

Research Progress on Mesenchymal Stem Cells-Derived Small Size Vesicles for the Treatment of Liver Diseases

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Abstract: Liver disease is a major public health challenge, accounting for a substantial burden of mortality and morbidity worldwide. Currently, liver transplantation is the gold standard for treating end-stage liver diseases. However, it is affected by the shortage of organ sources and immune rejection. Mesenchymal stem cells (MSCs) can be used as an alternative therapy for liver diseases, especially in cases of liver cirrhosis, liver failure and complications of liver transplantation. However, MSCs may have potential effects on tumor occurrence. MSCs derived small size vesicles are nanoscale extracellular vesicles (EVs) which secreted by MSCs. MSCs derived small size vesicles are characterized by a lipid bilayer membrane, which encapsulate and transport various biomolecules such as proteins and miRNAs. Compared to MSCs, MSCs derived small size vesicles showed more advantages including lower tumor occurrence and lower toxicity. It has been found that MSCs derived small size vesicles ameliorate liver diseases via regulating lipid metabolism, inhibiting apoptosis, promoting regeneration, suppressing inflammation, drug delivery and so on. Based on the important role of MSCs derived small size vesicles in liver diseases treatment, this review aims to summarize the biological processes of small size vesicles with focusing on the effect of MSCs derived small size vesicles on different liver diseases. Additionally, we also discussed the potential future directions for small size vesicles research, including the challenges to be overcome and the prospects for clinical applications. This review provides theoretical support of MSCs derived small size vesicles as a potential therapeutic agent for liver disease in humans.

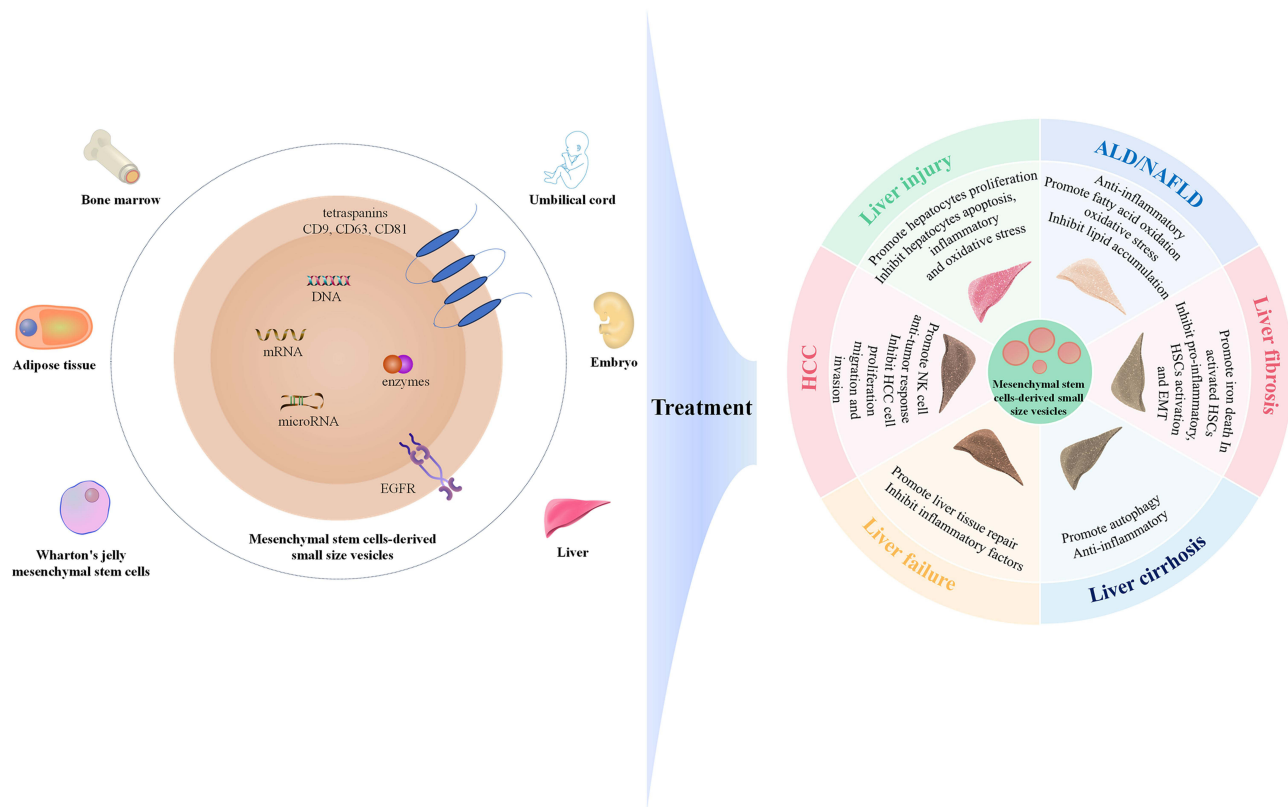
Keywords: liver disease, mesenchymal stem cells, MSCs, small size vesicles

Introduction

Various cells can release extracellular vesicles (EVs), which is bounded by a lipid bilayer and cannot replicate by themselves.¹ According to the characteristics including the size, density, composition and so on, EVs can be divided into different subtypes, such as microvesicles and apoptotic bodies.² As a subtype of EVs, small size vesicles usually origin from the endosomal system and are released via the multivesicular bodies (MVBs).³ So far now, small size vesicles have been found to be widely present in the vast majority of body fluids and tissues.⁴ Small size vesicles are approximately smaller than 200 nm in size and generally considered to be vesicles containing proteins, nucleic acids, and lipids.⁵ The proteins in small size vesicles exhibit excellent biological activities in physiological processes.⁶ There are many types of proteins on small size vesicles, such as metabolic enzymes phosphatolycerate kinase 1, NADP dependent male enzymes, heat shock proteins, cytoskeleton, secret proteins, etc.⁷ Several proteins in small size vesicles serve as biomarkers, such as CD9, CD63, lactadherin, glycosyl-phosphatidyl-inositol and so on, which may explain the targeting and selective



Graphical Abstract



characteristics of small size vesicles that entry into the target cells.⁶ In 2007, Valadi et al demonstrated that there were mRNA and miRNA in small size vesicles originated from the mast cells.⁸ In addition, it was also found that there were non-coding RNAs and mtDNAs presented in small size vesicles.⁹ Though the definition of small size vesicles is being updating in real-time, it is been widely recognized that small size vesicles and the contents of small size vesicles participate in several physiological and pathological biological processes.¹⁰ Additionally, the miRNA of small size vesicles can be absorbed by neighboring cells, which then may affect the target cells bioactivities.¹¹ The miRNA of small size vesicles can perform negative regulation and cause changes in the expression levels of target genes.¹² For example, the exosomes miR-92a-derived from K562 cells can reduce the expression of integrin $\alpha 5$ in human umbilical vein endothelial cells (HUVEC) and enhance endothelial cell migration.¹³ It has been reported that non-coding RNA from mesenchymal stem cells-derived exosomes (MSCs-exos) has great potential in the treatment of wound healing.^{14,15} For example, miR-135a in the human amniotic mesenchymal stem cell-derived exosomes can promote fibroblast proliferation and migration.¹⁶ The lncRNA KLF3-AS1 on bone marrow mesenchymal stem cell-derived exosomes (BMSC-exos) also can accelerate wound healing by upregulating vascular endothelial growth factor A.¹⁷

As a digestive organ, liver serves as a hub for several chemical and physiological reactions that occur in the body, such as the nutrients metabolism, blood volume and endocrine regulation, the synthesis and decomposition of lipids and cholesterol.¹⁸ As is well known, liver is composed of hepatocytes, cholangiocytes, hepatic stellate cells (HSCs), Kupffer cells, and liver sinusoidal endothelial cells (LSECs).¹⁸ These cells work together to regulate liver function at multiple levels and dimensions.¹⁹ Hepatocytes are the most common cells in the liver, and the regeneration of the liver is tightly associated with the proliferation of hepatocytes.²⁰ Regulating the hepatocytes cell cycle can effectively inhibit the senescence of hepatocytes and stimulate their proliferation.¹⁹ Cholangiocytes are highly specialized cells and can collaborate with hepatocytes to achieve liver regeneration.²¹ When one of the two kind cells fail to regenerate,

hepatocytes or cholangiocytes can function as facultative stem cells and switch to each other for liver regeneration.²² Different from hepatocytes and cholangiocytes, HSCs have four phenotypes: quiescent, activated, inactivated, and senescent.²³ Activated HSCs cause liver damage and induce liver fibrosis.²⁴ Kupffer cells are liver-derived macrophages that are commonly found in the hepatic sinusoids.²⁵ Kupffer cells can resist bacteria and endotoxins, playing an important role in immunity.²⁶ LSECs are well-known for their ability to form the hepatic sinusoidal wall. LSECs are well-known for their ability to form the hepatic sinusoidal wall.²⁷ LSECs usually form an open fenestrae and lack of an organized basement membrane, which play a pivotal role in maintaining hepatic homeostasis.²⁸

Liver disease is a relatively broad concept, specifically including infections of hepatitis B virus (HBV) and hepatitis C virus (HCV), alcoholic liver disease (ALD), non-alcoholic fatty liver disease (NAFLD), and end-stage liver diseases such as liver cirrhosis, liver failure, and hepatocellular carcinoma (HCC).²⁹ Generally speaking, if early-stage liver disease patients have not received systematic treatment, all types of chronic hepatitis may develop into cirrhosis, chronic liver failure, and HCC.³⁰ It has been reported that approximately 2 million people die each year due to liver disease, with the vast majority of deaths caused by cirrhosis and liver cancer.³¹ Many risk factors can trigger liver disease, including alcohol, high fat diet and so on.²⁹ Meanwhile, some other organs and diseases are also tightly associated with the occurrence of liver disease.^{32,33} For examples, diabetes has been demonstrated to be closely related with liver disease³⁴ and heart disease may cause acute liver damage, congestive liver disease, and chronic liver disease.³² Several chemical drugs have been demonstrated the positive effect on the treatment of liver disease, for example, lamivudine improves the prognosis of patients with chronic HBV.³⁵ Resveratrol and silymarin can attenuate the development of NAFLD.^{36,37}

This article will detailly introduce the biological processes of exosomes in body and summarizes the research progress of MSCs derived small size vesicles for the treatment of liver diseases.

Biological Processes of Small Size Vesicles

The Biogenesis of Small Size Vesicles

The biogenesis of small size vesicles is related to cell endocytosis. The generation of small size vesicles usually concludes budding, invagination, MVBs formation, and secretion.³⁸ When the plasma membrane buds and enters into the cell, an initial vesicle, which is also called early endosome, is formed. The early endosome is responsible for the initial sorting of internalized cargo.³⁹ The fate of early endosomes is different. Some early endosomes will be recycled by being fused with the Golgi apparatus network or plasma membrane, while some early endosomes are destined to mature into late endosomes.⁴⁰ During the maturation process of the endosomes, the endosomes membrane is also undergoing changes. A significant feature is the alteration of the membrane composition. For example, sphingolipids in membrane are replaced by ceramides, and Rab5 is substituted by Rab7.⁴¹ These changes are necessary for late endosome sorting and play an important role in the transportation of late endosomes. Following with the endosome mature, regions of the endosomal membrane invaginate into the intraluminal space and form multiple intraluminal vesicles (ILVs), which are the precursors of small size vesicles. ILVs transform the compartment into MVBs, however, some MVBs fuse with lysosomes, where the ILVs are degraded. Others MVBs fuse with the plasma membrane and release the ILVs into the extracellular space, which are known as small size vesicles (Figure 1).^{42,43}

Due for the important role of ILVs on the formation of exosomes, the mechanisms for regulating the formation of ILVs attracts more and more attention. So far now, they are mainly summarized into two main categories: those that rely on endosomal sorting complex required for transport (ESCRT) and those that do not rely on ESCRT.⁴⁰ ESCRT can be divided into ESCRT-0, I, II and III, which is essentially composed of class E vacuolar protein sorting (Vps). The main function of ESCRT-0, ESCRT-I, and ESCRT-II is to sort the cargos that need to be wrapped into functional microstructural domains on the inner membrane to promote signal transduction. Different from them, ESCRT-III induces budding and scission of the microstructural domains, leading to the production of ILVs.⁴⁴ Notably, some auxiliary proteins participate in the formation process of ESCRT, such as multi-vesicular body of 12 kDa, tumor susceptibility gene 101 (TSG101) and so on.^{44,45} Tony et al found that multi-vesicular body of 12 kDa is a component of ESCRT-I complex, which binds to the coiled helical domain of Vps23 to ensure the ordered assembly of ESCRT-I and II.⁴⁶

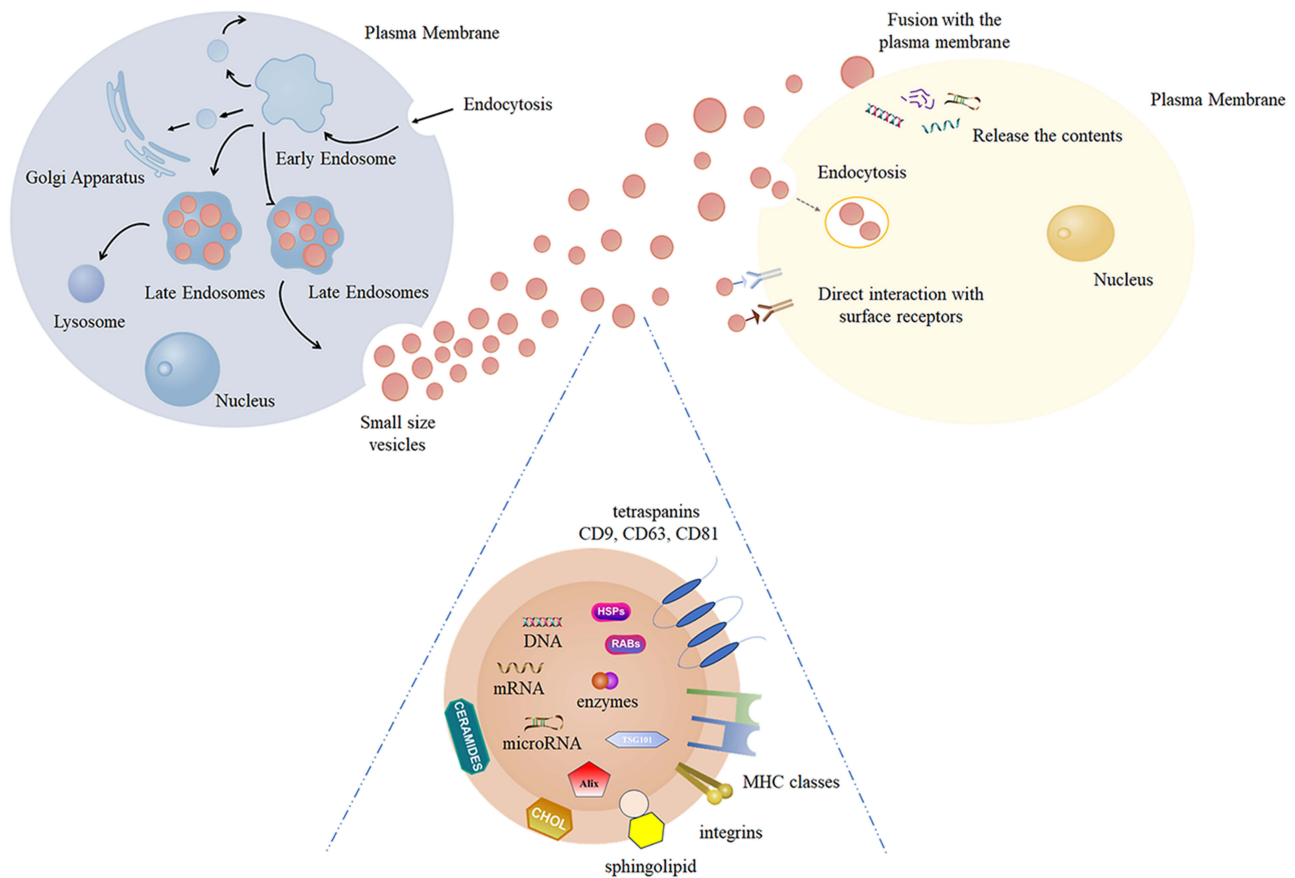


Figure 1 The biogenesis of small size vesicles. The biogenesis of small size vesicles begins with the plasma membrane invaginating to form early endosomes. Part of early endosomes recycle their cargo by fusing with the Golgi apparatus network or the plasma membrane. At the same time, others early endosomes mature into late endosomes. Ultimately, some late endosomes are degraded by fusion with lysosomes, while others release small size vesicles fuse with the plasma membrane and then release small size vesicles. After the release of small size vesicles, small size vesicles directly fuse with the target cell membrane and release the contents. Small size vesicles also enter target cells through endocytosis and direct interaction with target cells surface receptors.

Strickland et al reported that TSG101 is a component of ESCRT-I and mainly responsible for the recognition of monoubiquitin.⁴⁷

Exclude the classical ESCRTs take part in the ILVs generation, there are other mechanisms involved in the process by an ESCRT-independent manner.³⁹ In mammalian cells, ILVs form within the MVBs lumen when ESCRT is depleted, indicating the existence of an ESCRT-independent pathway to form ILVs.⁴⁸ As is reported, the generation of ceramides, phosphatidic acids, or lipid rafts can facilitate the non-dependent ESCRT pathways.⁴⁹ For example, ceramides, which have structural and physical properties that promote vesicle biogenesis, works together with membrane sphingolipids to promote the lipid raft microdomains formation and then trigger the spontaneous negative curvature of the lipid raft microdomains membrane to initiate the ILVs generation.^{41,42}

The Release of Small Size Vesicles

It is well known that MVBs can be transported to lysosomes or the plasma membrane. Factors involved in MVBs transport, such as cortactin, Rab proteins, ISGylation, and soluble N-ethylmaleimide-sensitive factor attachment protein receptors, are attracting increasing attention.

The interaction between MVBs and actin with microtubule cytoskeleton has been found to be crucial for the MVBs transport to the plasma membrane.⁵⁰ Cortactin, a protein that presents in all actin-based structures, participates in the processes including endocytosis, vesicle transport, adhesion, migration, and invasion.⁵¹ Known cortactin has been shown

to reduce the release of small size vesicles. In addition, Sinha et al reported that cortactin facilitates the transport and docking of MVBs with the plasma membrane, playing a pivotal role in the release of EVs.⁵²

Rab proteins are a key family of small GTPases. As the key regulators of eukaryotic membrane trafficking, Rab proteins play critical roles in vesicle fission and fusion. Pararray reported that Rab proteins take part in nearly every stage phase of membrane trafficking, including the formation of transport vesicles, the movement of vesicles and the docking of vesicles with their target compartments.⁵³ It has been reported that silencing Rab27a increases the size of MVBs, while silencing Rab27b leads to regional redistribution of MVBs.⁵⁴ Hsu et al confirmed that inhibiting Rab35 leads to the accumulation of endosomal vesicles and reduces the secretion of exosomes.⁵⁵ Additionally, other members of the Rab family such as Rab11 and Rab35 are also demonstrated the critical role in regulating the release of small size vesicles.^{50,56}

Meanwhile, ISGylation, a post-translational ubiquitin like modification, was found to be associated with the small size vesicles release. It has been found that the induction of ISGylation promotes the fusion of MVBs with lysosomes, leading to the degradation of MVBs and preventing their transport to the plasma membrane.^{50,57}

After MVBs are transported to the plasma membrane, some molecules, such as soluble N-ethylmaleimide-sensitive factor attachment protein receptor, promote the fusion of vesicles with plasma membrane or organelle membrane.⁴⁴ With the help of microtubules, Rabs, and tethering factors, one vesicle soluble N-ethylmaleimide-sensitive factor attachment protein receptor and two target soluble N-ethylmaleimide-sensitive factor attachment protein receptors form four-helix bundles to drive the fusion between MVBs and plasma membranes and lead to the small size vesicles secretion.³⁹

The Uptake of Small Size Vesicles

When small size vesicles directly fuse with the target cell membrane, their contents are released into the cytoplasm, completing their uptake process. Although this direct fusion pathway is thought to be one of the most effective means of cargo delivery into target cells, other mechanisms also exist for small size vesicles uptake, such as endocytosis and direct interaction with target cells surface receptors (Figure 1).^{6,40} Endocytosis is considered to be the main way for cells to take in small size vesicles.¹ Small size vesicles undergo uncoating and subsequently fuse with plasma membrane to enter the cells.⁵⁸ The contents in exosomes need to be released from the vesicles and enter the cytoplasm to exert their functions. Otherwise, they are degraded by lysosomes, recycled within the cell or secreted into the extracellular space.⁵⁹ Due to the poor predictability of this process among different cell types, there are still many unknown aspects regarding the mechanism by which small size vesicles release their contents into the cytoplasm.¹ A proposed mechanism is that small size vesicles fuse with endosomes in a pH-dependent manner, making the plasma membrane permeable and allowing the contents in small size vesicles to reach the cytoplasm.⁶⁰

Physiological Roles of Exosomes

Garbage Carrier

The formation of small size vesicles originates from endocytosis within cells and carries biological information from the parent cells.¹ Due to the characteristics of small size vesicles formation, small size vesicles are regarded as garbage disposers which eliminate unnecessary cellular components, including redundant or misfolded proteins, molecules and metabolic wastes.⁶¹ Surya Shrivastava and Kevin V Morris found that overexpressed RNA and DNA fragments were also collected in small size vesicles and expelled outside the cells, which maintained cellular homeostasis.⁶² These studies indicates that small size vesicles can also be regarded as the garbage carrier in the cells.

Cell Communication

Small size vesicles trigger signal transduction mainly through two primary mechanisms, one is directly interacting with extracellular receptors, the other is internalized by receptor cells and then released cargo.⁴⁰ For directly interaction, the transmembrane ligands on the surface of small size vesicles can directly bind to surface receptors on target cells for signal transduction. For example, human DEX activate T cells through MHC peptide complexes.⁶³ Meanwhile, small size vesicles can also be internalized via various pathways, including clathrin-mediated endocytosis, lipid raft mediated endocytosis, caveolin mediated endocytosis, phagocytosis and macropinocytosis. These processes are not mutually

exclusive and can operate concurrently.⁴⁰ It has been reported that rat bone marrow MSCs internalize PC12-derived exosomes through clathrin-mediated endocytosis and micropinocytosis, which transfers miR-21 from exosomes to bone marrow MSCs and reduces the expression of TGF β RII and TPM1 in bone marrow MSCs.⁶⁴

Cell communication is tightly associated with various disease. For instance, small size vesicles have been found to alleviate the symptoms of metabolic diseases.⁴⁰ Metabolic disorders are characterized by pathogenesis driven by lipotoxicity, chronic inflammation, and oxidative stress, underpinned by dysregulated interorgan communication involving the liver, pancreas, adipose tissue, and immune system.⁶⁵ Metabolic organs, such as pancreas, adipose tissue, skeletal muscle, and liver, secrete small size vesicles by autocrine or paracrine secretion, which transfer to other metabolic organs. These small size vesicles facilitate the intercellular communication and mediate the cross-talk among multiple metabolic organs, finally alleviating the progression of metabolic diseases.⁶⁶

Cell Proliferation and Maturation

Small size vesicles contribute to the reproduction and development of organisms, especially in the formation of germ cells. Studies have shown that supporting cells-derived exosomes in the testes facilitate the entrance of seminiferous tubules to mediate sperm differentiation.^{67–69} Human seminal plasma-derived exosomes can participate in the occurrence, modification, and fertilization of sperm, meanwhile, they are also related to sperm motility, fertilization, and acrosome reactions.⁶⁷ Human follicular fluid-derived exosomes in female ovaries play an important role in physiological processes including cumulus dilation, fertilization, and embryonic development.^{67,70} Human endometrial cells-derived exosomes promote implantation by regulating the proliferation and adhesion of trophoblast cells.⁷⁰ Human endometrial exosomes play an important role in embryonic dormancy. During early embryonic development, endometrial epithelium-derived exosomes induce embryonic diapause by inhibiting the myelocytomatosis viral oncogene homolog / mechanistic target of rapamycin1 and 2 signaling pathways.⁷¹ Human placenta contains-derived exosomes various growth factors, miRNAs and mRNAs, which can participate in regulating the physiological functions of the maternal uterus and fetal development, and are important indicators for detecting placental activity.⁷²

Inflammatory Response

Small size vesicles can also effectively suppress inflammatory response. Human BMSC-exos has been demonstrated to downregulate interleukin-1 β (IL-1 β), interleukin-6 (IL-6), and TNF- α to alleviate the symptoms of Alzheimer's disease in mice.⁷³ Meanwhile, miRNAs in hUCMSC-exos regulates the canonical inflammatory NF- κ B signaling pathway to alleviate chronic skeletal muscle inflammation.⁷⁴ However, not all small size vesicles can alleviate inflammation. Deng et al reported that after peripheral blood monocytes take up exosomes released from adipose tissue of obese mice, they differentiate into activated macrophages, with increased the secretion of TNF- α and IL-6.⁷⁵

Immunoregulation

As a type of nano-vesicle, small size vesicles can activate and regulate immune responses.⁷⁶ DEX contain MHC-I, MHC-II, CD86 and CD86, which contribute to activate CD4+and CD8+T cells, finally leading to anti-tumor immunity.⁷⁷ Small size vesicles secreted by B lymphoma cells subjected to heat shock contained high level of HSP60 and HSP90, along with enhanced expression of immunogenicity-related molecules including MHC-I, MHC-II, CD40 and CD86. These small size vesicles effectively active CD8+ T cells, leading to a potent anti-tumor immune response.⁷⁸ NK cells-derived small size vesicles can directly diffuse into tumor tissues to exert their cell lytic activity and participate in immune responses.⁷⁹ Mast cell-derived small size vesicles upregulate the expression of MHC-II, CD40, CD80 and CD86 in immature DCs, promote antigen-presenting ability of T cells, ultimately activate antigen-specific immune responses.⁸⁰

Small size vesicles are also associated with the migration of tumor cells. Zhao et al reported that miR-934 in human colorectal cancer cells-derived exosomes active macrophages and promote liver metastasis of colorectal cancer.⁸¹ Meanwhile, small size vesicles can also serve as a diagnosis biomarker to detect the type and progression of cancer. A promising diagnostic approach for early cancer detection and metastasis monitoring is non-invasive identification of the location and expression levels of biomarkers in cancer cells. Looking for highly specific and sensitive biomarkers remains a central focus in non-invasive cancer diagnosis.⁸² Advances in small size vesicles research discovered that some

proteins on exosomes can serve as promising biomarkers for cancer. For instance, Melo et al found that Glypican-1 on exosomes in patients' blood had high sensitivity and specificity in the early stage of pancreatic cancer.⁸³ ECM and protease related proteins were highly expressed in non-small cell lung cancer cells-derived exosomes.⁸⁴ All these studies indicated the potential role of small size vesicles in cancers.

Miniature Nanoreactors

It is well known that miniature nanoreactors, which can be prepared by combining enzymes with natural or artificially synthesized materials, displays a significant advantage in treating diseases such as cancer and rare genetic disorders.⁸⁵ Due for the natural nanostructures, small size vesicles may be applied in nanoreactors, carrying a variety of bioactive molecules and possessing strong biocatalytic potential.⁴⁰ Wan et al found that miniature exosomes nanoreactors enhanced the catalytic efficiency of enzymes and improved therapeutic efficacy. They combined M1 phenotype macrophages derived exosomes and zeolitic imidazolate framework-8 nanostructure to construct miniature nanoreactors, which inhibited the activity of *P. gingivalis* by increasing the biosynthesis of nitric oxide and effectively increased the degree of alveolar bone resorption in periodontal disease rats.⁸⁶ Though applied in miniature nanoreactors, small size vesicles fully exerted their biochemical characteristics and strengthened their therapeutic effects. Although studies on miniature small size vesicles nanoreactors remain limited, this application offers a powerful solution for future nano-therapeutic engineering.

Interact with Extracellular Matrix

Extracellular matrix is a proteinaceous network that provides a dynamic and heterogeneous microenvironment.⁸⁷ It has been demonstrated that ECM could mediate the small size vesicles secretion and uptake, meanwhile small size vesicles cargo affects ECM remodeling.⁸⁸ The physical properties of ECM, such as elasticity, also affects the secretion of small size vesicles.⁸⁸ For instance, the number of small size vesicles released by MSCs cultured on soft hydrogel is higher than that released cultured on hard hydrogel or on tissue culture plastic.⁸⁹ Some substances in small size vesicles, such as MMPs and insulin-degrading enzyme, are released into the ECM to participate in the remodeling of the matrix.⁸⁸ Hoshino et al discovered cancer-associated fibroblasts derived exosomes released MT1-MMP, leading to ECM degradation and promoting the maturation and stability of the invadopodia.⁹⁰ Insulin-degrading enzyme in microglia derived exosomes enhanced the degradation of A β in mice treated with lovastatin.⁹¹ All these studies suggest the interaction between small size vesicles and extracellular matrix.

The Application of Mesenchymal Stem Cells-Derived Exosomes in Liver Diseases

Liver is a digestive and metabolic organ and involved in several physiological processes. As a public health problem, liver disease accounts for high morbidity and mortality worldwide.⁹² Liver disease occurs throughout the human lifespan. For example, cholestatic liver disease commonly caused by structural and genetic factors in newborns.⁹³ In pregnant women, hepatitis can be caused by hepatitis virus or herpes simplex virus.⁹⁴ There are many methods used to diagnose liver diseases nowadays, including hematological indicators (alanine aminotransferase, aspartate aminotransferase), liver biopsy, ultrasound, magnetic resonance spectroscopy and transient elastography.⁹⁵

Nowadays, there are many emerging biological therapies for liver diseases treatment, such as miRNA and antibody-drug conjugates.^{96,97} Among these, growing research is exploring small size vesicles therapy as a novel treatment approach for liver diseases. It has been reported the therapeutic effects of small size vesicles on liver diseases, including hemp sprout-derived exosome, tangerine peel-derived exosome and milk-derived exosomes.⁹⁸⁻¹⁰⁰ Additionally, the components of small size vesicles, such as some specific miRNAs and protein, were also found to directly stimulate or inhibit certain genes to alleviate liver disease.¹⁰¹ In Table 1, we summarized the therapeutic effects of contents from some small size vesicles on liver diseases to illustrate their functions.

Small size vesicles also displayed potential capability for the diagnose in liver diseases. In the development of novel, reliable and non-invasive diagnostic strategies for liver diseases, human blood-derived exosomes and human urine-

Table 1 The Role of Contents in Small Size Vesicles in the Treatment of Liver Diseases

Liver Disease	Animal Model	Source	Contents	Function	References
Acute liver injury	CCl ₄ or LPS induced acute liver injury mice model	Human umbilical cord mesenchymal stem cells	miR-455-3p	Inhibit macrophage activation and reduce the expression of inflammatory factors	[102]
Liver injury	Portal vein ligation rat model	Rat liver tissue	Jagged1 protein	Promote liver regeneration through the YAP-Notch signaling pathway	[103]
Liver injury	Cecal ligation and puncture induced septic liver injury mice	Human bone marrow mesenchymal stem cells	ATG2B	Promote mitochondrial absorption and inhibit the release of mtDNA into the cytoplasm and the STING signal transmission in macrophages	[104]
Liver injury	CCl ₄ induced liver injury mice model	Mouse adipose mesenchymal stem cell	Hepatocyte growth factor	Inhibit the expression of liver fibrosis-related proteins and collagen fiber accumulation	[105]
NAFLD	High-fat and high-fructose induced NAFLD rat model	Human umbilical cord mesenchymal stem cells	miR-627-5p	Inhibit rat weight, liver steatosis and improve the liver injury and glucose homeostasis	[106]
NAFLD	High-fat diet induced NAFLD mice model	Honey	miR-5108, miR-5119	Inhibit NF-κB and c-Jun pathway	[107]
NAFLD	High-fat diet induced NAFLD mice model	Human adipose mesenchymal stem cells	miR-223-3p	Inhibit <i>E2F1</i> to reduce lipid accumulation and fibrosis	[108]
NAFLD	Western diet induced NAFLD mice model	Human umbilical cord mesenchymal stem cells	miR-24-3p	Regulate macrophage polarization, promoting the increase of anti-inflammatory macrophages	[109]
Liver fibrosis	CCl ₄ induced liver fibrosis mice model	Human umbilical cord mesenchymal stem cells	miR-148a	Inhibit the occurrence of pro-inflammatory macrophages	[110]
Liver fibrosis	CCl ₄ induced liver fibrosis mice model	Human umbilical cord mesenchymal stem cells	miR-27b-3p	Inhibiti liver fibrosis to reduce YAP activated LOXL2 expression	[111]
Liver fibrosis	CCl ₄ or thioacetamide induced liver fibrosis mice model	Human umbilical cord mesenchymal stem cells	miR-499a-5p	Inhibit GPX4 and promote ferroptosis in activated HSCs	[112]
Liver fibrosis	CCl ₄ induced liver fibrosis mice model	Human Wharton's jelly mesenchymal stem cells	miR-124	Inhibit HSCs activation and switching of splenic monocytes from inflammatory Ly6C ^{hi} to restorative Ly6C ^{lo}	[113]

(Continued)

Table 1 (Continued).

Liver Disease	Animal Model	Source	Contents	Function	References
Liver fibrosis	CCl ₄ induced liver fibrosis mice model	Human liver stem cells	miR-142a-5p	Inhibit the occurrence of pro-inflammatory macrophages and promote the increase of anti-inflammatory macrophages	[114]
Liver fibrosis	CCl ₄ induced liver fibrosis mice model	RAW 264.7 cells	lncRNA Snhg6	Inhibit NF-κB pathway and the activation of HSCs	[115]
Liver fibrosis	Thioacetamide induced liver fibrosis mice model	Human bone marrow mesenchymal stem cells	circCDK13	Inhibit the activation of the PI3K/AKT and NF-κB signaling pathways	[116]
Liver fibrosis	/	Serum of patients with liver failure	circDIDO1	Inhibit the activation of HSCs through miR-141-3p/PTEN/AKT pathway	[117]
Liver failure	LPS/D-galactosamine induced liver failure mice model	Human adipose tissue mesenchymal stem cells	miR-17	Inhibit inflammasome activation in hepatic macrophages	[118]
HCC	Hep3B cell xenograft tumor model in nude mice	Human bone marrow mesenchymal stem cells	miR-15a	Inhibit tumorigenicity and metastasis of HCC cell	[119]
HCC	/	Human umbilical cord mesenchymal stem cells	miR-451a	Inhibit ADAM10 and suppress cell cycle transition, proliferation, migration and invasion of HCC cell	[120]
HCC	LM3 cell xenograft tumor model in nude mice	CSQT-2 and LM3 cells	p120-catenin	Inhibit the proliferation and migration of HCC cells through STAT3 pathway	[121]
HCC	/	Sorafenib-resistant HCC cells	circUPF2	Promote the formation of the circUPF2-IGF2BP2-SLC7A11 ternary complex and reduce iron death sensitivity	[122]
HCC	/	Huh7 cells	GTF2H2	Inhibit the survival ability, migration, tube formation and permeability of HUVECs cells	[123]

derived exosomes have emerged as promising tools for early detection and prognosis.^{101,124} The specific protein and nucleic acid in human blood-derived exosomes and human urine-derived exosomes traverse the blood-brain barrier, effectively mirroring the underlying physiological and pathological status of the liver.¹⁰¹ In Table 2, we summarized the small size vesicles as possible biomarkers in different liver diseases.

From the aspect of liver disease treatment, utilizing stem cells derived small size vesicles from the liver as a treatment method for liver diseases seems to be the best option. Indeed, liver-derived small size vesicles exhibits biological activity and there are several published articles about the function of EVs from liver cells on liver disease.^{143,144} We believe that liver-derived small size vesicles will play a significant role in the diagnosis and treatment of liver diseases in the further. Notably, as one of the most available primary stem cells in the human body, MSCs also exhibits excellent biological activity including anti-inflammatory, anti-fibrotic, immunosuppressive effects and so on. MSCs are an ideal source of small size vesicles due to their less adverse reactions.¹⁴⁵ Interestingly, exosomes secreted by MSCs was also been found

Table 2 Potential of Small Size Vesicles as Biomarkers for Liver Disease Diagnosis

Liver Disease	Source	Small Size Vesicles Contents	Expression	Reference
HBV-Induced liver cirrhosis	Serum	adenosylhomocysteinase	Up	[125]
HBV-Induced HCC	Serum	hsa_circ_0028861	Down	[126]
HBV-related acute-on-chronic liver failure	Plasma	miR-23b-3p, miR-223b-3p, miR-339-5p, tsRNA-20, tsRNA-46	Down	[127]
HCV	Serum	miR-122, miR-199a	Down	[128]
Liver injury	Urine	CD26, CD81, Slc3A1, CD10	Down	[129]
NAFLD	Serum	miR-21	Up	[130]
NAFLD	Serum	miR-192	Down	[131]
NAFLD	Hepatocytes	GLUT1	Up	[132]
ALD	Serum/ plasma	miR-122, miR-155	Up	[133]
ALD	Serum	miR-30a, miR-192	Up	[134]
Liver fibrosis	Serum	miR-103-3p	Up	[135]
Liver fibrosis	Serum	miR-138, miR-143	Up	[136]
Liver fibrosis	Serum	miR-155	Up	[137]
liver cirrhosis	Urine	SC5AC, SC5AA, NPT4, SC5A1, AT1A1	Up	[138]
HCC	Serum	miR-146a, miR-192, miR-221	Up	[139]
HCC	Serum	LINC00635, ENSG00000258332.1	Up	[140]
HCC	Serum	miR-18a, miR-221, miR-222, miR-224	Up	[141]
HCC	Serum	miR-101, miR-106b, miR-122, miR-195	Down	[141]
HCC	Serum	miR-99a	Down	[142]

excellent therapeutic effects in liver disease.^{146,147} Here, we attempt to summary recent advances in the therapeutic applications of MSCs derived small size vesicles for liver diseases.

Effect the Mesenchymal Stem Cells Derived Small Size Vesicles on Liver Injury

Drugs and exogenous toxins may cause liver damage and lesions.¹⁴⁸ Acute liver injury usually refers to damage to liver cells caused by excessive use of drugs, toxic foods and so on. Due to the availability of over 1000 drugs on the market and the development of many new drugs and the synthesis of new chemical substances in recent years, drug-induced liver injury attracts more and more attention. According to statistics, the most common drugs involved in liver injury are anti infection drugs, psychotropic drugs and lipid-lowering drugs.¹⁴⁹ Exclude the drug abuse induced liver injury, other risk factors such as chemical poison also may induce liver injury. Liver injury is usually accompanied by oxidative stress. In the process of liver drug or chemical poison metabolism, there is a decrease in ATP synthesis and an increase in reactive oxygen species (ROS) levels.¹⁵⁰ These drugs or toxic substances interact with cytochrome P450, generating toxic products such as free radicals, which will further bind to proteins and nucleic acids, or cause lipid peroxidation, leading to cell necrosis.^{151,152} When the risk factors persist for a long time, the acute liver injury may develop into liver fibrosis and HCC.¹⁵³

Nowadays, MSCs derived small size vesicles have become a potential therapeutic strategy for alleviating liver injury. MSCs derived small size vesicles promote the hepatocyte proliferation by regulating the cell cycle. Human embryonic stem cell-derived exosomes upregulated the expression of cell proliferation markers in the G1 and S phases to promote cell proliferation in acetaminophen (APAP) or hydrogen peroxide (H₂O₂) stimulated hepatocytes.¹⁵⁴ MSCs derived small size vesicles reduce inflammation by targeting inflammatory-related genes through the miRNAs or suppress the protein levels of inflammatory factor-related signaling pathways to mitigate inflammation response. For example, in the carbon tetrachloride (CCl₄) or lipopolysaccharide (LPS) induced acute liver injury mice, miR-455-3p in hUCMSC-exos inhibited macrophage activation and the production of inflammatory factors to alleviate liver injury.¹⁰² Wu et al found that hUCMSC-exos downregulated the expression of inflammatory factors with upregulation of the ERK1/2 and PI3K/AKT signaling pathway to inhibit oxidative stress-induced cell apoptosis in APAP induced liver injury mice.¹⁵⁵ Recent report suggests that the combined cross-effect of ferroptosis and lactate accumulation stimulated the inflammatory response in acute liver injury mice.^{156,157} Tian et al found that BMSC-exo can also be used as a delivery carrier. By loading MnO₂ and dexamethasone, MSCs-exo/MnO₂@dexamethasone decreased ROS production, which promoted the redox homeostasis and inhibited ferroptosis in the liver. MSC-exo/MnO₂@dexamethasone also reduced lactate levels by generating oxygen and alleviate inflammation.¹⁵⁸ Most engineered small size vesicles can be used for the treatment of liver disease by acting on multiple targets. Just like the small size vesicles nanoparticles designed by Tian et al, they not only regulated the lactate levels to alleviate inflammation, but also reduced oxidative stress and ferroptosis to achieve multi-targeted treatment.

Effect the Mesenchymal Stem Cells Derived Small Size Vesicles on ALD

ALD is a liver disease caused by long-term alcoholism. According to incomplete statistics, men consume 60–80 g of alcohol per day or women consume 20 g of alcohol per day for 10 years or longer, which usually develop to ALD.¹⁵⁹ Individual variation in ALD is primarily attributed to a complex interplay of genetic and environmental factors. ALD involves multiple mechanisms, the most prominent of which is the toxic effect mediated by acetaldehyde, a metabolite of alcohol. In addition, oxidative stress and cytokine mediated inflammatory responses also involved in the progression of ALD.¹⁶⁰ The ethanol metabolism in the liver is mediated by alcohol dehydrogenase and generates acetaldehyde, which is then converted to acetate.¹⁶¹ This progression generates a large amount of nicotinamide adenine dinucleotide (NADH). Excessive NADH lead to metabolic disorders, inhibit the oxidation of fatty acids, and cause alcoholic fatty liver.¹⁶²

Alcohol abuse affects the transcription factors related to lipid metabolism in the liver, including the hepatic fatty acids production and oxidation. It has been found that alcohol increased the synthesis of fatty acids in hepatocytes by upregulating sterol regulatory element-binding protein 1c (SREBP-1c). Knocking off *SREBP-1c* reduced ethanol-induced fatty liver in mice.¹⁶³ Alcohol can also inhibit ECM, leading to a decrease in the activity of p-ACC and carnitine palmitoyltransferase1.¹⁶⁴

Additionally, gut microbiota is also tightly associated with the alcohol metabolism. Kong et al found that when mice suffer from alcoholic fatty liver, the proportion of *Muribaculaceae* significantly decreases, while the number of *Lachnospiraceae*, *Runminococcaceae* and *Erysipelotrichaceae* increases significantly.¹⁶⁵ They also found that alcohol induced gut microbiota imbalance and reduced the expression of enterohepatic protective factors with activating the NF-κB signaling pathway to produce inflammation in the liver.¹⁶⁵

The therapeutic potential of MSCs derived small size vesicles for ALD has not been well explored. Up to now, there are only some small size vesicles from other sources exhibited great potential for ALD treatment. Kim et al found that pomegranate-derived exosomes effectively prevent liver damage by reducing hepatic oxidative stress and apoptosis in mice with alcohol abuse.¹⁶⁶ *Pueraria lobata* root-derived exosomes inhibited alcohol induced ferroptosis and decreased liver lipid accumulation.¹⁶⁷ These results indicate the use of MSCs derived small size vesicles as a potential treatment for ALD.

Effect the Mesenchymal Stem Cells Derived Small Size Vesicles on NAFLD

NAFLD is a metabolic disease with high prevalence worldwide, which has become a parallel epidemic to obesity.¹⁶⁸ NAFLD ranges from the non-alcoholic fatty liver (NAFL) to the more severe non-alcoholic steatohepatitis (NASH).

NAFL typically manifests as steatosis and mild lobular inflammation. NASH includes hepatocyte ballooning degeneration, diffuse lobular inflammation and almost always fibrosis.¹⁶⁹ Without intervention, NAFLD can advance to liver fibrosis, which may further progress to liver cirrhosis and HCC.¹⁷⁰ In addition, NAFLD is closely associated with other diseases. There is evidence to suggest that NAFLD can affect extrahepatic organs and increase the risk of type 2 diabetes mellitus (T2DM), cardiovascular disease (CVD) and chronic kidney disease.¹⁷¹ In NAFLD, the accumulation of lipid in liver is driven by ceramides and diacylglycerols, which have been demonstrated to inhibit insulin signaling and cause hepatic insulin resistance.¹⁷² In addition, lipid overload in pancreatic beta-cells promotes insulin secretion and alters the expression of PPAR α , glucokinase, glucose transporter 2 and proinsulin, which enhances insulin resistance in human body.¹⁷³ Individuals with NAFLD exhibit a specific lipid profile, including elevated triglyceride-rich lipoproteins, low HDL-cholesterol and increased proportion of circulating small-dense particles of LDL-cholesterol. This specific lipid profile causes the occurrence of CVD.¹⁷¹ Exclude T2DM and CVD, there are also studies found the association between NAFLD and colorectal cancer and osteoporosis.¹⁷⁴

Many factors, such as lipid accumulation, endoplasmic reticulum stress, mitochondrial dysfunction, oxidative stress and inflammation, are involved in the progression of NAFLD. These complex and intertwined factors lead to significant individual and clinical differences in NAFLD.¹⁷⁵ Lipid accumulation in NAFLD is characterized by excessive synthesis of triglycerides (TG) in hepatocytes. Many substrates account for TG synthesis such as white adipose tissue, de-novo lipogenesis and the consumption of a high-fat and/or high-sugar diet. However, a significant increase of hepatic lipid accumulation lead to mitochondrial dysfunction, which results in the inflammation response and oxidative stress in the liver. Also, driven in part by lipid accumulation, endoplasmic reticulum stress results in an accumulation of unfolded proteins, triggering the unfolded protein response and ultimately cause cell apoptosis. Similarly, when the levels of lipophilic substances in hepatocytes continue increasing, liver damage will worsen and progress to more severe disease.¹⁷⁶

The therapeutic effect of MSCs derived small size vesicles on NAFLD is manifested through multiple mechanisms, including reducing lipid accumulation, lowering inflammation and reducing oxidative stress. Yang et al found that the calcium/calmodulin-dependent protein kinase 1 in hUCMSC-exos reduced lipid accumulation by inhibiting SREBP-1C mediated fatty acid synthesis and enhancing peroxisome proliferator activated receptor α mediated fatty acid oxidation in NAFLD mice.¹⁷⁷ Nie et al discovered that apical papilla stem cells derived exosomes promoted the expression of genes related to fatty acid oxidation and transport in the liver and simultaneously inhibited genes expression related to fatty acid synthesis to regulate fatty acid metabolism in NASH mice model.¹⁷⁸

In addition, MSCs derived small size vesicles suppress inflammation response in NAFLD. Zhao et al isolated and obtained ADSC-exos from mice epididymal adipose tissue and administered them to obese mice Results found that STAT3 in ADSC-exos activated arginase-1 and induced the anti-inflammatory M2 phenotype in white adipose tissue.¹⁷⁹ Similarly, in mice fed a high-fat high cholesterol (HFHC) diet, hUCMSC-exos treatment decreased the frequency of F4/80⁺ macrophages in the liver and altered macrophage polarization, reducing the M1 pro-inflammatory phenotype with increasing the M2 anti-inflammatory phenotype.¹⁸⁰

In NAFLD, the accumulation of triglycerides leads to excessive production of ROS and oxidative stress. Multiple reports have confirmed that nuclear factor erythroid 2-related factor 2 (Nrf2) participates in cellular antioxidant response.¹⁸¹ Nrf2 translocates from the cytoplasm to the nucleus and activates the expression of antioxidant genes, such as quinone oxidoreductase 1 and superoxide dismutase. Kang et al demonstrated that hUCMSC-exos promote the phosphorylation of Nrf2 to activate quinone oxidoreductase 1 expression in NAFLD mice.¹⁸⁰ Du et al found that hUCMSC-exos reduced ROS production and superoxide dismutase content with decreasing kelch like ECH associated protein 1 and NADPH oxidase 2 expression to inhibit oxidative stress in mice primary hepatocytes stimulated with palmitic acid They also found that miR-24-3p derived from hUCMSC-exos inhibited lipid accumulation, ROS production and inflammatory response in palmitic acid stimulated mice primary hepatocyte.¹⁸²

Exclude these, MSCs derived small size vesicles are found to be involved in some other mechanisms in liver disease. For example, Moayedfard et al discovered that mice ADSC-exos reduce endoplasmic reticulum stress indicators, apoptotic markers and restore autophagy processes in NASH mice.¹⁸³

Though some studies have only reported the therapeutic effects of small size vesicles themselves, some studies found the specific cargo components of the small size vesicles attributed to regulating the underlying mechanism, such as the miRNAs and proteins. Therefore, subsequent therapeutic studies may be conducted by enriching for these components.

Effect the Mesenchymal Stem Cells Derived Small Size Vesicles on Liver Fibrosis

Liver fibrosis is the advanced progression of hepatitis and fatty liver. Several risk factors induce liver fibrosis such as chronic HCV infection, alcohol abuse and NASH.¹⁸⁴ Advanced liver fibrosis results in cirrhosis and liver failure, which often requires liver transplantation.¹⁸⁵ The main characteristic of liver fibrosis is the accumulation of ECM. There are significant alterations about the quantity, quality and distribution of ECM in liver fibrosis. Fibrosis alters the composition of the hepatic ECM, replacing the normal collagen IV/VI in the space of Disse with a collagen I/III- and fibronectin-rich matrix, which triggers sinusoidal capillarization.¹⁸⁴ HSCs activation accounts for the excessive production of ECM. In a physiological state, HSCs are quiescent and non-proliferative. When the liver is stimulated, HSCs differentiate from a quiescent phenotype to an activated phenotype. During this process, a large amount of ECM components and pro-inflammatory mediators are produced. In addition, activated HSCs express a large amount of alpha smooth muscle actin and tissue inhibitor of metalloproteinases 1, promoting the formation of fibrosis.²⁴ Some cytokines, such as transforming growth factor- β (TGF- β), platelet-derived growth factor, chemokines, leptin and adiponectin, are also involved in liver fibrosis. TGF- β is a key factor in the progression of liver fibrosis. TGF- β activate HSCs, transforming them into myofibroblast like cells. This progression stimulates the synthesis of ECM proteins with blocking their degradation pathways. It has been widely accepted that TGF- β signaling pathway is a promising target for treating liver fibrosis.¹⁸⁶ Platelet-derived growth factor is an effective mitogen in HSCs and is upregulated in fibrotic liver. Inhibiting platelet-derived growth factor activity was found to reduce liver fibrosis formation.¹⁸⁷ Chemokines drive inflammation response with promoting the migration of fibrotic cells to the injury site, thereby exacerbating the severity of the injury in liver fibrosis. The interaction between chemokine receptors and ligands plays different role in liver fibrotic pathology. For example, the C-C motif chemokine receptor 5, the C-C motif chemokine receptor 1 and the chemokine (C-X-C motif) ligand 4 act synergistically to promote fibrosis, while the interaction between the chemokine (C-X-C motif) ligand 9 and the chemokine (C-X-C motif) receptor 3 prevents fibrosis.¹⁸⁸ Some cytokines in adipose tissue also regulate the progression of liver fibrosis. Studies have shown that leptin inhibit the progression of liver fibrosis, while adiponectin possess anti-fibrotic activity.¹⁸⁹

Studies indicate that MSCs derived small size vesicles exhibit efficacy in ameliorating liver fibrosis, such as preventing the activation of HSCs and reducing collagen formation. Human ADSC-exos alleviated collagen and liver damage through inhibiting the p38 MAPK/NF- κ B pathway in mice.¹⁹⁰ Wu et al found that human ADSC-exos treatment inhibited HSCs activation and improved glutamine and ammonia metabolism, thereby improving liver ammonia circulation in CCl₄-induced liver fibrosis mice.¹⁹¹ Zhang et al found that human ADSC-exos suppressed LX2 cell activation and inhibited liver fibrosis progression in mice by regulating choline metabolism and inhibiting the PI3K/AKT/mTOR signaling pathway.¹⁹² In addition, hUCMSC-exos was found to downregulate the expression of smoothed and inhibited the HSCs activation, exerting anti-fibrotic effects in the liver.¹⁹³ In line, hUCMSC-exos induced ferroptosis of HSCs by regulating the xCT/GPX4 axis.¹⁹⁴ Human BMSC-exos reduced collagen accumulation and inhibited Wnt/ β -catenin pathway in HSCs.¹⁹⁵

Given the therapeutic role of small size vesicles in liver fibrosis, the small size vesicles miRNA exhibited potential effects on the disease progression.¹⁹⁶ MiRNAs in small size vesicles have shown significant efficacy in treating liver fibrosis. Tian et al found that hUCMSC-exos alleviated liver damage and reduced fibrosis-related phenotypes and they also found that miR-148a enriched in hUCMSC-exos targeted Kruppel like factor 6, which further inhibited the STAT3 signaling pathway to suppress the generation of pro-inflammatory macrophages, thereby contributing to protect against liver fibrosis in mice.¹¹⁰ Qu et al reported that human ADSC-exos alleviated liver fibrosis markers such as collagen I and α -SMA in CCl₄-induced liver fibrosis mice, while, miR-181-5p in human ADSC-exos downregulated Stat3 and Bcl-2 to activate autophagy in HST-T6 cells.¹⁹⁷ Human placenta stem cell-derived exosomes alleviated fibrotic phenotype, and miR-378c in exosomes targeted E3 ubiquitin ligase S phase kinase associated protein 2 to inhibit epithelial mesenchymal transition, inactivating HSCs in mice.¹⁹⁸ 3D human embryonic stem cells-derived exosomes enriched with miR-6766-3p

suppressed HSCs activation through targeting the TGF β RII-SMADS pathway, which ameliorated the liver fibrosis in mice.¹⁹⁹

In conclusion, miRNAs in MSCs derived small size vesicles alleviate liver fibrosis through multiple pathways, including regulating the polarization of M1 macrophages to M2 macrophages, inhibiting the activation of HSCs, reducing TGF- β secretion and promoting autophagy.²⁰⁰ Utilizing miRNAs from small size vesicles as therapeutic strategy provides new insights into the prevention of liver fibrosis.

Effect the Mesenchymal Stem Cells Derived Small Size Vesicles on Liver Cirrhosis

According to statistics, approximately 2 million people worldwide die from liver disease each year, with an estimated 1 million dying from cirrhosis and the other 1 million from other liver diseases such as HCC. Liver cirrhosis is one of the common causes of death worldwide and the third leading cause of death among people aged 45–64.²⁰¹ Liver cirrhosis causes the metabolic disorders in the body leading to an increase in bilirubin and portal vein pressure, along with a decrease in coagulation factors and thrombopoietin.²⁰² Many factors are associated with the progression of liver cirrhosis, such as alcoholism, metabolic liver disease, viral hepatitis, autoimmune hepatitis, primary biliary cholangitis, etc. In some patients, there are more than one factors that accelerates the progression of the disease.²⁰³

There are some studies reported the preventive and therapeutic effects of MSCs derived small size vesicles on liver cirrhosis. MSCs derived small size vesicles reduce the expression of fibrosis-related factors to alleviate liver cirrhosis. Abd Allah et al found that hUCMSC-exos reduced the expression of TGF- β , MMP 9 and α -SMA, which contributed to the amelioration of liver cirrhosis in rat.²⁰⁴ Mardpour et al demonstrated that human embryonic stem cells-derived exosomes alleviated liver cirrhosis in rats by upregulating the expression of collagenase, BCL-2 and anti-inflammatory cytokines, while concurrently downregulating α -SMA and TIMP1.²⁰⁵

It is widely known that MSCs are able to exert their effects via exosomes. There are not enough examples of MSCs derived small size vesicles used for treating liver cirrhosis, however, there are many successful cases of MSCs clinical therapy for liver cirrhosis. Shi et al recruited 219 patients with HBV related decompensated liver cirrhosis for their study Their research results indicate that human umbilical cord MSCs treatment can significantly improve the long-term survival rate and liver cirrhosis in patients.²⁰⁶ Lu et al analyzed 11 clinical trials and found that human MSCs therapy reduced MELD scores compared to the liver cirrhosis group.²⁰⁷ Jang et al demonstrated that transplantation of autologous human BMSCs can treat alcoholic liver cirrhosis They showed that 54.5% patients observed histological improvement according to the Laennec fibrosis system, demonstrating the therapeutic potential of MSCs in liver cirrhosis.²⁰⁸ Though there are several examples about MSCs in liver cirrhosis. MSCs derived small size vesicles exhibits more advantages over MSCs in terms of liver cirrhosis treatment. For instance, MSCs derived small size vesicles eliminate the potential tumor formation, unnecessary differentiation and infection transmission.²⁰⁹ All these indicates the therapeutic potential about potential MSCs derived small size vesicles in liver cirrhosis.

Effect the Mesenchymal Stem Cells Derived Small Size Vesicles on Liver Failure

Liver failure is an acute decompensation of liver function with multiple organ dysfunctions in body and its clinical features are manifested as coagulation disorders, jaundice and ascites.^{210,211} Liver failure divided into four categories: acute liver failure, subacute liver failure, acute non chronic liver failure and chronic liver failure.²¹² The common causes of liver failure include HBV, HAV, hepatitis E virus (HEV) infection, alcohol abuse and bacterial infection.²¹³ Although liver has regenerative ability, liver failure is a serious and irreversible disease.²¹⁴ At present, the main methods for treating liver failure are comprehensive medical care, artificial liver support systems and liver transplantation, but the mortality rate of liver failure is still high.²¹⁰

Although the treatment of liver failure is difficult, there are some examples that demonstrated the great potential of MSCs derived small size vesicles in treating liver failure. MSCs derived small size vesicles alleviate inflammation response in liver failure. For example, mice ADSC-exos reduced the secretion of inflammatory factors by inhibiting the activation of inflammasomes in LPS/GalN induced acute liver failure mice model.¹¹⁸ Zhang et al found that pretreatment of hUCMSC-exos attenuated inflammatory damage in liver and promoted liver tissue repair by inhibiting the activation of the PYD domains-containing protein 3 pathway.²¹⁵ MSCs derived small size vesicles also induced liver regeneration

by upregulating angiogenesis. Human placenta stem cell-derived exosomes effectively increased C-reactive protein levels and upregulated Wnt signaling pathway, which promoted liver regeneration and alleviated liver failure in rat.²¹⁴ Pyroptosis is a form of programmed cell death in hepatocytes and is regarded as an important pathological feature of liver failure.²¹⁶ Xie et al found that miR-423-5p in hUCMSC-exos targeted ZBP1 to inhibit ATP and LPS-induced hepatic pyroptosis in LO2 cells.²¹⁷ MSCs derived small size vesicles restored autophagic flux to protect hepatocyte against death. Lin et al found that let-7a-5p in human BMSC-exos accelerated the nuclear translocation of transcription factor EB (TFEB) and induced the expression of lysosomal genes to promote autophagy, which contributed to the prevention of hepatocyte death.²¹⁸

It is worth noting that liver transplantation remains the only cure for end-stage liver disease.²¹⁹ However, liver transplantation often leads to immune rejection and inflammatory responses. Small size vesicles possess immunoregulatory and anti-inflammatory functions, which alleviate the immune rejection and inflammation after the liver transplantation.²²⁰ For instance, donor DEX, enriched with immunosuppressive factors such as TGF- β 1 and CTLA-4, inhibited the activation of T cells and regulated other immune cells to maintain the immune tolerance.²²¹ DEX also promoted the polarization of macrophages from the pro-inflammatory M1 phenotype to the immunosuppressive M2 phenotype, thereby enhancing the local immunosuppressive microenvironment and alleviating the immune exclusion response.²²² Tian et al discovered that mouse MSC-exos inhibited the expression of pro-inflammatory cytokines and chemokines in the non-heart-beating liver transplantation mouse model and suppressed the pyroptosis of Kupffer cells by regulating the TXNIP-NLRP3-Caspase1 signaling pathway.²²² Liver transplantation is also susceptible to the risk of ischemia-reperfusion injury. Severe ischemia-reperfusion injury requires retransplantation.²²³ Deng et al found that human bone marrow MSCs derived exosomes delivered miR-16-5p to liver and inhibited the expression of SLC39A14 to reduce cell ferroptosis in ischemia-reperfusion injury mice.²²⁴ All these studies suggest that small size vesicles is a potential therapeutic approach to increase the survival rate by alleviating the immune rejection reaction, inflammation and liver damage after liver transplantation.

Effect the Mesenchymal Stem Cells Derived Small Size Vesicles on HCC

HCC is a primary malignancy of the liver and constitutes a major global health problem.²²⁵ Due for the poor prognosis and high mortality rate, HCC is listed as the sixth most common tumor and the third largest cancer. According to statistics, the number of HCC cases continues increasing every year.²²⁶ The risk factors associated with HCC include infection with HBV or HCV, exposure to aflatoxin B1, alcohol abuse, metabolic dysfunction associated steatohepatitis and so on.²²⁷ HCC involves sustained inflammatory damage, hepatocyte necrosis and fibrotic deposition. The development of liver cirrhosis markedly increases the risk of HCC.²²⁸ Nowadays, the treatment methods for HCC include liver resection, liver transplantation, radiation therapy and chemotherapy.²²⁷

Recently, MSCs derived small size vesicles therapy is a promising treatment strategy for HCC. Gao et al found that human ADSC-exos reduced tumor volume, promoting natural killer T-cell anti-tumor response and inhibiting HCC in rat.²²⁹ Xu et al found that miR-451a in hUCMSC-exos suppressed paclitaxel resistance, proliferation, epithelial-mesenchymal transition and cell cycle progression in Hep3B and SMMC-7721 cell through inhibiting ADAM10.¹²⁰ lncRNA FAM99B in hUCMSC-exos inhibited malignant cellular phenotypes and tumorigenesis in HCC.²³⁰ Ma et al found that miR-15a in human BMSC-exos impeded HCC progression via downregulation of SALL4.¹¹⁹ Ding et al found that human BMSC-exos inhibited the migration, invasion and proliferation in HCCLM3 and SK-Hep-1 cells They also reported that miR-374c-5p in human BMSC-exos suppressed epithelial mesenchymal transition in hepatocellular carcinoma by inhibiting the Wnt/ β -catenin pathway.²³¹

Notably, MSCs derived small size vesicles can also be used as a delivery nano-material for the treatment of HCC. Prior study showed that loading miR-122 into ADSC-exos promoted HepG2 and Huh-7 cell sensitivity to sorafenib and 5-fluorouracil.²³² Lou et al found that ADSC-exos effectively delivered miR-199a to Huh-7, SMMC-7721 and PLC/PRF/5 cells to inhibit the development of HCC through mTOR signaling pathway.²³³ MSCs derived small size vesicles can also deliver drugs and others through engineered modification. For example, Liang et al loaded the anti-cancer drug NCTD into human BMSC-exos through electroporation They found that the BMSC-Exos-NCTD delivery system arrested the cell cycle of HCC cells and increased cell apoptosis in rats.²³⁴ Lou et al loaded miR-122 into human ADSC-

exos, which enhanced the growth inhibitory effect of sorafenib on HCC cells, providing a new therapeutic strategy for HCC.²³² In summary, MSCs derived small size vesicles achieve the therapeutic effect on HCC, at least partly, by delivering various types of drugs and others.

Challenges and Future Perspectives

Although the research on EVs has developed rapidly, recent studies have found that not all substances secreted into the extracellular space are carried by EVs.²³⁵ Non-vesicular extracellular nanoparticles (NVEPs) are a type of diverse membrane particles released from cells, and they lack a lipid bilayer membrane.²³⁶ NVEPs can carry and release proteins, RNA and DNA, which have been proven to be the main carriers for the release of bioactive substances.²³⁵ NVEPs include a variety of types, such as albumin, exomeres, lipoproteins, supermers, vault and viral particles. Albumin can bind and transport endogenous molecules, including long-chain fatty acids, bilirubin, heme and vitamin D, which has antioxidant, antithrombotic and immune-regulating functions.²³⁷ Schleicher's research indicates that some patients with liver cirrhosis and acute kidney injury respond positively to albumin treatment, such as a decrease in serum creatinine levels, which provides a basis for subsequent clinical predictions.²³⁸ Lipoproteins are widely known to transport lipids, but they are associated with nucleic acids transportation. High-density lipoprotein and low-density lipoprotein may carry small RNAs such as miRNAs and sRNAs to the target cells.²³⁹ Very low-density lipoproteins transport endogenous lipids to adipose tissue and muscle tissue, while chylomicrons transport exogenous lipids from the intestine to the circulation system.²⁴⁰ Vaults are 41×73 nm particles that contain multiple copies of three proteins and small untranslated RNA.²⁴¹ Infected cells release virus particles.²³⁵ The virus particles are nanostructures composed of one or more structural proteins.²⁴² Biller et al confirmed that virus particles, like EVs, mediated horizontal gene transfer.²⁴³ Exomeres are membrane-like nanoparticles ranging from 28 to 50 nanometers, containing RNA and DNA and metabolic enzymes.²⁴⁴ Exomeres are released under both physiological and pathological conditions in most cells and tissues.²⁴⁵ The size of supermers is smaller than exomeres (22–32 nm), and supermers can efficiently cross the blood-brain barrier.²³⁵ Meanwhile, the metabolic enzyme enriched in the supermers is higher than in the exomeres.²⁴⁶ Many clinically relevant proteins, such as amyloid precursor protein, intercellular mesenchymal epithelial transfer factor and TGF β -induced protein are also highly expressed in supermers.²⁴⁵

Recently, the discovery of supermers promotes the researchers to pay more attention on re-evaluating the function of EVs in eukaryotic cells.²⁴⁷ The enhanced capacity of NVEPs in transporting biological information has raised doubts about the therapeutic efficacy of EVs. However, during the secretion process, cells simultaneously release both EVs and NVEPs, making it difficult to distinguish between them.²³⁵ Therefore, it is necessary to re-examine whether the efficacy of the EVs reported previously comes from the EVs themselves or from NVEP. Nowadays, how EVs can be effectively distinguished from NVEPs remains a challenge. Some scientists have developed various methods in an attempt to solve this problem. A microfluidic platform technology was employed by Mehraji et al for the separation and purification of EVs. This technology effectively separated the EVs according to their size and markedly enhanced the EVs recovery rate.²⁴⁸ At the same time, the nanofluidic cytometry can also identify the size of EVs and sort EVs with specific characteristics.²⁴⁹ Super-resolution microscopy provides higher sensitivity, enabling nanoscale analysis of the size and morphological heterogeneity of EVs.²⁵⁰ 3D hollow fiber bioreactors combined with fast-protein liquid chromatography-based size-exclusion chromatography achieve the separation of EVs and NVEPs.²⁵¹ Although these methods effectively separated and purified EVs and NVEPs, a gold standard method still needs to be established urgently, which then can be used to verify the efficacy of EVs.

Due for the ability of penetrating natural barriers and low toxicity, small size vesicles attract more and more attention both as drugs and drug deliveries.²⁵² However, natural small size vesicles also have significant drawbacks. For instance, their clearance rate in the blood is high, which limits the therapeutic efficacy and application of small size vesicles.²⁵³ Research have shown that modification of small size vesicles or combining small size vesicles with biocompatible and highly adhesive biomaterials maximized the retention of small size vesicles without their rapid clearance.²⁵⁴ Xing et al used heat-sensitive cell-free extracellular matrix hydrogel to sustained release of rat ADSC-exos, which regulated the matrix synthesis and alleviated intervertebral disc degeneration in rats.²⁵⁵ Wan et al utilized light-crosslinked spherical hydrogels to encapsulate bone marrow MSCs derived exosomes from rat, which prevented the exosomes rapid clearance and destruction in blood.²⁵³ Modifying EVs with albumin can also prolong the circulation time of EVs. Liang et al decorated albumin on the surface of EVs and injected them to mice. They found these engineered EVs bound to mouse

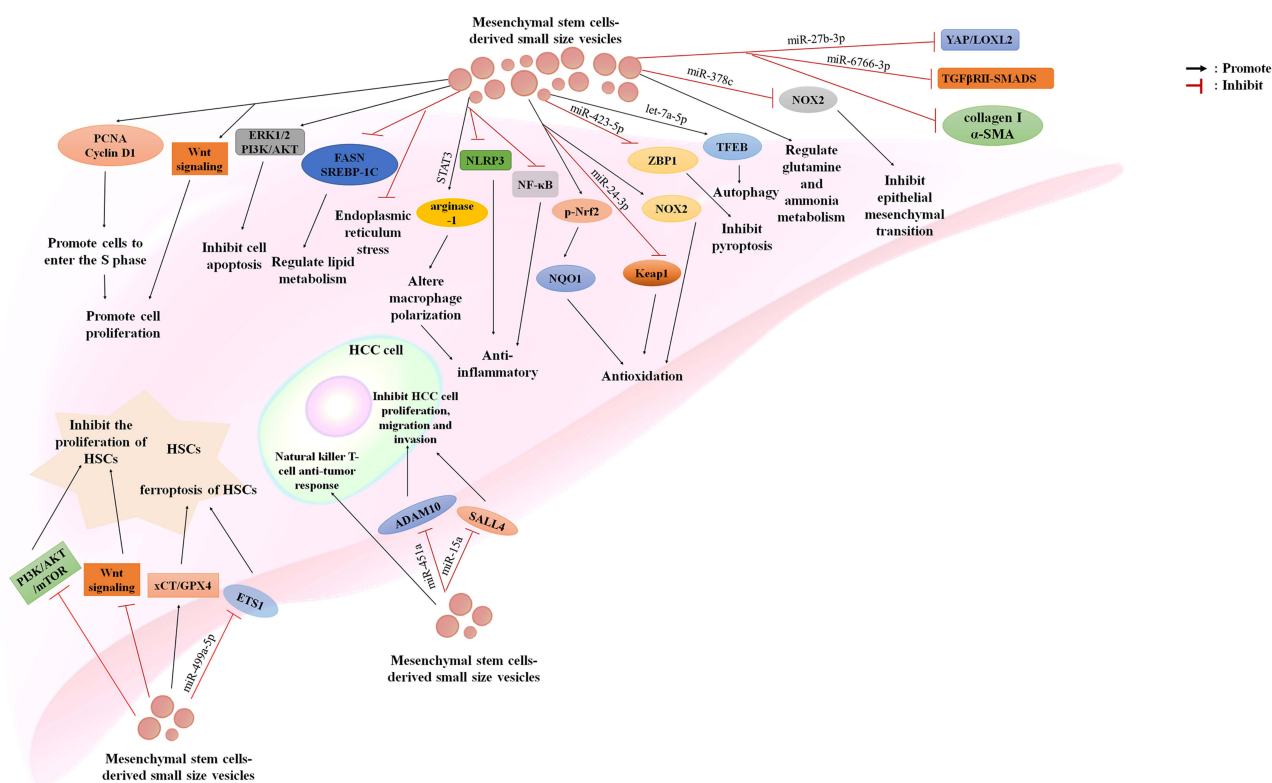


Figure 2 Summary of underlying mechanisms about MSCs derived small size vesicles in the treatment of liver diseases. MSCs derived small size vesicles protect against liver diseases through various mechanisms, such as promoting cell proliferation, inhibiting cell apoptosis and pyroptosis, regulating lipid metabolism and glutamate metabolism, inhibiting endoplasmic reticulum stress, reducing inflammation and oxidative stress, promoting autophagy, inhibiting epithelial-mesenchymal transition, inhibiting the activation of HSCs, promoting ferroptosis of HSCs, inhibiting the proliferation, migration and invasion of HCC cells and promoting the anti-tumor response of nature killer T cells. The black arrows indicate increased levels; the red arrows indicate decreased levels.

Abbreviations: HCC, hepatocellular carcinoma; HSCs, hepatic stellate cells; TGF- β , transforming growth factor- β ; α -SMA, alpha-smooth muscle actin.

serum albumin, significantly prolonging the circulation time of EVs.²⁵⁶ Based on these studies, the rapid clearance of EVs in the blood may be solved by engineered modification. In conclusion, combining EVs with biomaterials enables their continuous release, meanwhile modifying the surface can extend their circulation time. These methods for modifying small size vesicles should be given full attentions to enhance the application of small size vesicles.

As an emerging therapeutic approach for liver diseases, clinical applications based on MSCs derived small size vesicles require more preclinical studies for comprehensive evaluation. The safety and potential side effects of MSCs derived small size vesicles in liver disease treatment should also be thoroughly considered.²⁵⁷ At present, there is no unified standard for the preparation of small size vesicles.²⁰⁰ Therefore, efficient separation and purification methods are needed to distinguish exosomes from NVEPs and others. In conclusion, MSCs derived small size vesicles exhibits promising potential for clinical translation and application. In the future, MSCs derived small size vesicles may be applied as drugs or delivery vectors for multi-targeted therapy, which could not only deepen existing treatment strategies for liver diseases but also emerge as a novel therapeutic option.

Conclusion

Emerging as a promising diagnostic and therapeutic candidate in clinic, small size vesicles have attracted extensive attention in recent years. Liver diseases constitute a significant global health burden and are a leading cause of mortality worldwide. In-depth investigations into cell-free therapy have demonstrated that MSCs derived small size vesicles possess broad application potential in liver diseases, including drug delivery and liver transplantation. MSCs derived small size vesicles ameliorate liver diseases through multiple mechanisms. For instance, MSCs derived small size vesicles promote liver regeneration, regulate lipid metabolism, inhibit inflammation and oxidative stress, suppress the activation of HSCs and reduce the growth of HCC cells (Figure 2). MSCs derived small size vesicles in the treatment of

liver diseases involves multiple types of stem cells, such as human umbilical cord MSCs, human bone marrow MSCs, human adipose MSC, mouse or rat bone marrow MSCs and adipose MSCs. MSCs derived small size vesicles are also be recognized as a kind of biological carrier that can enter target cells, such as HSCs and HCC cells. By transporting cargo, MSCs derived small size vesicles exhibited more potential as an alternative treatment option for various liver diseases. Though there are lots of studies demonstrated the positive effects of MSCs derived small size vesicles on liver disease, the usage of MSCs derived small size vesicles in liver disease is still at the stage of in vitro research and animal levels. Clinical research on MSCs derived small size vesicles in the treatment of liver diseases is still relatively scarce. Exclude the therapeutic effect, small size vesicles can serve as markers for diagnosing the pathological progression of liver diseases. All these underscores the importance of MSCs derived small size vesicles as a key player in the treatment of liver diseases.

Abbreviation

ADSC-exos, adipose mesenchymal stem cell-derived exosomes; ALD, alcoholic liver disease; APAP, acetaminophen; BMSC-exos, bone marrow mesenchymal stem cell-derived exosomes; CCl₄, carbon tetrachloride; CVD, cardiovascular disease; DCs, dendritic cells; ECM, extracellular matrix; EMT, epithelial-mesenchymal transition; ESCRT, endosomal sorting complex required for transport; EVs, extracellular vesicles; H₂O₂, hydrogen peroxide; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; HEV, hepatitis E virus; HFHC, high-fat high cholesterol; HSCs, hepatic stellate cells; hUCMSC-exos, human umbilical cord mesenchymal stem cell-derived exosomes; HUVEC, human umbilical vein endothelial cells; IL-1 β , interleukin-1 β ; IL-6, interleukin-6; ILVs, intraluminal vesicles; LPS, lipopolysaccharide; LSECs, liver sinusoidal endothelial cells; MHC-I, major histocompatibility complex-I; MHC-II, major histocompatibility complex-II; MMP 9, matrix metalloproteinase 9; MSCs, mesenchymal stem cells; MSCs-exos, mesenchymal stem cells-derived exosomes; MVBs, multivesicular bodies; NADH, nicotinamide adenine dinucleotide; NAFL, non-alcoholic fatty liver; NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; NVEPs, non-vesicular extracellular nanoparticles; NK, natural killer; Nrf2, nuclear factor erythroid 2-related factor 2; ROS, reactive oxygen species; SREBP-1c, sterol regulatory element-binding protein 1c; T2DM, type 2 diabetes mellitus; TEXs, tumor-derived exosomes; TGF- β , transforming growth factor- β ; TNF- α , tumor necrosis factor α ; TSG101, tumor susceptibility gene 101; Vps, class E vacuolar protein sorting; α -SMA, alpha-smooth muscle actin.

Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

Funding

This research was supported by Hebei Natural Science Foundation (grant number H2025205003) and Science Foundation of Hebei Normal University (grant numbers L2024K03).

Disclosure

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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