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MSC-derived exosomes for small RNA delivery in disease treatment: a narrative review

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Highlights:

- Summary of the biological functions of MSC-EXO.
- Summary of the latest research progress of MSC-EXO as a drug delivery vector.
- This review focuses on the clinical application of MSC-EXO in the treatment of various diseases.

Abstract: Mesenchymal stem cells (MSCs) are known for their ability to differentiate and self-renew, playing a critical role in tissue homeostasis and repair. Despite their therapeutic potential, clinical applications of MSCs face challenges, including safety concerns and uncertain effects on tumors. In contrast, MSC-derived exosomes (MSC-EXOs) have shown comparable or superior efficacy across various diseases, primarily due to their cargo of functional RNAs and proteins. These natural nanovesicles offer a promising drug delivery platform, combining the advantages of both MSCs and exosomes. Genetic engineering approaches, such as surface modification and drug loading, further enhance their therapeutic capabilities. Small RNA drugs present novel opportunities for expanding therapeutic targets, but efficient delivery remains a significant challenge. MSC-EXOs, either natural or engineered, provide a safe and effective solution for delivering small RNA drugs, holding great promise for both research and translational applications. However, large-scale production of MSC-EXOs remains a key hurdle, and ongoing efforts focus on optimizing strategies for producing high-quality MSC-EXOs in sufficient quantities for industrial and clinical use. This review examines the role of MSC-EXOs in small RNA drug delivery, highlighting the associated challenges and potential solutions for scalable production.

Keywords: mesenchymal stem cell-derived exosomes (MSC-EXOs); small RNA; drug delivery systems; large-scale production

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1. Introduction

Mesenchymal Stem Cells (MSCs), a type of adult stem cell, also known as multipotent mesenchymal stromal cells, are multipotent stem cells with the capacity for differentiation and self-renewal [1]. According to the definition by the International Society for Cell and Gene Therapy (ISCT), MSCs must meet three criteria: (1) adherence to plastic culture dishes during *in vitro* cultivation; (2) expression of surface antigens CD73, CD90, and CD105, while lacking CD34, CD45, CD14 or CD11b, CD79α or CD19, and HLA-DR; (3) ability to differentiate into various mesodermal cell types, such as adipocytes, chondrocytes, and osteoblasts, during *in vitro* culture [2]. MSCs derived from the bone marrow were first isolated by Friedenstein and colleagues in 1996. They constitute a functional component of the hematopoietic stem cell niche, promoting hematopoiesis and participating in the maintenance of hematopoietic stem cell homeostasis [3]. In addition to the bone marrow, MSCs can be isolated from various tissues or organs, such as the umbilical cord, placenta, lungs, muscles, and bones. Overall, MSCs play a crucial role in regulating tissue homeostasis and maintaining tissue integrity [1].

MSCs are easily accessible, genetically stable, immunogenically low, and exhibit significant therapeutic potential [4]. For instance, in a murine model of myocardial infarction, local injection of MSCs has been demonstrated to promote cardiomyocyte and vascular regeneration [5]. Systemic administration of MSCs via tail vein injection can restore neurological function in stroke rats through the release of trophic factors and induction of neurogenesis [6]. In a collagen-induced arthritis mouse model, a single intraperitoneal injection of MSCs can prevent severe, irreversible damage to bone and cartilage [7].

In clinical research, MSCs have been applied in the treatment of severe osteogenesis imperfecta, acute and severe graft-versus-host disease, and other diseases, yielding promising results [3]. However, MSCs still exhibit limitations. Firstly, MSCs are not a homogeneous population but rather a heterogeneous mixture of cells with different morphologies and functions. These cells may exert opposing immunomodulatory effects and possess varying proliferative and differentiation capacities. This high heterogeneity may weaken the overall therapeutic efficacy of MSCs or complicate the interpretation of their therapeutic mechanisms [1]. Secondly, the pro-inflammatory and anti-inflammatory phenotypes of MSCs demonstrate opposing effects on tumors. Pro-inflammatory MSCs induce anti-tumor immune responses, suppressing tumor growth by inhibiting Wnt, AKT, and PI3K pathways to slow cancer cell proliferation. Conversely, anti-inflammatory MSCs can inhibit anti-tumor immunity, induce epithelial-mesenchymal transition, stimulate tumor angiogenesis, and promote drug resistance in tumor cells [1].

MSC-derived exosomes (MSC-EXOs) is a crucial component secreted by MSCs. Similar to MSCs, MSC-EXOs exhibits characteristics of immune regulation, promotion of angiogenesis, and tissue regeneration. It has been demonstrated to possess equivalent or even superior therapeutic efficacy compared to MSCs in various injury and disease models [8]. For example, Zhi *et al.* highlighted that umbilical cord-derived MSC-EXOs (UC-MSC-EXOs) can ameliorate clinical symptoms of colitis in mice, alleviate colon damage, and reduce inflammatory states. Shao *et al.* found that in a rat model of acute myocardial infarction, MSC-EXOs demonstrated superior cardiac repair effects compared to MSCs. In a rabbit skin wound model, the efficacy of subcutaneously injected adipose and bone marrow-derived MSC-EXOs was superior to that of intravenous injection of MSCs. Another preclinical study

suggested that MSC-EXOs has beneficial effects in improving neurogenesis and cognitive function in a mouse model of Alzheimer's disease [9].

Interestingly, the impact of MSC-EXOs on tumors appears to be a double-edged sword. MSC-EXOs from different sources, administered at varying doses or through different routes, may exert completely opposing effects on different types of malignant tumors. For instance, Zhu *et al.* found that in a xenograft mouse model of gastric and colorectal malignancies, human bone marrow-derived MSC-EXOs (BM-MSC-EXOs) stimulated the ERK1/2 and p38 MAPK pathways, increasing the expression of VEGF and CXCR4 in tumor cells, thereby promoting angiogenesis and tumor growth [10]. Conversely, Wu *et al.* discovered that UC-MSC-EXOs induced apoptosis and cell cycle arrest in T24 bladder cancer cells by upregulating caspase-3 expression and reducing Akt phosphorylation [11].

MSC-EXOs, possessing the advantages of exosomes while inheriting the characteristics of MSCs, has been widely utilized as a nanocarrier for drug delivery in the treatment of various diseases, including tumors, neurodegenerative disorders, and immune-related conditions, yielding promising results in recent years. Furthermore, an increasing number of studies are dedicated to modifying MSCs or their derived exosomes to load small RNA drugs, addressing the challenges associated with small RNA drug delivery to a certain extent and optimizing the therapeutic potential of MSC-EXOs.

In this review, we will first introduce the functional components within MSC-EXOs that contribute to therapeutic effects, followed by an overview of the advantages and limitations of MSC-EXOs as a novel drug delivery system. Subsequently, we will summarize and discuss the progress in research on modifying MSC-EXOs as small RNA drug carriers in disease treatment, as well as the bottlenecks and breakthroughs faced by MSC-EXOs in clinical applications.

Our review offers several key novel contributions. First, we systematically analyzed the potential of MSC-EXOs in the treatment of various diseases, particularly in cancers, spinal cord injury, and chronic wounds. Second, we highlighted the unique advantages of MSC-EXOs based on comparing the properties of several nanoscale drug delivery systems. Furthermore, we discussed new methods to enhance the yield and functionality of MSC-EXOs, which are vital for optimizing exosome-based therapeutic delivery.

Currently, researches on MSC-EXOs develop rapidly, but there are still challenges in translating them from laboratory to clinical settings. Our review timely summarizes the latest research progress of MSC-EXOs and points out the potential path from bench to bedside. In addition, we emphasize the importance of standardized protocols, which is crucial for the progress of future research.

Our review not only summarizes the existing knowledge of MSC-EXOs but also proposes new research directions and challenges. For instance, we discuss how to improve the targeting capabilities of exosomes through engineering approaches, and also raise concerns that the expression of certain therapeutic small RNA might also modify other properties of MSC-EXOs. We also emphasize the challenges in the large-scale production and purification of exosomes and propose possible solutions.

2. Functional components of MSC-EXOs

MSC-EXOs primarily exerts its effects through the transmission or modulation of various regulatory factors, including mRNA, miRNA, growth factors, cytokines, chemokines, among others [12] (Figure 1). Tomasoni *et al.* discovered that the proliferative effects of MSC-EXOs on proximal tubular cells are partially mediated by insulin-like growth factor-1 receptor mRNA [13]. MSC-EXOs contains a

substantial amount of angiopoietin-1 (Ang-1) mRNA, playing a crucial role in vascular stabilization and anti-inflammatory processes. Tang *et al.*, using Ang-1 mRNA-depleted MSC-EXOs, demonstrated that the therapeutic effects of MSC-EXOs on acute lung injury and their immunomodulatory effects on macrophages are partially mediated by Ang-1 mRNA for the first time. However, the mechanism of MSC-EXOs impact on macrophages modulation will need to be further elucidated and the crosstalk between MSC-EXOs and other immune cells remains to be studied [14]. Collino *et al.*, through the use of MSCs with Drosha knockout, obtained MSC-EXOs with widespread downregulation of miRNA, proving that miRNA is a key effector in the therapeutic action of MSC-EXOs [15]. Wang *et al.*'s research indicated that miR-223 is highly enriched in MSC-EXOs. Wild-type MSC-EXOs exhibited significant protective effects against cecal ligation and puncture (CLP)-induced cardiac dysfunction, cell apoptosis, and inflammatory responses. However, the protective effects disappeared in miR-223 knockout MSC-EXOs, leading to exacerbated inflammation and cell death due to the upregulation of miR-223 target genes Sema3A and Stat3. Their findings for the first time suggest that MSC-induced protective effects on sepsis may be largely dependent on exosomal miR-223. However, other miRNAs/proteins include in MSC-EXOs may also be a contributor, which should be further evaluated [16]. Jahangiri *et al.* found that BM-MSC-EXOs is rich in miR-100 and miR-143. The transfer of miR-100 from BM-MSC-EXOs to colon cancer cells effectively downregulated the expression of mTOR, Cyclin D1, K-RAS, HK2, and upregulated p-27. Through the miR-100/mTOR/miR-143 axis, there was a significant inhibition of epithelial-mesenchymal transition-related factors (such as SNAIL and TWIST) and metastasis-related genes (such as MMP2 and MMP9), effectively suppressing proliferation, migration, and invasion of colon cancer cells while inducing apoptosis. Their study advances the current understanding of the molecular crosstalks between the exosome contents and cancer cell behavior. However, they mainly focused on the miR-100/mTOR/miR-143 axis. An investigation on other genes/proteins that play a role in colon cancer progression may be necessary [17].

In addition to RNA, the abundant proteins in MSC-EXOs are equally crucial factors mediating their therapeutic effects. Wnt4 present in UC-MSC-EXOs can promote β-catenin nuclear translocation, enhancing its activity, thereby stimulating the proliferation and migration of skin cells. MSC-EXOs lacking Wnt4 fails to activate β-catenin, significantly diminishing their *in vivo* therapeutic efficacy. Nevertheless, the exosomal components are very complicated, so it requires further studies to determine whether there are other molecules enhancing the activity of Wnt/β-catenin signaling in wound healing [18]. Ahn *et al.* demonstrated that MSC-EXOs effectively alleviates neonatal hyperoxic lung injury, with this protective effect primarily mediated by vascular endothelial growth factor (VEGF) present in MSC-EXOs. This is the first study demonstrating that MSC-EXOs are as effective as MSCs in attenuating neonatal hyperoxic lung injuries, indicating MSC-EXOs may be used as a safe and effective cell-free therapy for bronchopulmonary dysplasia. However, whether the transplantation of VEGF-overexpressing MSC-EXOs enhance the beneficial effects of MSCs in bronchopulmonary dysplasia model remains to be studied [19]. Shen *et al.* found that downregulating the membrane receptor CCR2 weakens the protective effect of MSC-EXOs on renal ischemia-reperfusion injury. CCR2 may serve as a decoy to inhibit CCL2 function, suppressing the recruitment and activation of macrophages, playing a critical role in inflammation regulation and renal injury repair. The role of the CCR2 positive exosomes may increase our understanding of the protective effect of MSC-EXOs and may provide a novel target for therapeutic studies [20]. Vrijsen *et al.* reported that the potent pro-angiogenic effect of MSC-EXOs is mainly

mediated by extracellular matrix metalloproteinase inducer (EMMPRIN). EMMPRIN is a mediator in cell migration and angiogenesis, by activating and upregulating MMPs and VEGF. As a coreceptor for VEGFR2, EMMPRIN also positively mediate VEGF signaling. Increased levels of EMMPRIN on the recipient endothelial cells by exosomal uptake could therefore increase the levels of MMPs and VEGF, enhance VEGF signaling and thus induce angiogenesis [21].

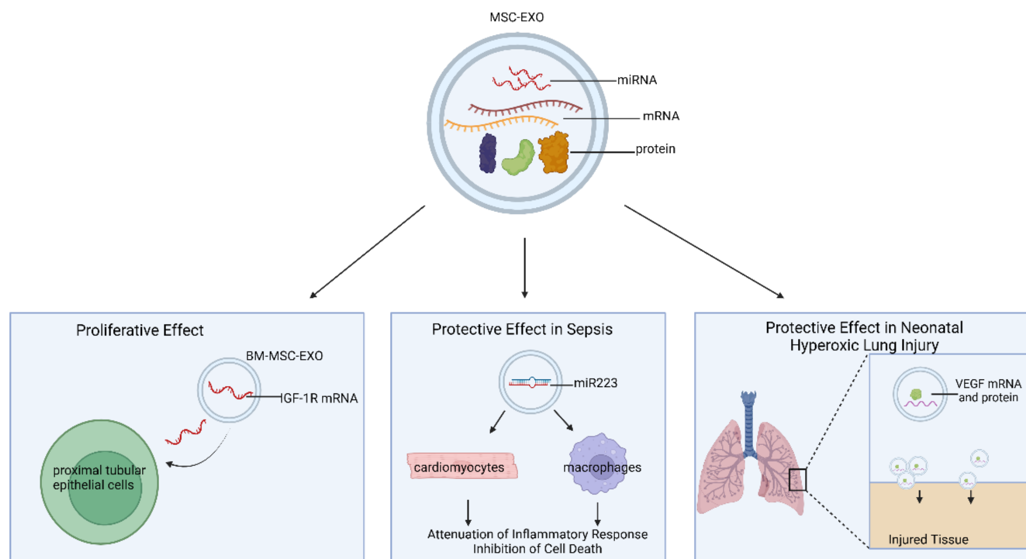

Figure 1. Functional components of MSC-EXO include miRNA, mRNA and protein. There are some examples of how MSC-EXO exert its effects through the transmission of various functional components [12,13,16,19].

3. MSC-EXOs as a novel drug delivery system

Exosomes, as naturally released nanovesicles from cells, represent an excellent drug delivery system. Firstly, they possess high penetrability, capable of traversing various physical and biological barriers such as the blood-brain barrier (BBB), blood-retinal barrier, and various barriers within the tumor microenvironment. Therefore, they hold immense potential in the treatment of ophthalmic conditions, central nervous system diseases, and cancers. Secondly, in comparison to traditional artificial nanocarriers like liposomes and lipid nanoparticles, exosomes have the advantages of low immunogenicity and high safety [22]. Exosomes share significant structural and molecular similarities with cell membranes, enabling them to evade phagocytosis by immune cells [23]. Thirdly, the lipid bilayer membrane structure of exosomes also serves to protect loaded drugs from degradation [24]. Fourthly, exosomes are easily amenable to engineering modifications, including surface alterations through genetic engineering, chemical modifications, and the loading of therapeutic substances such as drugs, proteins, miRNA, siRNA, mRNA, *etc.*, into their interiors through methods like co-culturing, electroporation, ultrasonication, freeze-thaw cycles, and others [25]. Instead, liposomes primarily deliver their cargos through passive accumulation, and their complex functionalization has not succeeded in clinical trials [26].

MSC-EXOs has become a “darling” in the academic community, with a rapidly growing body of research in recent years on its mechanisms of action and therapeutic potential against various diseases. As an emerging drug delivery system, MSC-EXOs possesses several unique advantages:

(1) Abundant Source and High Ethical Feasibility: Exosomes can be produced by various cell types, such as immune cells, tumor cells, and MSCs, with MSCs being particularly rich in exosome production. Besides, MSCs have significant expansion potential for scalable, commercially viable exosome manufacturing and can be derived from various ethically uncontroversial human tissues [26].

(2) Inherent Tumor Homing Properties: MSCs naturally possess the ability to homing towards tumor tissues or the tumor microenvironment, attracted by various chemotactic factors released by the tumor. MSC-EXOs inherits this property, making them promising in anti-cancer therapies [26].

(3) Immunomodulatory Effects: MSC-EXOs exhibit immunosuppressive activities and immunomodulatory properties, which would prolong the shelf life of MSC-EXOs-based drug delivery vehicles and enhance the bioavailability of their payloads [26].

(4) High Safety: Compared to MSCs, MSC-EXOs do not undergo self-replication, eliminating potential tumorigenic risks associated with MSCs. Additionally, MSC-EXOs are smaller in size, exhibit lower or no expression of membrane surface tissue antigens, and are less prone to induce immune responses. The immunoevasive property of MSC-EXOs allows for repeated treatments without patients developing immunity to the carriers [26].

Despite the numerous advantages of MSC-EXOs as a new drug delivery system, several challenges still exist. For instance, exosomes may be susceptible to viral contamination during the extraction process. Due to the similar sizes of viruses and exosomes, viral contamination may occur during the extraction process, with viruses integrating their genetic material and proteins into exosomes. Furthermore, standardization of clinical-grade MSCs *in vitro* culture still needs improvement. Studies have indicated that the use of fetal bovine serum in the *in vitro* culture of MSCs may alter their phenotype, rendering them immunogenic. Additionally, the substances loaded into MSC-EXOs may vary significantly under different culture conditions [24].

RNA delivery efficiency is another issue that needs to be considered. For RNA in exosomes to be functionally active within recipient cells, they must enter the cytoplasm. However, if taken up through endosomal pathway, they often face degradation in lysosomes, suggesting endosomal escape is crucial. It's possible that exosomes have natural mechanisms to facilitate this escape. Additionally, modifying exosome membranes (such as incorporating fusion proteins) to enhance fusion with the cell membrane may facilitate direct release of RNA into the cytoplasm, thereby improving RNA’s escape from endosomes. Ideas can also be sought from bacteria and viruses. Viruses like adeno-associated virus and influenza virus, along with some bacteria, use pH-sensitive fusion proteins or pore-forming toxins to exit the endosome after uptake. Such proteins could be engineered into exosome membranes [27,28].

To the best of our knowledge, there is currently no systematic comparison of MSC-EXOs and other carriers (like exosomes from other cells or artificial nanocarriers) in terms of RNA delivery efficiency, especially endosomal escape efficiency. However, given their distinct lipid and protein profile, exosomes can naturally integrate with cellular membranes to directly release their contents into the cytoplasm. The complex surface proteins of MSC-EXOs allow it to be easily modified, further enhancing

the endosomal escape efficiency of the delivered RNA. Therefore, we speculate that MSC-EXOs represent a promising avenue for coping with the delivery dilemma of small RNAs [27,28].

4. Modified MSC-EXOs as a small RNA drug delivery system in disease treatment

siRNA, upon loading onto the RNA-induced silencing complex (RISC) through its antisense strand, binds to target mRNA, leading to the degradation of the target RNA. On the other hand, miRNA mimics, resembling endogenous miRNAs, function by degrading target mRNA or inhibiting its translation, thereby interfering with its function. Compared to traditional drugs, small RNA drugs have advantages such as endogenous control, strong targeting, a rich pool of candidate targets, and simplified design [29,30]. MSCs possess the capability to secrete a significant amount of exosomes. In higher mammals, a naturally occurring exosomal small RNA transport system is present in the body. Consequently, the use of MSC-EXOs for delivering small RNA drugs has gained substantial attention due to its safety, efficiency, and convenience. Ongoing research in this area continues to advance [31] (Tables 1–3).

4.1. Application in antitumor therapy

4.1.1. siRNA drug delivery

MSC-EXOs have been widely used as vehicles for the delivery of siRNAs in the treatment of various malignancies (Table 1). Take pancreatic cancer as an example. As the "king of cancers" in the digestive system, pancreatic cancer is characterized by insidious onset, low early diagnosis rate, high surgical mortality, and a propensity for metastasis. Pancreatic cancer has one of the lowest five-year survival rates and poorest prognoses among malignant tumors. In recent years, there has been a significant increase in incidence and mortality rates, posing a significant burden on patients and society. Therefore, there is an urgent need to develop effective new therapies [32].

Zhou *et al.* utilized modified MSCs derived from the bone marrow as a siRNA drug delivery system for immunotherapy in pancreatic cancer. The exosomes were isolated from the supernatant of bone marrow derived-MSCs, followed by differential centrifugation and sucrose gradient separation. siRNA targeting galectin-9 was loaded into exosomes by electroporation using a Gene Pulser X Cell Electroporation System, and oxaliplatin (OXA) was modified on the surface of exosomes, resulting in iEXO-OXA. OXA was used to trigger immunogenic cell death (ICD) at the tumor site, and it killed tumor cells by inhibiting DNA synthesis and repair. The siRNA was employed to reverse the immunosuppression caused by M2-type tumor-associated macrophages. They demonstrated enhanced cellular uptake efficiency and improved primary tumor-targeting ability of BM-MSC-EXOs. Several possible mechanisms of exosome internalization have been reported in literature, including vesicle-cell fusion, endocytosis, micropinocytosis and phagocytosis, but what mechanism mediates exosome internalization in their setting needs further exploration. In vivo and *in vitro* experiments indicated that iEXO-OXA induced antitumor immunity and exhibited superior therapeutic effects. Subsequent preclinical and clinical studies are highly anticipated [33].

For a long time, the presence of the BBB has posed significant challenges in delivering drugs to the brain. Therefore, the treatment of brain tumors remains a crucial topic in current research. Haltom *et al.*, by modifying BM-MSC-EXOs to encapsulate siRNA targeting Myc, created iExo-Myc. Specifically, up

to 4×10^9 total exosomes were mixed with 4μg Myc siRNAs via electroporation. In their previous studies, they observed a siRNA loading efficiency of approximately 100% [34]. While precise quantification of siRNA loading per exosome remained a challenge, based on these data, they anticipated that the majority of siRNA was associated with exosomes. They confirmed the inhibitory effects of iExo-Myc on the growth, proliferation, and angiogenesis of late-stage glioblastomas. Transcriptomic analysis of the tumor showed that the inhibition of Myc affected the mesenchymal transition and estrogen receptor signaling pathways. Single-nucleus RNA sequencing (snRNA-seq) revealed that iExo-Myc treatment led to the transcriptional suppression of various growth factor and interleukin signaling pathways, causing the transformation of tumor cells from a hazardous mesenchymal phenotype to a more favorable pro-neural phenotype. Interestingly, their data also suggest that MSC-EXOs may pass through the BBB, as they demonstrated iExo-Myc localized to tumors after intravenous administration [35]. Currently, our understanding of how exosomes are trafficked *in vivo* are limited, thus it may be necessary to evaluate the ability of exosomes to penetrate the BBB and the possibility of exosomes accumulating in the brain without crossing the BBB.

The tumor homing property of MSCs is largely mediated by chemotactic factors on their surface, with CXCR4 being one such factor. Xu *et al.* transfected 293T cells with a plasmid encoding the CXCR4 gene, collected the cell supernatant containing CXCR4 RNA for lentivirus infection of normal MSCs, obtaining exosomes with high CXCR4 expression. Subsequently, they loaded siRNA targeting the Survivin gene into these exosomes via electroporation (80 μL of 1 mg mL^{-1} exosome solution was mixed with 4.8 μL of 20 mM siRNA in an RNA-free tube), resulting in $CXCR4^{high}$ Exo/si-Survivin. In vitro and *in vivo* experiments demonstrated that $CXCR4^{high}$ Exo/si-Survivin could accumulate at the tumor site and effectively inhibit tumor growth. Moreover, they explored the cellular targeting and cellular escape mechanism of $CXCR4^{high}$ Exo. They found that $CXCR4^{high}$ Exo tend to migrate to tumour cells in large amounts and effectively release the encapsulated drug for a long period of time. To explore how exosomes accomplish drug release, tumour cells were cocultured with exosomes and stained for lysosomes at different times. They found that in the early stage, exosomes enter the cells, most of the exosomes overlap with lysosomes, and a decrease in the overlapping portion of exosomes and lysosomes appears after 4 h, which suggests the escape of exosomes. Exosomes continue to escape from lysosomes as time progresses, and most of the exosomes gather in the inner side of the cell membrane and barely overlap with lysosomes by 24 h. To some extent, this result confirmed that exosomes released the loaded siRNA through the lysosomal pathway [36].

Furthermore, siRNA has been reported to exert antitumor effects by enhancing tumor cell sensitivity to drugs. For example, GRP78 has been shown to be associated with sorafenib resistance in hepatocellular carcinoma (HCC). Li *et al.* modified bone marrow-derived MSCs to express the exosomal siGRP78 by transfection using lipofectamine 2000. The resulting exosomes can overcome sorafenib resistance in liver cancer cells, and the combination of these exosomes with sorafenib inhibits the growth and invasion of liver cancer cells [37]. These data offer a solid foundation for employing siGRP78-modified exosomes in a therapeutic approach aimed at overcoming Sorafenib resistance in HCC patients. Moreover, results from this study might have an impact on other types of tumors, such as colon cancer and gastric cancer, where GRP78 is abundantly expressed.

Table 1. Examples of siRNAs delivered by MSC-EXOs in different cancers' treatment.

Cancer type	siRNA	Effects	References
Pancreatic Cancer	Galectin-9 siRNA	Reverse immunosuppression	[33]
Glioblastomas	Myc siRNA	Inhibit growth, proliferation, and angiogenesis	[35]
Lung and gastric cancer	Survivin siRNA	Inhibit tumor growth	[36]
Hepatocellular carcinoma	GRP78 siRNA	Suppress sorafenib resistance	[37]

4.1.2. miRNA drug delivery

In addition to siRNA, MSC-EXOs overexpressing various miRNAs are increasingly being applied in anti-tumor therapy (Table 2). For instance, Vakhshiteh *et al.* transduced dental pulp-derived MSCs using XMIRXpress-34a lentivectors and confirmed that dental pulp-derived MSC-EXOs (DP-MSC-EXOs) overexpressing miR-34a significantly promoted apoptosis in breast cancer cells MDA-MB-231, inhibiting their invasion and migration. However, the miR-34a-enriched exosomes are still required to be investigated for *in vivo* function and tumor-homing capacities [38]. O'Brien *et al.* obtained exosomes enriched with the effective breast cancer inhibitor miR-379 (EXO-379) by infecting MSCs with lentivirus. In vivo experiments showed that MSCs overexpressing miR-379 did not significantly inhibit tumor growth, while EXO-379 exhibited a good inhibitory effect, which raise interest in the potential for therapeutic application. Nevertheless, it is important to note that in this research, the particle dosage was determined by using Nanoparticle Tracking Analysis (NTA) to ensure greater precision and reproducibility. However, this may not reflect a clinically relevant dose. Dose escalation studies are crucial for establishing an effective and clinically relevant dosage of exosomes [39]. Liu *et al.* identified miR-138-5p as a suppressor of bladder cancer. Through lentiviral infection, they obtained adipose-derived MSCs stably expressing miR-138-5p and their derived exosomes (Exo-miR-138-5p). In vitro experiments demonstrated that Exo-miR-138-5p could inhibit the proliferation, migration, and invasion of bladder cancer cells. In a nude mouse subcutaneous tumor model, intravenous and intratumoral administration of Exo-miR-138-5p reached the tumor tissue, delivering miR-138-5p and significantly inhibiting tumor growth [40]. It is noteworthy that they carefully compared the therapeutic effects of intratumoral and intravenous administration in the same tumor and found that the intratumoral approach yielded a more pronounced efficacy. This could be attributed to the local enrichment of exosome-carried drugs resulting from the targeted injection. Furthermore, in order to increase the concentration of therapeutic exosomes in tumor tissues, it may be useful to conjugate a specific bladder cancer-targeting sequence to exosome membranes. Katakowski *et al.* transfected MSCs with a miR-146b expression plasmid, and harvested exosomes released by the MSCs. They confirmed that intratumoral injection of MSC-EXOs overexpressing miR-146b (miR-146b-exo) significantly inhibited the growth of primary brain tumors in rats. Nevertheless, further research is required to fully characterize the delivery of exosomal miRNA to brain tumors. In addition, the expression of miR-146b might also modify the non-miRNA properties of MSC-EXOs. Since these alterations could influence the therapeutic efficacy of miR-146b-exo, the effect of miR-146b expression upon MSC-EXOs warrants further investigation [41]. Li *et al.* first identified that adipose-derived MSCs with low CD90 expression and their derived exosomes significantly inhibited tumor growth in mice. They loaded miR-16-5p mimic

into CD90 low-expressing adipose-derived MSC-EXOs (ADSC-EXOs) using membrane fusion via liposomes, confirming that the modified exosomes had a stronger anti-tumor efficacy. The clinical relevance of this study is highlighted by the accessibility of its active therapeutic elements. Apart from breast cancer, the results may also have broad applicability to a variety of solid tumors [42].

Additionally, certain miRNAs, although not directly exerting anti-tumor effects, can promote anti-tumor immunity by regulating the fate of immune cells. Overexpression of these miRNAs in MSC-EXOs can showcase significant potential in tumor therapy. For example, miR-10a regulates the fate of naïve T cells. Bolandi *et al.* loaded miR-10a mimics into ADSC-EXOs via electroporation. The resulting exosomes can induce the expression of RORγt and Foxp3 in naïve T cells, inhibit the expression of T-bet, thereby promoting the differentiation of naïve T cells into Treg and Th17 and inhibiting differentiation into Th1. Recently, many studies have suggested that Th17 cells, when transferred into mice, exhibit better tumor inhibitory effects than Th1 cells [43], necessitating further investigations on the role of miR-10a-overexpression MSC-EXOs in tumor therapy. Methodologically, it is noteworthy that they stored MSC-EXOs in −70 °C prior to use. However, they clearly demonstrated the stability of preserved and miRNA electroporated exosomes in their setting and indicated that MSC-EXOs could effectively (~250 fold) deliver miR-10a to $CD4^+$ T cells [44].

Conversely, inhibiting certain pro-tumor miRNAs is also an effective therapeutic approach. For instance, miR-142-3p is highly expressed in various breast cancers. It induces cancer cell over-proliferation by activating the Wnt pathway and transactivating the expression of miR-150. Naseri *et al.* loaded MSC-EXOs with locked nucleic acid (LNA)-modified anti-miR-142-3p oligonucleotides by electroporation. Modified exosomes efficiently deliver anti-miR-142-3p, reducing the expression of miR-142-3p and miR-150 in breast cancer cells or tumor tissues. Moreover, they investigated the tumor tropism and biodistribution of the MSC-EXOs in tumor-beating mice, claiming that MSC-EXOs mainly excrete by spleen and liver and secondarily by lung and kidneys. 48 hours post tail vein injection, they were almost cleared from lung, liver, spleen, and kidneys, but were still detectable in the tumor site [45]. In another study by them, LNA-anti-miR-142-3p delivered through MSC-EXOs significantly reduced the tumorigenicity of breast cancer stem cells, suggesting the potential use of modified MSC-EXOs for targeting cancer stem cells in anti-cancer therapies [46].

Table 2. Examples of miRNAs or miRNA inhibitors delivered by MSC-EXOs in different cancers' treatment.

Expression	miRNA	Cancer type	Targets/Pathways	References
Overexpressed	miR-34a	Breast cancer	Bcl2, c-MET	[38]
	miR-138-5p	Bladder cancer	Survivin	[40]
	miR-379	Breast cancer	COX-2	[39]
	miR-146b	Glioma	EGFR, NF-κB	[41]
	miR-34c-5p	Acute myeloid leukemia	CDK/Cyclin pathway	[48]
Inhibited	miR-142-3p	Breast cancer	miR150, APC, P2X7R	[45,46]

In addition to loading anti-cancer or pro-cancer miRNA oligonucleotides into the interior of MSC-EXOs, combined surface modification can further enhance the therapeutic efficacy of MSC-EXOs. For instance, Wen *et al.* innovatively developed a multistep-modified MSC-EXOs for the treatment of relapsed acute myeloid leukemia (AML). Leukemia stem cells (LSCs) are a significant factor in AML

relapse, and LSCs overexpress the interleukin-3 receptor. Wen *et al.* transduced lamp2b-il3 into MSCs using lentivirus to obtain exosomes (IL3OE-EXO) with the ability to target LSCs. Additionally, their previous research indicated that miR-34c-5p induces senescence to eliminate LSCs, offering a potential treatment for AML [47]. Therefore, they loaded miR-34c-5p agomir into IL3OE-EXO using electroporation. Furthermore, they observed high expression of CD44 on the surface of IL3OE-EXO. To enhance the bone marrow homing capability of IL3OE-EXO, they performed an enzyme reaction mediated by FVII to modify CD44 into a hematopoietic cell E-selectin/L-selectin ligand (HCELL), ultimately obtaining HCELL-IL3OE-exo loaded with miR-34c-5p. This type of exosome demonstrated enhanced bone marrow localization and selective targeting ability toward LSCs. In an AML humanized mouse model, it selectively promoted the clearance of LSCs, delaying the progression of AML. This study is the first study on glycan engineering of exosomes; similar modification has been mainly limited to cells. Moreover, utilizing simultaneous two-step modifications to engineer MSCs and MSC-EXOs was achieved for the first time [48].

4.2. Application in other diseases

Apart from cancers, MSC-EXOs enriched with RNAs can be advantageous for a range of additional diseases (Table 3).

4.2.1. Spinal Cord Injury

The spinal cord, responsible for relaying commands from the brain to various regions of the body, plays a critical role in motor and sensory function. Injury to the spinal cord can lead to severe deficits in the lower limbs below the affected segment, potentially resulting in paralysis. Such injuries not only inflict profound physical and psychological damage on patients but also present a considerable economic burden on society. Currently, there is a lack of effective therapeutic interventions, particularly for complete spinal cord injuries. In recent years, a growing body of research has focused on the modification of MSC-EXOs as a promising strategy for spinal cord injury treatment. For instance, Yu *et al.* transfected bone marrow-derived MSCs with miR-29b recombinant lentiviral vector and further discovered that the stable expression of miR-29b in BM-MSC-EXOs significantly ameliorated spinal cord injury in rat models [49]. Guo *et al.* demonstrated that MSC-EXOs loaded with PTEN-siRNA could cross the BBB via intranasal administration, localize to the injured spinal cord, downregulate PTEN expression, enhance axonal growth and angiogenesis, inhibit the proliferation of oligodendrocytes and astrocytes, and promote functional recovery in rats with complete spinal cord injuries [50]. Additionally, Lu *et al.* transfected Netrin-1 modRNA (chemically modified messenger RNA, synthesized *in vitro*) into bone marrow-derived MSCs to obtain exosomes enriched with netrin-1 (EXO-netrin1). They reported that EXO-netrin1 attenuated inflammation and necrosis in spinal cord injuries through the Unc5b/PI3K/AKT/mTOR signaling pathway, thereby promoting axonal regeneration. However, from a methodological standpoint, there are several limitations in their study that should be acknowledged for future research. For example, their study primarily concentrated on the Unc5b receptor, neglecting to examine other potential receptors, which could also significantly influence the effects of netrin-1 treatment for spinal cord injury. Moreover, while they measured axon growth induced by netrin-1, their assessment was limited to macroscopic length measurements. They did not conduct a

microscopic analysis of proteins which are critical for axonal growth and regeneration. Including such analyses could offer deeper insights into the cellular and molecular changes induced by netrin-1 [51].

4.2.2. Chronic wounds

Chronic wound healing poses a significant clinical challenge. Tao *et al.* discovered that synovium MSCs strongly promote fibroblast proliferation, and synovium MSCs overexpressing miR-126-3p by lentivirus transfection significantly enhance angiogenesis. In vitro experiments demonstrated that exosomes derived from miR-126-3p-overexpressing synovium MSCs (SMSC-126-EXO) stimulate the proliferation of human dermal fibroblasts and skin microvascular endothelial cells (HMEC-1) and enhance the migration of HMEC-1. In a diabetic rat model, SMSC-126-EXO accelerates epidermal cell regeneration and promotes vascularization, demonstrating enormous potential for enhancing wound healing [52]. Born *et al.* found that the long non-coding RNA HOTAIR can mediate the pro-angiogenic effect of endothelial cell-derived exosomes. To load HOTAIR into exosomes, they utilized non-viral plasmid transfection to promote overexpression within the producer MSCs, resulting in ~1000-fold increased exosomal HOTAIR content. These HOTAIR-enriched exosomes significantly promote vascularization and wound healing in diabetic mice. However, analysis of angiogenesis, which is a 3D process, using immunohistochemistry on 2D tissue sections has limitations. Furthermore, the transfection of exosomes-producing cells to load HOTAIR could also lead to additional complex alterations in exosome composition and bioactivity, which should be studied in the future [53]. Lv *et al.* loaded miR-21-5p mimics into ADSC-EXOs by electroporation and demonstrated the resulting exosomes promote keratinocyte proliferation and migration *in vitro* through the Wnt/β-catenin signaling pathway. In vivo, these exosomes accelerate diabetic wound healing by increasing re-epithelialization, collagen remodeling, vascularization, and vascular maturation. Notably, this research utilized a diabetic full-thickness wound model in rats to investigate the wound-healing process. However, it is important to recognize that these wounds, which were deliberately created in a laboratory setting, may not fully replicate the complexities of actual wounds encountered in diabetic patients. In clinical scenarios, chronic nonhealing wounds in diabetics evolve gradually with the progression of the disease and involve intricate interactions among various pathogens, immune cells, and tissue cells. Further research should explore the detailed mechanisms influencing wound microenvironments in diabetic patients [54].

4.3. Others

In addition, MSC-EXOs modified with small RNAs have been shown to be beneficial for various other diseases. Ma *et al.* electroporated MSC-EXOs with miR-132 mimics and discovered that intramyocardial injection of miR132-overexpressing MSC-EXOs in a mouse model of acute myocardial infarction significantly enhances the formation of new blood vessels in the peri-infarct region and protects cardiac function. This suggests that MSC-EXOs can serve as delivery vehicles for functional miRNAs in ischemic diseases therapies, especially in those focusing on promoting vascular formation [55].

Xin *et al.* found that MSC-EXOs mediate the transfer of miR-133b to astrocytes and neurons, regulating downstream gene expression, thereby promoting axonal remodeling and functional recovery after stroke. They confirmed that MSCs overexpressing miR-133b have better efficacy compared to naive MSCs in a rat middle cerebral artery occlusion (MCAo) model. However, the effects of miR-133b

overexpression upon MSC-EXOs content and miRNAs changes in the recipient cells warrants further investigation [56].

Wen *et al.* transfected plasmids encoding shFas and anti-miR-375 into human bone marrow-derived MSCs to generate exosomes that effectively silenced Fas and miR-375 in human islets. The simultaneous delivery of siFas and anti-miR-375 via human bone marrow-derived MSCs and their exosomes has been shown to reduce early apoptosis in transplanted human islets. Moreover, the suppression of further immune responses can be achieved through the intravenous administration of human bone marrow-derived MSCs and peripheral blood mononuclear cells co-cultured exosomes. This combined gene and cell therapy approach may represent a powerful strategy to enhance the success of islet transplantation [57].

Numerous other examples exist. For example, MSCs were transiently transfected with miR-10a or miR-486 mimics by electroporation to obtain exosomes enriched with these miRNAs. These engineered exosomes effectively promote kidney regeneration [58]. Exosomes enriched with miR-375 were generated from human adipose-derived MSCs stably overexpressing miR-375 after lentiviral transfection. These exosomes demonstrated excellent bone regeneration capabilities in a rat calvarial injury model [59]. Adipose-derived MSCs were transfected with agomir-23a-3p and seeded on porous Gelma hydrogel to produce exosomes enriched with hsa-miR-23a-3p. These engineered exosomes can promote chondrocyte proliferation, survival, and cartilage formation by activating the PI3K/AKT/mTOR signaling pathway [60]. Exosomes were extracted by ultracentrifugation from the culture supernatant of miR-26a-modified bone marrow-derived MSCs. These engineered exosomes can promote bone formation and inhibit bone loss *in vitro* and *in vivo* [61].

Table 3. Examples of RNAs delivered by MSC-EXOs in different diseases' treatment.

Disease type	RNAs	Effects	References
Spinal cord injury	PTEN-siRNA	Promote functional recovery	[50]
	Netrin-1 modRNA	Attenuate inflammation and necrosis, and promote axonal regeneration	[51]
	miR-29b	Alleviate histopathological damage and promote neuronal regeneration	[49]
Chronic wounds	miR-126-3p	Accelerate epidermal cell regeneration and promote vascularization	[52]
	HOTAIR	Promote vascularization	[53]
	miR-21-5p	Promote re-epithelialization, collagen remodeling, vascularization, and vascular maturation	[54]
Acute myocardial infarction	miR-132	Promote angiogenesis and protect cardiac function	[55]
Immune rejection	Fas shRNA, anti-miR-375	Suppress immune response after transplantation	[57]
Acute kidney injury	miR-10a, miR-486	Promote kidney regeneration	[58]
Calvarial injury	miR-375	Promote bone regeneration	[59]
Congenital microtia	hsa-miR-23a-3p	Promote chondrocyte proliferation, survival, and cartilage formation	[60]
Periodontitis	miR-26a	Promote bone formation and inhibit bone loss	[61]

5. Opportunities and challenges in the clinical application of MSC-EXOs

One of the major issues in the translational application of MSC-EXOs is the difficulty in large-scale production. Here, we summarize various strategies to enhance the yield and functionality of MSC-EXOs.

5.1. 3D culture

The traditional 2D culture yields a very limited number of cells, whereas the emerging 3D culture technology effectively addresses this issue. Yuan *et al.* inoculated MSCs in ultra-low attachment (ULA) plates and used wave-motion to spontaneously form 3D aggregates with diameters ranging from 100 to 300 micrometers. They found that MSCs cultured in 3D secreted approximately twice as many exosomes as those cultured in 2D. Moreover, compared to 2D-MSC-EXOs, 3D-MSC-EXOs exhibited elevated expression of cytokines and anti-inflammatory factors, along with stronger immunomodulatory functions. However, demonstrating the large-scale production of 3D-MSC-EXOs in bioreactors, such as wave or vertical wheel systems, for preclinical applications is yet to be achieved and it is also crucial to thoroughly characterize the exosomes secreted within these bioreactors. Additionally, ensuring the consistency of the protein and miRNA content within 3D-MSC-EXOs across different bioreactor batches is essential for maintaining quality control standards [62]. Similarly, bone marrow-derived MSCs cultured using Avitene Ultrafoam collagen hemostat secreted twice the amount of exosomes compared to those cultured under 2D conditions, and demonstrated better therapeutic efficacy in a rat model of traumatic brain injury [52]. For the first time, Chen *et al.* used porous gelatin methacryloyl (GelMA) as a medium for 3D culture of adipose-derived MSCs. The pores of GelMA ranged from 100 to 200 micrometers, with suitable expansion and degradation rates that promote cell metabolism, survival, and exosome secretion. The exosomes obtained using this method were 3.68 to 6.64 times those from 2D culture, significantly reducing the culture space and medium volume, shortening the culture time, and thereby lowering production costs, making large-scale production feasible [60].

It is noteworthy that rotating culture bottles with microcarriers and hollow fiber bioreactors, both of which have larger surface areas, are currently used for large-scale 3D culture of MSCs [63]. However, microcarriers require extensive preparation before cell seeding, including weighing, hydration, equilibration, and coating. These time-consuming and cumbersome steps hinder the large-scale production of MSCs. Yan *et al.* innovatively utilized a decomposable and dissolvable porous microcarrier, 3D TableTrix, for the rapid expansion of MSCs. 3D TableTrix is a macroporous, highly absorbent, elastin-based gelatin microcarrier, packaged into sterile tablets of specific weight. Upon contact with aqueous solution, it absorbs a large amount of liquid and disintegrates into individual microcarriers, eliminating the complex preparatory steps before cell seeding. Adipose-derived MSCs cultured with 3D TableTrix expanded more than 500-fold within 11 days, maintaining immunophenotypic characteristics, differentiation potential, and genomic stability, with almost no signs of senescence. Using this high-performance microcarrier, high-quality MSCs or MSC-EXOs are likely to achieve cost-effective industrial-scale production or clinical application. Nevertheless, further exploration of theoretical and practical aspects is required to harness the full potential of 3D TableTrix microcarriers. This includes but not limits to assessing potential aggregation risks, ensuring the efficient removal of any chemical residues associated with the microcarriers from the final cellular products, and conducting a more thorough analysis of cell quality [64].

5.2. Appropriate cell types and passages

To develop large-scale isolation methods for MSC-EXOs, Haraszti *et al.* compared the doubling times and exosome yields of MSCs from different sources, finding that umbilical cord-derived MSCs had the shortest doubling time and the highest exosome yield per cell compared to other types of MSCs, such as bone marrow-derived MSCs and adipose-derived MSCs [65]. Additionally, the passage number of cells affects the yield and function of exosomes. Chen *et al.* discovered that fourth-passage ADSC-EXOs (EXO4) had significantly higher yields than second-passage (EXO2) and sixth-passage ADSC-EXOs (EXO6). EXO2 exhibited stronger abilities to promote chondrocyte proliferation and inhibit apoptosis, while EXO4 and EXO6 showed greater chondrogenic potential. Excessively high passages (e.g., above 10 passages) may lead to MSC senescence, making the selection of appropriate, non-senescent passages crucial for obtaining therapeutically potent exosomes [60].

5.3. Suitable isolation methods

The gold standard for exosome isolation, ultracentrifugation, relies on differences in sedimentation coefficients to separate exosomes from cells, cell debris, and organelles. However, this method has several drawbacks, including yield being highly dependent on operator skill, potential vesicle breakage and aggregation, and poor scalability. Consequently, researchers have developed various alternative isolation techniques. One promising method is tangential flow filtration (TFF), which is highly suitable for processing large volumes and yields samples of high purity, making it attractive for preclinical and clinical applications. Haraszti *et al.* found that exosomes isolated from microcarrier-based 3D cultures of MSCs using TFF (3D-TFF-EXO) were seven times more abundant than those isolated using ultracentrifugation (3D-UC-EXO). Moreover, compared to 2D-UC-EXO, 3D-TFF-EXO exhibited a fivefold increase in the efficiency of siRNA delivery to neurons and silencing of the Huntingtin gene, suggesting that the combination of microcarrier-based 3D culture and TFF could be a powerful approach to enhance the yield and therapeutic potential of MSC-EXOs [65].

5.4. Other methods

Mechanical force and high-frequency ultrasound, among other chemical and physical stimuli, may represent another category of effective methods [66]. For instance, Du *et al.* found that nitric oxide (NO) stimulation enhanced the pro-angiogenic capacity of human placenta-derived MSC-EXOs (PL-MSC-EXOs) both *in vitro* and *in vivo* [67]. Modifying the microenvironment of MSCs during *in vitro* culture, such as using certain bioactive substances, may also help increase exosome yield. For example, Park *et al.* developed positively charged nanoparticles based on iron oxide and PLGA, known as PLGA-PEI PCS NPs. These nanoparticles are transported to lysosomes via clathrin-mediated endocytosis and promote exosome release by stimulating autophagy-related factors [68].

Induced pluripotent stem cell (iPSC)-derived MSCs also offer advantages in exosome production. For instance, Zhu *et al.* suggested that exosomes secreted by iPSC-derived MSCs (iMSC-EXOs) have a greater therapeutic effect on osteoarthritis than exosomes secreted by synovial membrane MSCs (SM-MSC-EXOs) [69]. Kim *et al.* claimed that compared to MSC-EXOs, a more significant increase in human keratinocytes (HaCaT) proliferation was observed with iMSC-EXOs [70].

By employing these innovative approaches, it is possible to further enhance the production and functionality of MSC-EXOs, thus facilitating their large-scale application and clinical translation.

6. Conclusion

Exosomes are natural nanovesicles containing various bioactive molecules, and they offer significant advantages as drug delivery vehicles due to their low immunogenicity and high permeability. MSCs possess distinctive characteristics such as immunomodulatory capacity, pro-angiogenic effects, and the ability to promote tissue repair and regeneration, making them widely employed in clinical applications. MSC-EXOs combine the advantageous properties of both exosomes and MSCs, and they can be engineered through surface modification or the encapsulation of therapeutic agents, enhancing their therapeutic potential. This highlights the growing significance of MSC-EXOs in clinical drug delivery and disease treatment.

A major obstacle in the development of small RNA therapeutics is the delivery system. Utilizing natural or engineered MSC-EXOs to deliver small RNA drugs provides a safe, efficient, and convenient method with substantial value for both basic research and translational applications. However, MSC-EXOs-based therapies face several challenges. First, large-scale production of MSC-EXOs is essential for clinical use, yet most research remains focused on small-scale laboratory studies involving individual active components of MSC-EXOs, with limited data on efficacy and safety from large-scale preclinical or clinical trials. Second, standardized protocols for the extraction, isolation, purification, and storage of MSC-EXOs have not yet been established. Additionally, the composition and mechanisms of action of MSC-EXOs are highly complex, and our understanding of the processes involved—from their production and transport to uptake by target cells—remains incomplete. Moreover, the effects of MSC-EXOs vary widely depending on their source, dosage, and route of administration, and these factors must be carefully considered for different diseases.

In conclusion, achieving large-scale clinical applications of MSC-EXOs requires significant further research and collaboration. Scientists, clinicians, investors, and policymakers must work together to address these challenges and unlock the full therapeutic potential of MSC-EXOs.

Abbreviations

ADSC-EXOs: adipose-derived MSC-EXOs; AML: acute myeloid leukemia; Ang-1: angiopoietin-1; BBB: blood-brain barrier; BM-MSC-EXOs: bone marrow-derived MSC-EXOs; CLP: cecal ligation and puncture; DP-MSC-EXOs: dental pulp-derived MSC-EXOs; EMMPRIN: extracellular matrix metalloproteinase inducer; GelMA: gelatin methacryloyl; HCC: hepatocellular carcinoma; HCELL: hematopoietic cell E-selectin/L-selectin ligand; ICD: immunogenic cell death; iMSC-EXOs: exosomes secreted by iPSC-derived MSCs; iPSC: induced pluripotent stem cell; ISCT: International Society for Cell and Gene Therapy; LNA: locked nucleic acid; LSCs: Leukemia stem cells; MCAo: middle cerebral artery occlusion; MSCs: Mesenchymal stem cells; MSC-EXOs: MSC-derived exosomes; NO: nitric oxide; NTA: Nanoparticle Tracking Analysis; OXA: oxaliplatin; PL-MSC-EXOs: placenta-derived MSC-EXOs; RISC: RNA-induced silencing complex; SM-MSC-EXOs: exosomes secreted by synovial membrane MSCs; snRNA-seq: Single-nucleus RNA sequencing; TFF: tangential flow filtration; ULA: ultra-low attachment; VEGF: vascular endothelial growth factor.

Conflicts of interests

The authors declare that they have no competing interests.

Authors' contribution

Conceptualization, Ruoyan Zhang and Xu Guo; methodology, Ruoyan Zhang; software, Ruoyan Zhang; validation, Ruoyan Zhang and Xu Guo; writing—original draft preparation, Ruoyan Zhang; writing—review and editing, Ruoyan Zhang and Xu Guo; supervision, Xu Guo. All authors have read and agreed to the published version of the manuscript.

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