue mainly through paracrine mechanisms. Extracellular vesicles (EVs), such as microvesicles (MVs) and exosomes (the smallest vesicles), probably have a distinct role in these mechanisms. Exosomes and MVs carry nucleic acids, proteins and lipids to communicate with the environment. Several studies have demonstrated that MSC-derived MVs and exosomes are promising therapeutic agents, although the exact mechanisms remained unclear. Next, the essay focuses on liver fibrosis, starting with an explanation of the function of MVs and exosomes during liver pathology and elaborating on the pioneering study of Li et al, using MSC-derived exosomes against liver fibrosis. Subsequently, the possible roles of exosomes in the treatment of liver fibrosis are discussed and finally guidelines for further research are provided.

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1. Introduction

1.1. Liver fibrosis

Liver disease is one of the most common diseases worldwide. Liver fibrosis is the precursor of liver cirrhosis. It is a frequent event in response to a variety of chronic events such as alcohol, drugs, obesity, metabolic diseases, viral hepatitis and autoimmune attack of hepatic cells^{1,2}. Liver fibrosis is a progressive disease. It is characterized by excessive proliferation of myofibroblasts and subsequent extracellular matrix (ECM) deposition in liver tissue, compromising normal hepatic structure and function²⁻⁴. Ultimately, liver fibrosis may lead to organ malfunction and death.

1.1.1. Obesity (NAFLD/NASH)

Nowadays, obesity and overweight are amongst the most common etiologies of liver fibrosis. Overweight and obesity are defined as abnormal or excessive fat accumulation that may impair health (WHO, 2016). People with a body mass index (BMI) of 25 or more are considered overweight and a BMI equal to or more than 30 are considered obese. Worldwide, obesity has more than doubled since 1980. In 2014, 1.9 billion people were overweight and of these 600 million were obese (WHO, 2016). Obesity is strongly associated with the development of comorbidities such as diabetes, cardiovascular diseases and non-alcoholic fatty liver disease (NAFLD)^{5,6}. NAFLD is a comorbidity that can ultimately lead to liver fibrosis. Excessive weight gain leads to increased liver fat. In the liver, this fat is stored as triglycerides and free fatty acids (FFAs)⁷. Lipotoxic effects of these FFAs as well as lipid intermediates impair not only the correct functions of liver cell organelles involving the production of reactive oxygen species (ROS), but also the infiltration of pro-inflammatory molecules and apoptosis of liver cells. This way a subset of 30-40% of NAFLD patients progresses to non-alcoholic steatohepatitis (NASH), which is characterized by fibrosis.

1.2. Pathology of liver fibrosis

After *acute* liver injury (e.g. acute viral hepatitis A), the wound-healing response causes parenchymal cells to regenerate and replace the apoptotic or necrotic cells (Yoon, Friedman & Lee 2016). This is a process associated with inflammation and with limited deposition of ECM. Liver fibrosis is the result of the wound-healing response of the liver to *chronic* injury of any etiology⁸ (Figure 1).

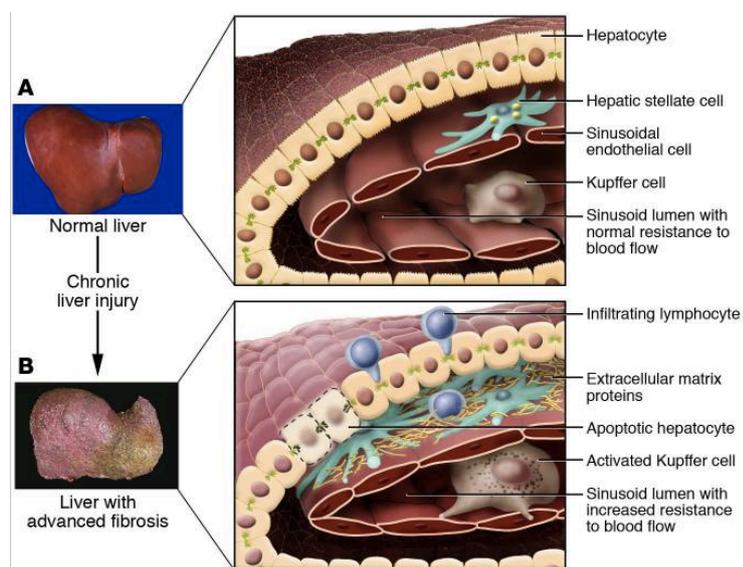


Figure 1. Changes in liver architecture associated with liver fibrosis. (A) Normal liver (B) Liver with advanced fibrosis⁹

If the damage to the liver is recurrent or persistent, eventually the liver regeneration fails and hepatocytes are replaced by abundant ECM. In an advanced stage, the liver contains approximately six times more ECM than normal, including collagens (I, III, IV), fibronectin, elastin, hyaluronan, laminin and proteoglycans^{8,9}. The fibrotic tissue is mostly located around portal tracts, causing increased blood flow resistance. The accumulation of ECM also results from a disbalance in synthesis and degradation; a decreased activity of ECM-removing matrix metalloproteases (MMPs) is measured during fibrosis, mainly thought to be due to the overexpression of their inhibitors (TIMPs, tissue inhibitors of MMPs)¹⁰.

1.2.1. Hepatic Stellate Cells (HSCs)

In liver fibrosis, the activation of hepatic stellate cells (HSCs) is a key pathological event. In healthy liver, HSCs are non-parenchymal quiescent cells that have physiological functions such as vitamin A storage, ECM homeostasis, and secrete mediators that may help to preserve hepatocyte mass¹¹. During inflammation associated with liver injury, Kupffer cells (the macrophages of the liver) as well as infiltrating bone marrow (BM)-derived macrophages and lymphocytes release reactive oxygen species (ROS) and cytokines activate HSCs to produce collagen⁹. During liver injury, HSCs activate or transdifferentiate into alpha-smooth muscle actin (α -SMA)-expressing myofibroblast-like cells, acquiring contractile, proinflammatory and fibrogenic properties^{11,12}. These activated HSCs (or myofibroblasts) in their turn secrete large amounts of ECM⁹. Other features of myofibroblasts are altered matrix degradation, mitogen-mediated proliferation and enhanced migration, allowing HSCs to navigate to sites of fibrogenesis.

The activation/transition of HSCs is seen as a key pathological event in liver fibrosis, a vision adopted in this paper. For sake of completeness, it must be mentioned that other sources of myofibroblasts have been identified. The activation of resident portal myofibroblasts (PMFs), mesenchymal progenitor cells, as well as fibrocytes recruited from the BM are mentioned as possible origins for myofibroblasts¹³. Several studies have also shown that myofibroblasts are derived from hepatocytes via epithelial-to-mesenchymal transition (EMT)^{14,15}. HSCs and PMFs however, are considered the major source of myofibroblasts. There is an ongoing discussion on which cell source has the most prominent role in liver fibrosis. The relative importance of each cell type in liver fibrogenesis probably also depends on the origin of the liver injury.

1.2.2. Main mediators during liver fibrosis

Transforming growth factor beta-1 (TGF- β 1) is recognized as one of the main profibrogenic mediators and plays a key role in the HSC activation and subsequent liver fibrosis⁹. HSCs contribute to the development of liver fibrosis through signaling of this TGF- β 1. This stimulates the transition to myofibroblast-like cells, as well as the synthesis of ECM proteins, and inhibits ECM degradation. Agents aimed at disrupting TGF- β 1 production or signaling pathways markedly decreased fibrosis in experimental models¹⁶. More recently, evidence has shown that connective tissue growth factor 2 (CCN2) is another important factor in the orchestration of fibrogenic pathways in active HSCs. Elevated levels of hepatic CCN2 is found in patients with liver fibrosis, with HSCs accounting for the majority of its production¹⁷. CCN2 promotes many of HSCs functions including activation, ECM production, adhesion and chemotaxis. Furthermore, the rapid proliferation of HSCs is found to be promoted by autocrine signaling of platelet-derived growth factor (PDGF) and vascular endothelial growth factor (VEGF)⁸. Besides the proliferation of HSCs, VEGF also stimulates angiogenesis, which is also associated with the progression of liver fibrosis. Due to fibrogenic tissue, the

increased resistance to the blood flow and subsequent hypoxia cause upregulation of VEGF and other pro-angiogenic factors, resulting in pathological angiogenesis¹⁸.

1.3. Treatment of liver fibrosis

At first, liver fibrosis was thought to be irreversible. Now it is known that even advanced fibrosis is reversible⁹. Reduction of liver fibrosis can occur after successful treatment of the underlying disease. However, it may take years before significant resolution is achieved, depending on (the severity of) the underlying disease. In obese patients suffering from liver fibrosis, weight loss is necessary. Weight loss can reduce fat in the liver, inflammation and fibrosis. Patients who lose weight after bariatric surgery (weight loss surgery) show attenuated fibrosis at two years postsurgery¹⁹. Weight reduction can reduce fibrosis and provides an important adjunct treatment but it does not completely resolve fibrosis. Therefore additional treatments are needed.

Currently, liver transplantation is the best option for patients with a dysfunctional liver. However, liver transplantation knows many limitations. Donors are scarce and post-transplant complications can occur, including immune rejection response and death of the donor or recipient in worst-case scenarios²⁰. Furthermore, immune suppressants markedly increase the chance of developing cancer and in overweight/obese patients receiving a new liver, the fibrosis may recur^{21,22}. Moreover, the chronic organ rejection after transplantation encompasses aspects of fibrosis, that eventually lead to organ failure: back to square one²³. Hence, effective therapies to replace liver transplantation are urgently needed.

The field of antifibrotic therapies is now emerging and therapies that can cure or suppress ongoing liver injury are being developed. As reviewed by Yoon and colleagues, therapeutic antifibrotic approaches target different aspects of the pathology: 1. Control or suppress the underlying (cause for) liver injury, 2. Antagonize receptor-ligand interactions and intracellular signaling, 3. Inhibit fibrogenesis, 4. Promote fibrosis resolution. Furthermore, decreasing inflammation and modulating the immune response are also important components of the therapeutic approach⁸. Although a growing number of agents are being tested for their antifibrotic activity in animals and humans, none have been approved for clinical use so far²⁴.

Recently, stem cell therapy has been introduced as an effective treatment strategy for patients suffering from liver fibrosis. Cell therapies are capable of complementing or replacing damaged liver cells and have shown promising benefits for liver fibrosis in experimental and clinical studies²⁵⁻²⁸. Mesenchymal stem cells (MSCs) have come forward as an excellent candidate for cell therapy, as they have practical advantages in regenerative medicine: MSCs are multipotent stem cells with a high capability of self-renewal, and low immunogenicity²⁹. A growing body of recent literature suggests that MSCs are promising for clinical use against liver fibrosis. Moreover, it is discovered that MSCs might exert their functions in a paracrine manner. The idea of replacing MSC cell transplantation therapy with a therapy using solely their secreted factors with the same effect has recently drawn much attention, as this would have major practical and safety benefits for clinical applications. It would be an *off-the-shelf* product that can be used in an allogeneic manner, without the risks of cell rejection.

1.4. Aim

In this conceptual literature review, the opportunities will be discussed of MSC-based and MSC exosome-based therapies for treatment of liver fibrosis.

2. MSC therapy for liver fibrosis

2.1. MSCs

To begin with, MSCs are capable of differentiating into a variety of mesenchymal cell lineages, including, bone, cartilage, neurons, muscle, skin and adipose tissue and therefore used for the regeneration of various tissues³⁰. MSCs can be isolated from various tissue sources. Initially these were derived from the BM, but currently similar cells are being obtained from many different adult tissues/organs, such as adipose tissue (AT), peripheral blood, lung, liver pancreas and more³¹. A major advantage of MSCs however is that these can be obtained from extraembryonic tissues after birth, including umbilical cord (UC), chorionic plate (CP), amnion and amniotic fluid^{32,33}

MSCs have the remarkable property to home to sites of tissue injury and institute repair, either by differentiating into tissue-specific cell phenotypes or, more importantly, by creating a milieu that increases the capacity of the endogenous cells to repair tissue and modulates the immune response^{34,35}. Early studies mainly focused on MSC differentiation. However, recent results demonstrate MSC's ability to repair tissues takes place without significant engraftment or differentiation. These findings have led to new concepts for MSC therapeutic effects, focusing on their instructive role. MSCs suppress the activity of immune cells, secrete a rich mixture of soluble trophic factors and specifically respond to the immediate needs of the injured tissues, which makes them one of the most useful cell therapies. This way, MSCs are also thought to play an important role in liver regeneration. *In vivo* experiments have shown that transplantation of MSCs of various origins into animal liver fibrosis models alleviated fibrosis.

2.2. Preclinical evidence for antifibrotic nature of MSCs in liver disease

2.2.1. Methods and in vivo models

In vitro studies are performed through co-culturing MSCs with hepatocytes or HSCs, either or not pre-treated with a profibrotic stimulus.

For most *in vivo* models of liver fibrosis, the hepatotoxin carbon tetrachloride (CCl₄) is used in rodents³⁶. CCl₄ comprises a model for acute liver damage, with a fibrotic phenotype. To induce liver fibrosis, CCl₄ is administered by subcutaneous injections twice a day during approx. 4-6 weeks. A less frequently used liver fibrosis model is the hepatotoxin and carcinogen dimethylnitrosamine (DMN), inducing chronic hepatitis-associated fibrosis³⁷. DMN is usually given intraperitoneally (i.p.) for 3 consecutive days of each week during approx. 4-6 weeks. Likewise, continuous administration of thioacetamide (TAA) is another less frequently used model of experimental liver fibrosis in rodents, given i.p. 3 times/week for 6 weeks³⁸.

2.2.2. General effect of MSCs on liver fibrosis (architecture and function)

A growing body of recent literature reports that the transplantation of MSCs of different origins into animal models of liver fibrosis ameliorates fibrosis. BM-MSc administration to rats suffering from liver fibrosis induced by CCl₄ ameliorated liver injury³⁹. The BM-MSc administration led to marked improvement of hepatic lobular architecture, a decreased area percentage of collagen fibers and improvements in liver function as indicated by serum liver tests, measured 4 weeks after MSC-administration. Completely Restored liver function and nearly normal liver architecture were shown 8 weeks after MSC-transplantation⁴⁰. Moreover, infusion of BM-MScs markedly reduced the mortality rates of CCl₄-induced fibrotic rats

(10% vs. 20%) and in DMN-induced fibrotic rats (20-40% vs. 90%)⁴¹. Human amniotic membrane-MSCs (hAMCs) reduced liver fibrosis in a CCl₄-model of liver cirrhosis as indicated by the decreased area of fibrosis and ameliorated liver function²⁸. Transplanted CP-MSCs also regressed fibrosis⁴². Hence, MSCs of various sources have been shown to ameliorate liver fibrosis. However, it has to be established exactly which fundamental aspects of MSCs biology and/or which therapeutic working mechanism of MSCs bring about this improvement.

2.2.3. Effect of MSCs on ECM

Excess production and decreased degradation of ECM are hallmarks of liver fibrosis. The effect of MSCs on collagen, the main component of the ECM, and other ECM proteins is of interest and has been thoroughly studied. In several CCl₄ liver fibrosis models, a decreased expression and concentration of collagen were found after MSC transplantation of MSCs of various origins^{25,28,39,42-44}. Decreased collagen could still have many different causes. In CCl₄-induced liver fibrosis in rats BM-MSC restored the expression levels of procollagen I and III (precursors of collagen) were restored⁴⁰. This indicated MSCs also inhibit the synthesis of collagen. Co-culture of BM-MSCs with activated HSCs reduced the secretion of procollagen type I C-peptide by HSCs, suggesting that the inhibition of collagen synthesis happens through modulating HSCs⁴⁵.

The imbalance of MMPs and TIMPs is one of the components responsible of impaired degradation of the pathological matrix during liver fibrosis. Changes in expression and concentration of these proteinases have been studied after MSC transplantation. In a CCl₄ mouse model, BM-MSCs increased the expression of MMP-9 and MMP-13^{46,47}. Also the expression of TIMP1 was found to be down-regulated in MSC-treated livers^{46,48}. As MMPs degrade ECM and TIMPs are their inhibitors, these findings suggest that MSCs also contribute to stimulating ECM degradation. However, in models for cardiac, lung and renal fibrosis, MMPs (-2, -9, -13) were found to have a lower expression and concentration following MSC treatment, while fibrosis was attenuated.⁴⁹

Taken together, these results indicate that MSCs both decrease ECM deposition as well as stimulate the degradation of the ECM, thereby decreasing the fibrotic area.

2.2.4. Effect of MSCs on myofibroblasts/activated HSCs

As stated earlier, the activation and proliferation of myofibroblasts, the culprit cell in fibrosis, is a key pathological event in liver fibrosis. A growing body of literature states that MSCs have antifibrotic effects through reducing myofibroblast/HSC activation. A specific marker for activated HSCs and myofibroblasts, α -SMA, is highly increased during liver fibrosis⁹.

Multiple *in vivo* experiments have shown that MSC transplantation decreased α -SMA expression after 2 weeks, with after 3-4 weeks almost normalized expression, indicating inhibition of HSC/myofibroblast activation and decreased fibrogenic functions^{28,42,46}.

However, 1 and 3 days after transplantation no significant decrease of α -SMA expression was measured, indicating that the antifibrotic effects were likely indirect²⁵. MSCs also inhibit TGF- β 1, the master pro-fibrogenic molecule: significant downregulation of TGF- β 1 was measured in livers 3 days and 2, 3 and 4 weeks after transplantation^{25,42,47}. The significantly lower expression of TGF- β 1 measured on day 3 implicates that this event takes place before the α -SMA decrease, which is in line with the idea that TGF- β 1 stimulated myofibroblast/HSC activation. In indirect co-culture experiments of HSCs with MSCs, a decrease in TGF- β 1 expression and protein was measured after 40 and 48hrs^{25,29}. This suggests that the decrease in TGF- β 1 is the result of paracrine signaling by MSCs, causing deactivation of HSCs and a decrease in α -SMA. Furthermore, MSCs significantly reduced

CCl₄-increased TGF-β1 and α-SMA protein in fibrotic mice when transplanted immediately, but not 1 week after, the start of exposure to CCl₄⁵⁰. This indicates that besides deactivating, MSCs can also prevent the activation of HSCs/myofibroblasts.

Whether the decrease in α-SMA is due to a reversion of activated HSCs to a quiescent state, a decrease in proliferation or increased HSC apoptosis (and just a lower number of cells), is unclear. Co-cultures of activated (with TGF-β1) HSCs with MSCs resulted in lowered HSC viability and a 3-fold increase in HSC apoptosis²⁹. Moreover, Parekkadan and colleagues found that at high MSC: HSC ratios, MSCs have the capacity to induce apoptosis and inhibit proliferation of HSCs, while not significantly decreasing levels of α-SMA expression⁴⁵. Only the 1:1 MSC to HSC ratio caused significantly decreased HSC proliferation, which may not be implementable *in vivo*. These results suggest that MSCs seem to lower the number of HSCs, mainly through induction of apoptosis, rather than deactivating them. Furthermore, transplantation of or co-culture with MSCs increased hepatocyte growth factor (HGF), which induces apoptosis in HSCs^{25,28,45,51}. Together, these results indicate that MSCs are capable of reducing the amount of myofibroblasts/activated HSCs, supposedly through paracrine signaling.

2.2.5. Effect of MSCs on hepatocytes

The ideal strategy of treating liver fibrosis would be to generate new hepatocytes replacing damaged or malfunctioning cells without causing excessive ECM deposition. MSCs have been shown to have their ameliorating effects on liver fibrosis also through positive effects on hepatocytes. To start with, MSCs stimulate hepatocyte regeneration by restoring or increasing proliferation of the cells^{25,28,52,53}. Whether or not this increase in hepatocyte proliferation is a result of the reduced activation of HSCs and/or the attenuation of liver fibrosis is not clear from these studies. Furthermore, hAMCs transplantation into CCl₄-treated mice suppressed hepatocyte apoptosis and senescence, measured 4 weeks after transplantation²⁸. The increase in hepatocyte proliferation and the decrease in apoptosis and senescence might be explained by the increase in HGF, induced by MSCs. Furthermore, HGF inhibits the EMT of hepatocytes to myofibroblasts, thereby preserving hepatocytes⁵⁴. In accordance with these data, co-culture of CP-MSCs with hepatocytes showed hepatoprotective effects, lowering CCl₄-induced necrosis and apoptosis⁵³. Interestingly, the CP-MSCs also upregulated the autophagic pathway, suggesting MSCs regulate hepatic regeneration through stimulating autophagy in damaged hepatocytes.

These studies proved evidence that MSCs can regenerate the liver through stimulating hepatocyte proliferation and anti-apoptotic and protective effects on hepatocytes.

2.2.6. Immunomodulation by MSCs

As shortly mentioned above, liver fibrosis is associated with an inflammatory response. Depending on the underlying etiology of liver fibrosis, different mediators initiate an inflammatory response, which further promote liver injury and fibrosis⁹. Many mediators and pathways have been identified for liver fibrogenesis. CD4+ T cells with Th2 polarization become activated and secrete Interleukin-4 (IL-4), IL-13, promoting fibrogenesis, while regulatory T cells can either favour or inhibit fibrogenesis by secreting TGF-β1 or IL-10 (antifibrotic).^{24,55} Natural killer (NK) cells reduce fibrosis by killing activated HSCs and producing IFN-γ.⁵⁶ Genetic studies in humans and rodents have identified IL-1b, IL-6, IL-10, IL-13 and IFN-γ as genes regulating fibrogenic response to injury.⁹ Fibrogenic growth factors (TGF-β1, FGF, Ccn2), adipokines (adiponectin and leptin), and vasoactive substances (angiotensin II, endothelin-1, norepinephrine) are each required for the development of fibrosis.^{9,57} TGF-β1 recruits Smad2/3, leading to its phosphorylation and stimulation of

fibrogenic gene expression.⁵⁸ Leptin has profibrogenic effects through suppression of proliferator-activated receptor γ (PPAR γ).⁵⁹ The vasoactive cytokine angiotensin II causes fibrogenesis through affecting the renin-angiotensin system, regulating the arterial pressure homeostasis in humans.⁹ Also several chemokines, such as CCL5 and CXCL4, play a pivotal role in liver fibrogenesis, promoting inflammation and the migration of HSCs to the site of injury⁶⁰. Activated HSCs itself also secrete pro-inflammatory chemokines and express cell adhesion molecules such as ICAM-1 and VCAM-1 through which they interact directly with immune cells.⁶¹

It seems that a vicious circle in which inflammatory and fibrogenic cells stimulate each other is taking place. Thus, therapeutic strategies able to modulate inflammation could help preventing liver fibrosis. It is well-known that MSCs are able to suppress the activity of both innate and adaptive immune cells. MSCs can exert a marked inhibitory effect on monocytes, (regulatory) T cells, NK-cells and antigen-presenting cells (APCs) including B cells, dendritic cells and macrophages^{31,57}. Soluble factors such as TGF- β 1, HGF, indoleamine 2,3-dioxygenase (IDO) and prostaglandin E2 (PGE2) seem to be implicated in this immunosuppressive activity⁶².

Interestingly, the immunoregulatory function of MSCs is highly plastic and the inflammation *status* seems to influence the immunosuppressive effect of MSCs⁶³. *In vivo* studies show that MSC transplantation increases HGF and anti-inflammatory molecule IL-10^{25,53}. Transplanted MSCs also decrease IL-6 expression, and when MSCs are pre-treated with IL-6 (cytokine secreted by HSCs during liver fibrosis) they secrete increased levels of IL-10, suggesting a dynamic response of MSCs in a pro-inflammatory environment⁴⁵. In addition, MSCs were found to secrete TNF- α , thought to inhibit the proliferative and fibrogenic function of activated HSCs together with IL-10. Figure 2 depicts a schematic model of some important effects of the soluble factors of MSCs on activated HSCs.

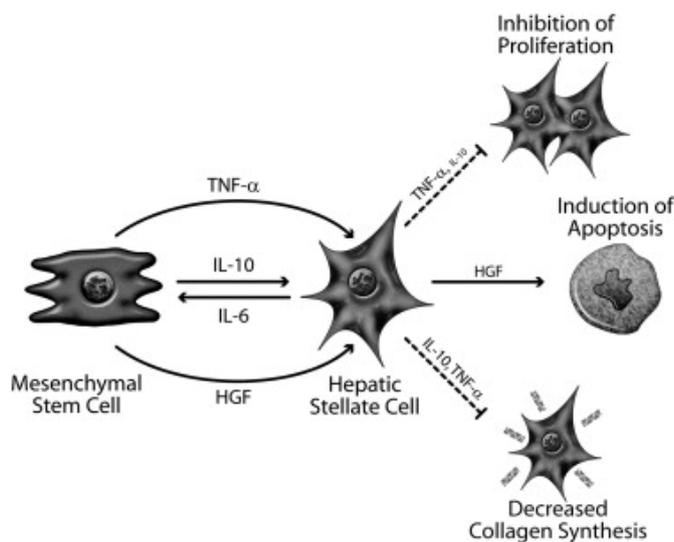


Figure 2. A schematic model of some of the paracrine effects of MSC-derived factors on activated HSCs. To get an idea of the complex paracrine stimulation of MSCs on HSCs, this figure shows some of the most important (immune) mediators and their effects. Release of IL-6 by activated HSCs leads to the secretion of IL-10 by MSCs. Induced IL-10, along with constitutively secreted TNF- α , inhibit HSC proliferation and collagen synthesis. The marginal effect of IL-10 on HSC proliferation is denoted by the smaller font size. HSCs undergo apoptosis after co-culture with MSCs due to increased levels of HGF. Figure taken from Parekkadan et al 2007⁴⁵

2.2.7. Differentiation into hepatocytes and engraftment

Many publications have reported that MSCs obtained from BM, AT and UC differentiated into hepatic-like cells *in vitro*^{30,64,65}. The question remains to what extent the MSCs differentiate and engraft in the tissue. This has been studied by *in vivo* tracking of transplanted MSCs. Using green fluorescent protein (GFP) as a marker, significant numbers of MSCs were shown present within liver fibrotic parenchyma at least during the 7 days after their cellular application, although diminishing over time²⁵. When MSCs stained with an infrared dye were systemically applied into TAA-treated animals, the MSCs present in the liver peaked after 1 day and decreased thereafter, with very little signal after 14 days. In most studies antifibrotic effects were still seen after 4 weeks. Another *in vivo* tracking study showed that MSCs survived within the liver for more than 4 weeks, however the numbers of cells with a hepatocyte phenotype were relatively low, not sufficient to reverse the injury⁵². Therefore, the differentiation of MSCs into hepatocytes can probably not explain the improvement of liver function. The role of MSCs does not seem limited to the simple, direct replacement of damaged cells and may even be even irrelevant regarding MSCs indirect antifibrotic effects.

Together, these data suggest that MSCs mainly exert their antifibrotic effects through paracrine mechanisms and only partly through differentiation into hepatocytes. An overview of the mechanisms discussed in this chapter is shown in figure 3.

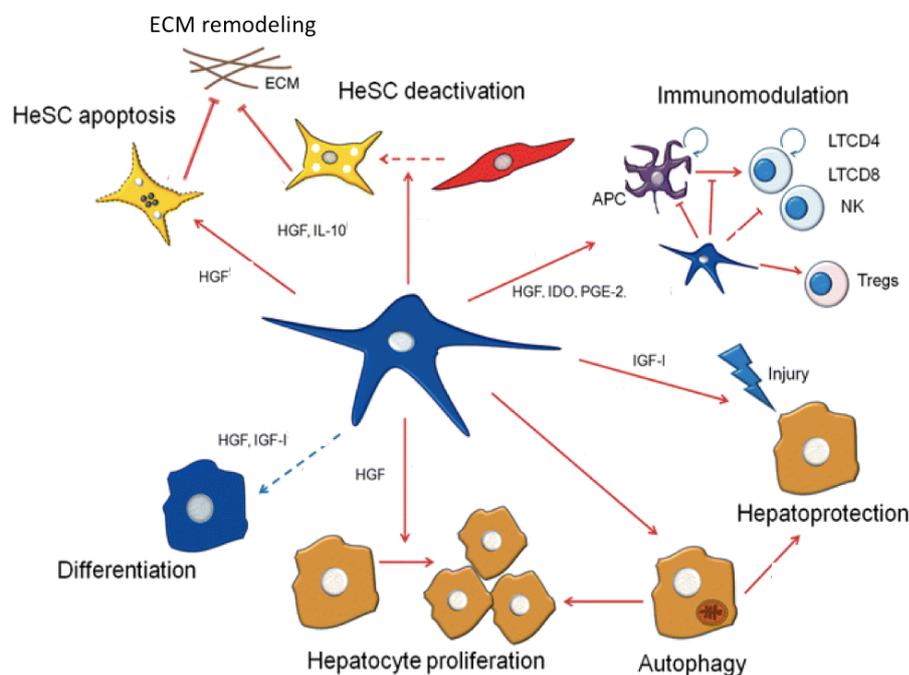


Figure 3. Mechanisms induced by MSCs contributing to liver fibrosis amelioration. According to available literature, MSCs likely ameliorate liver fibrosis through paracrine mechanisms (red arrows); Contribution of hepatic cell differentiation of MSCs has also been suggested, although this might be irrelevant for improvements observed in liver fibrosis (blue dashed line). Excreted molecules and growth factors likely mediate the paracrine mechanisms, including immunomodulation (i.e. inhibition of antigen presenting cell (APC) maturation, proliferation and activation; reduction of proliferation or maturation of lymphocytes (NK-cells, T cells) and induction of regulatory T-cells), enhanced hepatoprotection (i.e. through autophagy) and hepatocyte proliferation, HSC apoptosis and deactivation. Dashed line in red: it is unknown whether these cells can be restored to the quiescent and vitamin A reservoir phenotype. HGF=hepatocyte growth factor, IGF-1=insulin-like growth factor-1, IDO=indoleamine 2,3-dioxygenase, PGE-2= prostaglandin E2, ECM=, HeSC=HSC, APC=antigen presenting cell, LTCD4=lympocytic T cell CD4+, LTCD8= lymocytic T cell CD8+, NK=Natural Killer cell, Tregs= regulatory T cell. Modified figure from Fiore et al 2015³¹.

2.2.8. Secretome-mediated modulation of liver fibrosis by MSCs

Recent literature shows similar results when the secretome of MSCs is applied to fibrotic livers instead of MSCs itself. Different studies have shown that administration of MSC-cultured medium (CM) might function as alternative for MSC direct transplantation, in the treatment of liver fibrosis.

Indeed, systemic injection of MSC-CM leads to a marked survival benefit and a prevention of liver enzyme release in a D-galactosamine-induced rat model of fulminant hepatic failure (FHF)⁶⁶. The MSC-CM had anti-apoptotic effects (90% reduction) on hepatocytes and caused a 3-fold increase of proliferating hepatocytes *in vivo*, which was confirmed by *in vitro* experiments. The administration of MSC-CM also provided a significant survival benefit in rats with Gal-N-induced acute liver injury⁶⁷. After 12hrs, a remarkable reduction in infiltrating leukocytes as well protection of hepatobiliary pathological changes was measured. A proteomic screen of the MSC secretome revealed that a large fraction (30%) of MSC-CM was composed of chemokines, supporting their role as responsible mediators. When HSCs were incubated with the supernatant of BM-MSCs for 18h, a significant reduction in TGF- β 1 and the activation of HSCs was measured, while α -SMA and COL1A2 remained unchanged²⁵. After 40h however, a reduction in α -SMA and collagen1 α 2 was measured, suggesting that TGF- β 1 is responsible for the induction of α -SMA. When hepatocytes were incubated with the supernatant of MSCs engineered to produce insline-like growth factor-1 (IGF-1), a significant upregulation of IGF-1 and HGF expression was measured. Furthermore, indirect co-culture of activated HSCs with BM-MSCs decreased the production of TGF- β 1 by 75% and IL-6 by 16%, whereas the production of HGF and IL-10 were increased by 3-fold²⁹. These data suggest that factors released by MSCs are capable of modulating the expression of HSCs and hepatocytes in favour of liver regeneration. This supports the idea that the mechanism of MSCs to repair liver tissue is related to paracrine action rather than differentiation.

Paracrine modulation of liver pathology by the MSC secretome, traditionally comprises secreted proteinaceous factors as well as lipid mediators such as prostaglandins. However, in the past decade, cell-derived microvesicles (MV) and exosomes (discussed in the next section) emerge as yet another means of intercellular communication⁶⁸. MSC-derived MVs harbour a payload of therapeutically relevant molecules that may influence pathological processes including, cell death, inflammation and fibrosis. As exosomes have only recently been discovered, the function of exosomes is less well understood.

3. Exosomes in the treatment of liver fibrosis

3.1. Pioneering study of Li and colleagues

Li and colleagues were the first demonstrating that transplantation of exosomes derived from huc-MSCs can alleviate CCl₄-induced mouse liver fibrosis¹. Their experiments showed that MSC-derived exosomes alone were able to bring about multiple of the effects seen after MSC transplantation (further discussed in 3.5.2). Li and colleagues provided a novel mechanism for MSC-mediated tissue repair. Exosomes from MSCs are thought to be critical carriers of information, responsible for the regenerating and antifibrotic mechanisms. Because exosomes have high potential for clinical use, it is of great interest to investigate the current status and the possibilities of exosomes in the treatment of liver fibrosis.

3.2. Extracellular Vesicles (EVs)

Extracellular vesicles (EVs) are membrane-bound structures, which signal to paracrine or distal effectors. EVs are collectively represented by three subclasses of cell-derived vesicles, distinguished based on their size, biogenesis and markers (see figure 4)⁶⁹. Apoptotic bodies represent the largest EV subclass, ranging from 1-4 μ m in size. **Apoptotic bodies** are formed during the compartmentalization of apoptotic cells, and are generally assumed to carry inert particles destined for phagocytosis⁶⁹. **Microvesicles (MVs)** are EVs around 100-1000nm in size, which are shed directly from the cell membrane by a “budding” process⁷⁰. MVs deliver functional properties of the cell of origin through the transfer of proteins, mRNAs and particularly microRNAs (miRNAs) involved in epigenetic regulation⁷¹. MVs are generally identified by the expression of phosphatidylserine (PS) on their surface, indicating their release from activated or apoptotic cells as quiescent cells the membrane PS has a cytosolic orientation.⁶⁹ As Annexin V, a soluble protein used in the detection of apoptotic cells, binds to PS with high affinity, it is used as a marker of the MV subclass. However, there are controversies about their characterization as some groups have argued that a majority of circulating MVs is PS-negative. **Exosomes** are the smallest subclass of EVs, ranging between 30-100nm in diameter and generated by most, if not all, cells^{72,73}. Exosomes are formed within multivesicular bodies (MVB) that release their contents into the intercellular space upon fusion with the cell membrane. At first, exosomes were seen simply as means by which cells discard unwanted particles. Nowadays exosomes are believed to be used by cells as messengers or biological drug carriers, in order to communicate with the environment. The content of exosomes varies and can include proteins, lipids, mRNA, microRNA and other non-coding small RNAs, which can be transferred from the cell of origin to target cells to affect cellular function⁷⁴. Exosomes are characterized by the expression of membrane tetraspanins (CD63, CD9, CD81), integrins, cell adhesion molecules and proteins of the endosomal sorting complex required for transport (ESCRT)^{69,73}. Unfortunately, none of these markers are exosome specific, since they might be expressed on other vesicles of the MV size range as well.

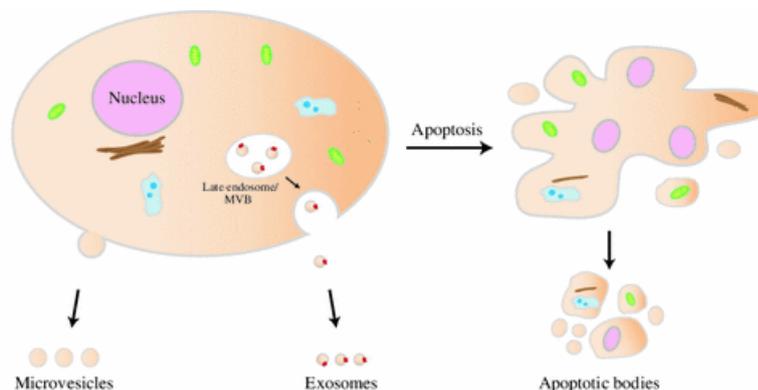


Figure 4. Schematic representation of the extracellular vesicles. Biogenesis and release of microvesicles (MVs), exosomes and apoptotic bodies. Modified figure from Akers et al 2013⁶⁸.

3.2.1. Differentiating between exosomes and MVs

Despite the fact that MVs are not of endosomal origin, they still share several features with exosomes including their molecular composition and cargo⁷². Currently there is no isolation method that results in a pure exosome population. Although differential centrifugation is the gold standard for isolating exosomes, it is still challenging to completely separate exosomes and MVs, as the sizes of MVs and exosomes mentioned here are somewhat arbitrary and subclass overlap may exist^{73,75}. For this reason many studies do not distinguish between both subclasses and they speak of “microvesicles” when meaning both exosomes and MVs. To make the terminology clear in this essay, the term “MVs” will be used for (a mix of) MVs (and exosomes) and the term “exosomes” will be used for exosomes only.

3.3. The use of MVs as disease therapy

There is a significant amount of research into how MVs can be used for the cure of several diseases. In most studies, “natural MVs” are used, derived from MSCs or other stem cells, and thereby mimicking the effect of that certain cell type on a tissue or disease. In addition, exosomes can be used as drug carriers, which can be artificially modified. Both types of application will be discussed in the following section.

3.3.1. MSC-derived MVs

To start with, MSC-derived MVs are demonstrated as promising therapeutic agents in a silica model of pulmonary fibrosis (Choi, Ban & Rhim 2014). A tail vein injection of MSC-derived exosomes reduced collagen I concentration and immune cell infiltration. Interestingly, the exosome injection was not as effective as MSC transplantation, which directly replaced fibrosis with hepatocytes and significantly reduced the fibrotic area and α -SMA (exosomes reduced these parameters non-significantly). However, it seems that the MSC treatment was started 2 weeks earlier (and lasted 2 weeks longer) than the exosome treatment, which does not provide a proper comparison.

MSCs have been reported with opposite effects on tumor growth and the same applies for their MVs. On one hand, MSC-derived MVs have been reported to exert inhibiting effects on tumor growth. *In vitro* experiments in three different tumor cell lines showed that MSC-MVs (size 145 ± 57 nm) induced apoptosis through activating negative regulators of cell cycle⁷⁶. Furthermore, *in vivo* intra-tumor administration of MSC-MVs in established tumors generated by subcutaneous injection of tumor cell lines in immune deficient mice markedly inhibited tumor growth. These data differ from the observation that BM-MSC-exosomes increased tumor incidence and growth, when tumor cells were mixed with exosomes before the injection in mice⁷⁷. The mechanism was ascribed to an enhancement of tumor cell proliferation and of angiogenesis that favours tumor engraftment. A similar discrepancy was also the case with MSC transplantation and seems to be caused by the difference in timing of MSC/MV-injection.

Currently, the company Esperite N.V. has started the first clinical project using MSC-derived EVs, including exosomes, in the treatment of inflammation responsible for Crohn’s disease (a form of inflammatory bowel disease) perianal fistulas (Reference: <http://www.esperite.com/?p=5810> ?). In 2017, the first in man experiments will be performed and the company expects that delivery of concentrated MSC-derived EVs directly to the site of injury would result in greater therapeutic effect when compared to allogenic MSC therapies.

3.3.2. Exosomes as drug-carriers

Exosomes are increasingly seen as possible drug delivery vehicles. Exosomes can be artificially loaded with certain signaling molecules in different ways⁷⁸. One approach is to stimulate or transfect the host cells and this way adjust the exosomal secretion, also called *passive loading*. Another strategy is *active loading*, in which the drugs of interest are loaded into purified exosomes using techniques such as electroporation and lipofection. Loaded with specific agents the exosomes can cross the plasma membrane to deliver their cargo into target cells. Exosomes have an intrinsic ability to home to target tissues and are amenable to membrane modifications that enhance cell type-specific targeting.

3.4. MVs in the pathogenesis of liver fibrosis

To get a better understanding of how MVs are able to influence other cells or even how these could influence a disease, we will discuss several studies in which MVs are found as mediators between hepatic cells in the liver. MVs have been suggested to be both fibrogenic

as well as fibrolytic. On the one hand, MVs can contribute to the induction of HSC fibrolytic activity.⁷⁹ *In vitro*, activated and apoptotic (CD4+ and CD8+) T-cells release MVs which fuse with HSCs and “horizontally transfer” membrane molecules that trigger the upregulation of MMPs (-1,-3,-9,-13), the downregulation of procollagen-a1 and weakened the profibrogenic activities of TGF-β1. Endothelial cell-derived exosomes can also positively regulate the phenotype of HSCs: exosomal sphingosine kinase 1 regulates HSC signaling and migration, which is dependent on exosome adhesion and endocytosis⁸⁰. When critical mediators of these exosome actions on HSCs are inhibited, chemotactic effects are blocked and mice were protected from CCl₄-induced liver fibrosis.

On the other hand, the important fibrogenic factor CCN2 is packaged by activated HSCs into exosomes which mediate its intracellular transfer to other quiescent or activated HSCs⁸¹. Furthermore, in a hypoxia-induced model of kidney injury, injured epithelial cells produce a significantly higher number of exosomes with specific genetic information, such as TGF-β1 mRNA that has the capacity to initiate activation and proliferation of myofibroblasts.⁸² Together these data demonstrate that MVs are released by several cell sources during liver fibrogenesis and play a role in both pro-fibrogenic and fibrolytic mechanisms.

3.4.1. MicroRNAs in the pathogenesis of liver fibrosis

Of the molecules that can be transferred by exosomes, miRNAs have attracted most attention as they are important epigenetic regulators. It has been shown that miRNAs regulate more than one-third of all human genes⁸³. MiRNAs have also been implicated in the development and progression of liver fibrosis: miRNAs play essential roles in HSC proliferation, apoptosis and migration as well as in ECM deposition and maturation⁸⁴. As a matter of fact, miRNAs involved in liver fibrosis can be broadly divided into pro-fibrotic or antifibrotic miRNAs, where pro-fibrotic miRNAs are upregulated and antifibrotic miRNAs are downregulated during fibrogenesis. Interestingly, quiescent HSC-derived exosomes suppress fibrogenic signaling in HSCs by transferring miRNA-214 and miRNA-199a-5p, negative regulators of CCN2^{85,86}. Thus, exosomes are involved in the epigenetic regulation of liver fibrosis.

3.5. Effect of liver stem cell-derived MVs in liver regeneration

So far, only one study is performed investigating MSC-derived MVs in the treatment of liver fibrosis. However, exosomes derived from human liver stem cells (HLSCs), a resident stem cell population in the liver, also accelerated liver regeneration in 70% hepatectomized rats⁸⁷. The intravenous injection of HLSC-derived MVs (80-1000nm) stimulated proliferation and resistance to apoptotic stimuli of mature hepatocytes by a horizontal transfer of specific mRNA and miRNAs associated with several cell functions involved in the control of transcription, translation and proliferation. The fact that HLSC-MVs have similar effects on liver regeneration as MSC-MV gives insights into the functions of MVs and suggests that other stem cells could also be used. However, a study comparing the MSC with LSCs transplantation, show that MSCs were more effective⁵².

3.6. MSC-derived exosomes: a novel possible treatment for liver fibrosis

3.6.1. Experiment using MSC-derived exosomes for the treatment of liver fibrosis

As mentioned at the beginning of this chapter, Li and colleagues showed that transplantation of successfully isolated exosomes (40-100nm, CD9+ and CD81+) derived from huc-MSCs (hucMSC-Ex) can alleviate CCl₄-induced mouse liver fibrosis¹. 2 weeks after transplantation, the hucMSC-Ex inhibited hepatocyte apoptosis and lobule destruction. Starting after 3 weeks, TGF-β1 and collagen type I and II levels were markedly (50%) reduced and significant alleviation of liver fibrosis was measured. Their findings suggest that hucMSC-Ex have their

effects through inactivating the TGF- β 1/Smad2 signaling pathway and thereby reversing the EMT of hepatocytes. Unfortunately, the effect of hucMSC-Ex on HSCs/myfibroblasts was not investigated and also they did not use a MSC-transplanted comparison. However, Li and colleagues were the first and the last to use MSC-derived exosomes in the treatment of liver fibrosis, leaving many questions unanswered.

3.6.2. Potential mechanisms of MSC-derived exosomes against liver fibrosis

Based on the knowledge we have of the effect of MSCs on liver fibrosis, and the idea that MSCs might exert these effects in a paracrine manner, several working mechanisms of MSC-derived exosomes on liver fibrosis can be hypothesized. Figure 5 sums up the possibilities of MSC-derived exosomes in the treatment of liver fibrosis. MSCs excrete exosomes, containing proteins, lipids, mRNA and non-coding RNAs, which are capable of exerting biological effects on recipient cells. As not much research has been done so far with MSC-derived exosomes in liver fibrosis, the effects (shown with the blue arrows) are mostly based on MSC cell therapy results. It would be of great interest to study the mechanisms shown in this figure when doing experiments with MSC-derived exosomes.

Besides the high potential of natural MSC-derived exosomes, there are more possibilities with exosomes such as stimulating the host cells in order to optimize the exosome-cargo (*passive loading*). Also the MSC-derived exosomes can be used as drug carriers, by *in vitro loading* of certain small molecules into the exosomes before injection. This way MSC-derived exosomes can be optimized or completely designed as ideal treatment vehicle for liver fibrosis.

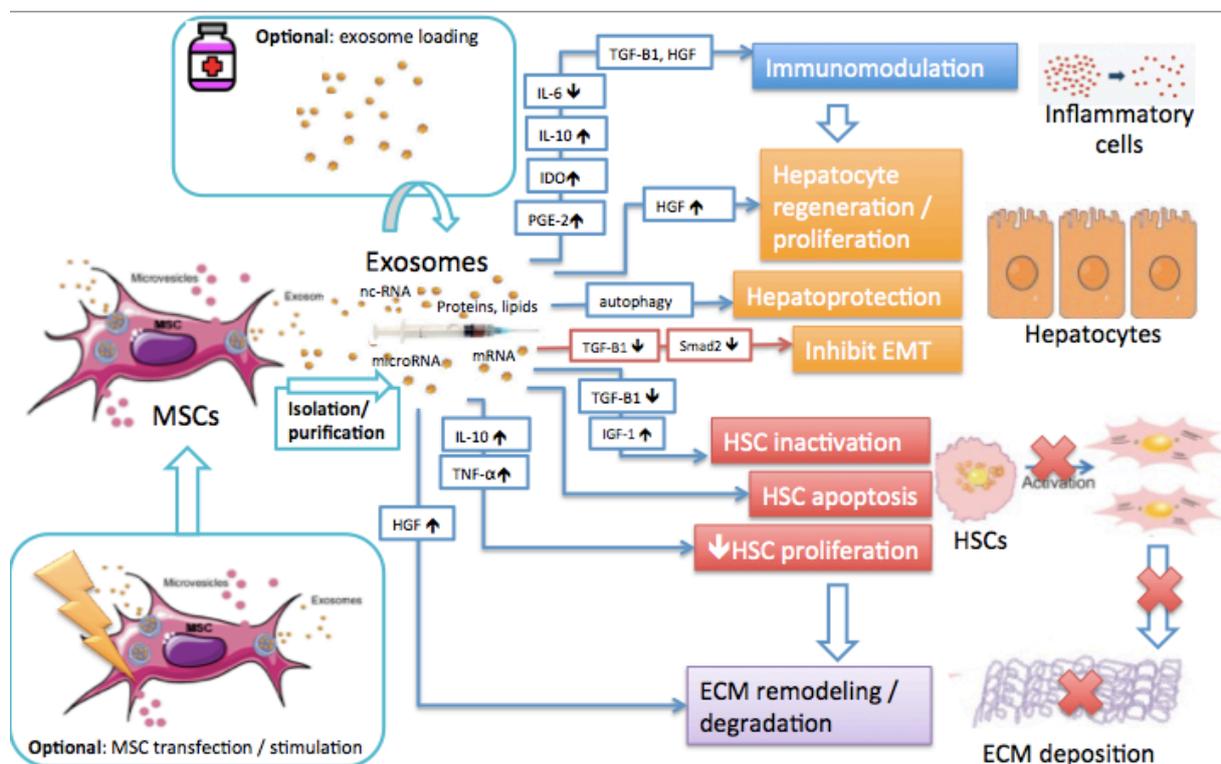


Figure 5. Potential use and mechanisms of MSC-derived exosomes in liver fibrosis. MSCs excrete exosomes, containing proteins, lipids, mRNA and non-coding RNAs which are capable of exerting biological effects on recipient cells. The exosomes have to be isolated and purified and can be injected intravenously or into the fibrotic liver. The blue arrows represent hypothetical working mechanisms through which MSC-derived exosomes might exert their antifibrotic effects. The red arrow shows the established mechanism by Li et al. 2013. Depicted are also some of the important mediators of that mechanism, found to be modified after MSC transplantation. Optional are MSC transfection/modulation (*passive loading*) before exosome isolation and *active* exosome loading after isolation. **Abbreviations:** MSC=mesenchymal stem cell; HSC=hepatic stellate cell; ECM=extracellular matrix; HGF=hepatocyte growth factor; TNF- α =Tumor necrosis factor- α ; TGF- β 1=transforming growth factor- β 1; IGF-1=insulin-like growth factor-1; IDO=indoleamine 2,3-dioxygenase, PGE-2= prostaglandin E2

4. Discussion – *what does the future hold?*

Liver fibrosis is a complex multifactorial disease and a growing problem worldwide due to obesity, alcohol-related liver disease and viral hepatitis. MSCs have come forward as promising candidates for cell therapy against liver fibrosis. As shown in chapter 2, MSCs are able to influence different aspects of liver fibrosis, likely through paracrine mechanisms that match the *common trophic mode of action* of MSCs in all disease models, as reviewed by Caplan.⁸⁸ Although the studies presented in this essay suggest that the source of MSCs does not influence the outcome, MSCs are described with different advantages. For example, AT-MSCs have biological advantages regarding the proliferative capacity, secreted proteins (FGF, IFN- γ , IGF-1), and immunomodulatory effects. BM-MSCs have advantages in osteogenic and chondrogenic differentiation potential and other secreted proteins (stem cell-derived factor-1 and HGF).⁸⁹ These differences are to be considered systematically when choosing the MSC source for liver fibrosis therapy.

Disadvantages of MSC transplantation, such as transformation into tumor cells, can be overcome when only using their secreted MVs. Moreover, MV treatment is cheaper (up to 10-times lower production costs), as they provide the great advantage of being produced in very large concentrations in highly homogenous preparations.⁹⁰ Furthermore, MV treatment also has a lower risk of immune response and the local administration is easier. At variance with transplanted MSCs, which have the capability to home in the site of the injury and ensure continued delivery of paracrine molecules, MSC-MVs can provide a one-hit stimulus. This provides a shorter, but more controllable treatment strategy.

Exosomes are intrinsically bioactive and have the capacity to serve as biovesicles for nucleic acids, proteins and lipids. Their molecular composition is not only cell-type dependent but can also differ, even when the exosomes originate from the same parental cells, suggesting that each exosome (subpopulation) exerts specific effects. Still, we have limited knowledge of the function of exosomes. Not much research has been done using MSC-exosomes and each study brings us a step closer to understanding their functions. Study by Li et al provided the first results of MSC-exosomes on fibrotic liver, showing inhibition of the EMT. Could this effect be restricted to (the epigenetic regulation of) exosomes? Or do MSC-exosomes fulfil a number of MSCs antifibrotic mechanisms, such as inhibiting HSC proliferation/activation? Figure 5 can be seen as a guideline for future research using MSC-exosomes against liver fibrosis. Further investigation should reveal whether exosomes have a distinct role in the paracrine fibrolytic mechanisms of MSCs. When studying the effects of MSC-exosomes, it is of great value to always use a control in which MSCs itself are transplanted, to compare the clinical values but also to reveal distinct functions of exosomes in intercellular communication.

MSC-derived exosomes definitely have potential as therapeutics. For further research however, the following developments are necessary. First, methods to isolate and purify exosomes (or MVs) should be optimized. To do this, specific exosome markers are needed and efforts should be made to enhance the selectivity of these isolation procedures. Second, most studies on exosome function have focused on miRNA content and effects, although recent findings show that other ncRNAs are significant exosomal components as well. Future efforts should focus on elucidating the functions of these less well-understood ncRNAs in exosomes. Also the possible contribution of lipids and proteins (whether or not incorporated into the membrane) to the biological effects of exosomes need further investigation. Third, if

exosomes will be used as drug-carriers, loading methods should be optimized as current methods are highly inefficient and mainly applicable to small RNAs (miRNAs, siRNAs).

Together, the future holds a lot of promise for the therapeutic use of MSC-derived exosomes against liver fibrosis. Thorough research into this specific application of exosomes will provide valuable knowledge of the various MSC functions that exosomes fulfil and of the regulatory mechanisms through which they act.

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