

## Review article

# MSC based gene delivery methods and strategies improve the therapeutic efficacy of neurological diseases

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## ABSTRACT

Mesenchymal stem cells (MSCs) are promising seed cells for neural regeneration therapy owing to their plasticity and accessibility. They possess several inherent characteristics advantageous for the transplantation-based treatment of neurological disorders, including neural differentiation, immunosuppression, neurotrophic, and safety. However, the therapeutic efficacy of MSCs alone remains unsatisfactory in most cases. To improve some of their abilities, many studies have employed genetic engineering to transfer key genes into MSCs. Both viral and nonviral methods can be used to overexpress therapeutic proteins that complement the inherent properties. However, to date, different modes of gene transfer have specific drawbacks and advantages. In addition, MSCs can be functionalized through targeted gene modification to facilitate neural repair by promoting neural differentiation, enhancing neurotrophic and neuroprotective functions, and increasing survival and homing abilities. The methods of gene transfer and selection of delivered genes still need to be optimized for improved therapeutic and targeting efficacies while minimizing the loss of MSC function. In this review, we focus on gene transport technologies for engineering MSCs and the application of strategies for selecting optimal delivery genes. Further, we describe the prospects and challenges of their application in animal models of different neurological lesions to broaden treatment alternatives for neurological diseases.

## 1. Introduction

Mesenchymal stem cells (MSCs) are types of multipotential adult stem cells that are characterized by high proliferation capacity, self-renewal, low immunogenicity, and multilineage differentiation. MSCs can be isolated from bone marrow (BMSCs), umbilical cord (UC-MSCs), peripheral blood (PB-MSCs), placenta, teeth (dental pulp stem cells, DPSCs), and adipose tissue (AD-MSCs) [1]. They can be induced *in vitro* to differentiate into neurocytes, chondrocytes, osteoblasts, adipocytes, and many other cell types [2]. Thus, they are often considered ideal seed

cells for cell transplantation and have critical research and clinical value. Several clinical trials have been conducted using MSCs for neurological, bone, cardiovascular, and autoimmune diseases [3,4].

Neurological diseases are characterized by nerve injury and loss of neurons, which are difficult to restore. Injury to the neural system usually results in the permanent loss of a number of neural functions, causing memory loss, cognitive defects, dyskinesia, paraplegia, and peripheral paralysis. This is because axons in the central nervous system (CNS) at the lesion can rarely regenerate after exposure to injury. Injuries to the CNS, such as spinal cord injury (SCI), stroke, traumatic

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brain injury (TBI), and various neurological disorders, are often related to regeneration failure, resulting in poor prognosis [5]. Because MSCs can differentiate into neurocytes, they have been used to promote neural regeneration and repair in several neurological diseases. The potential efficacy of MSCs for the restoration of neurological functions depends on two main mechanisms: (i) sustained neurogenic differentiation for cell replacement and (ii) release paracrine transmission of reparative factors or secondary effects after cell death [6]. Unfortunately, wild type (naïve) MSCs transplanted directly at the lesion site or injected into the vascular system frequently die within several days due to natural senescence [7], a hostile local microenvironment at the injury site, and (or) nutrient deprivation [8].

Transfecting MSCs with genes that can enhance resistance to cytotoxic conditions, such as hypoxia/ischemia, oxidative stress, and acute or chronic inflammation or promote local neurotrophs and neuroprotection may not only enhance MSC survival *in vivo* but also facilitate neuronal replacement, repair of surviving neurons, and the reconstruction of neural circuitry, potentially resulting in lasting restoration of neurological function.

In this review, we discuss the approaches used to genetically alter MSCs and gene selection strategies for optimal neural repair. We also summarize recent progress in using modified MSCs for the treatment of various neurological disease models (Fig. 1).

## 2. Tools for gene delivery

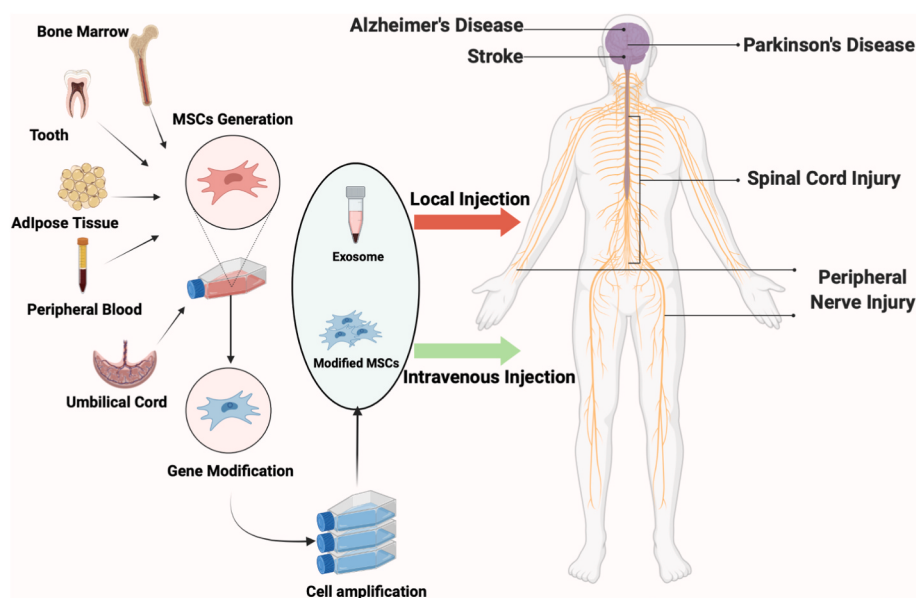
The tools for gene delivery are mainly divided into viral-based methods and nonviral-based methods. The former is considered to be used in constructing stably transfected MSCs, which leads the MSCs has more sustained exogenous gene expression time. The later nonviral-based methods always sustain transient gene expression in MSCs. Most previous works of gene edited MSCs are relied on viral-based methods for their high transfected efficiency and high expressed time. With the rapid development of nano gene engineering technology, more and more scientists have focused on finding new transfected vector or remolding the materials to elevate the transfected efficiency.

### 2.1. Advantages and drawbacks of virus-based gene transfer

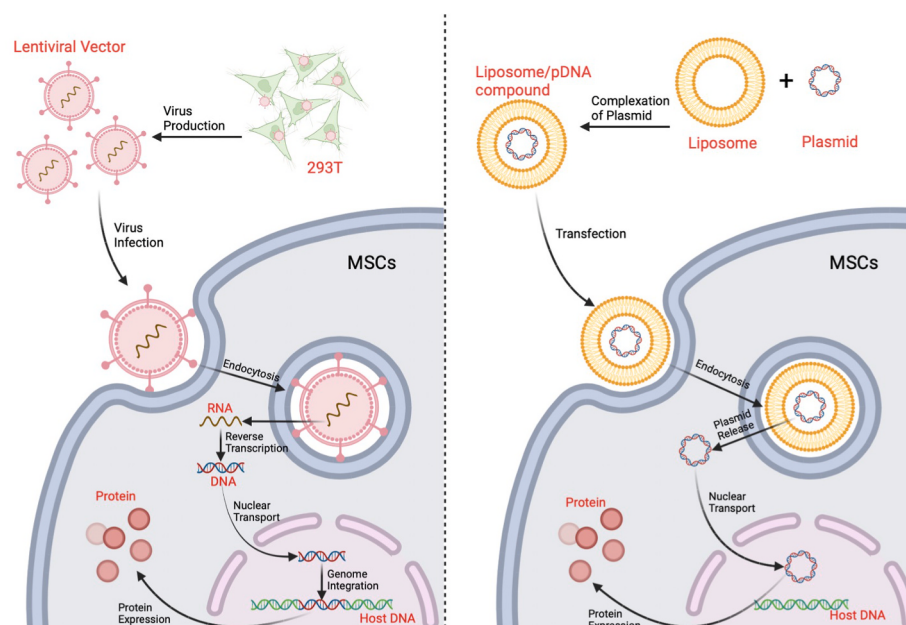
Numerous viral vectors are widely used to modify the gene expression profile of MSCs, including retroviruses [9,10], lentiviruses [11,12], adenoviruses, adeno-associated viruses (AAVs) [13,14], baculoviruses [15,16]. Gene delivery using viral vectors exploits the natural capacity of viruses to infect MSCs and insert viral genes into the host genome, including any genes of interest (transgenes) ligated to the viral genome or replacing viral gene(s) (Fig. 2).

Compared with nonviral vectors, viral vectors have several advantages for the genetic modification of MSCs: i) the inherent susceptibility of rapidly proliferating MSCs to viral infection, ii) stable long-term expression of integrated genes, iii) the capacity to carry large exogenous DNA fragments, and iv) intra-nuclear replication and transcription to express overlapping open reading frames.

However, there are a number of limitations to viral-based gene delivery that may restrict their widespread use in gene editing projects for MSCs in the nervous system. For lentiviral vectors lacking specificity, the main problem is that transduction is not required for the process of cell infection. It raises the potential risk of non-specific infection of nerve cells by local injection of MSCs with gene editing. In addition, most lentiviral vectors are derived from HIV, raising safety concerns for *in vivo* use [17]. Although gene therapies utilizing AAV have demonstrated safety and long-term efficacy in clinical trials for a variety of applications, there are evidences that these vectors can cause innate and humoral responses that result in limited transgene expression and can lead to severe inflammation. More specifically, numerous groups have identified significant toxicity and limitations of repeated administration of AAVs after intracranial injection for CNS gene delivery [18,19]. Significant inflammatory and expression of Class I poly-histocompatibility antigens was found, and the persistent state of the AAV vector remains a potential target for destructive immune responses that can lead to local demyelination of peripheral neurons [20]. Finally, there are a number of problems with the manufacturing and scalability of virus particles that have been struggling to overcome for the past two decades, including the restriction of the maximum gene size that AAVs can carry to 4.7 KB for packaging of foreign DNA, high manufacturing costs that call into question economic scalability, and the presence of cellular impurities in all currently used carrier production protocols [21]. A major problem in



**Fig. 1.** Schematic diagram of process of the gene modified MSC in treating neurological diseases. MSCs are isolated from bone, tooth, adipose et al. and cultured for amplification. Then MSCs are undergone with genetic modification with genes with different functions. The exosomes or modified MSCs are transplanted into lesion part or injected with vein.

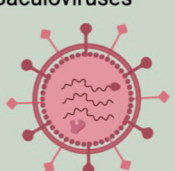


**Fig. 2.** The figure shows two typical gene delivery methods including viral vector (lentiviral vector, left) and non-viral vector (liposome, right). In the viral-based process, the viruses with target genes were always produced in 293T cells. Then, viruses infect host MSCs with endocytosis effect, and randomly insert the target gene is into the host genome. In non-viral methods, vectors encapsulated with plasmids are transported into MSCs, and release plasmids into nuclear without gene integration.

the use of adenoviral vectors is their immunogenicity. Direct injection of adenoviral vectors with transgene into host cells activated CD4<sup>+</sup>, CD8<sup>+</sup> and antigen presenting cells. Activation of this immune response against the transgene and viral capsid proteins may lead to virion clearance and transgene silencing [22]. Another disadvantage of adenoviral vectors is that the transgene cannot be continuously expressed [23]. In addition, some reports have suggested that the use of adenoviral vectors may have

adverse effects on cell differentiation [24].

We summarize the main disadvantages of using viral vectors to transfect MSC for the treatment of nerve injury (Fig. 3): (i) Virus transfection has irreversible damage to the proliferation, metastasis and repair of mesenchymal stem cells. (ii) Inflammation caused by inherent immunogenicity may cause damage to nerve cells. (iii) Risk of integration and insertional mutations of viral genomes. (iv) There are lethal and

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|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|--|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|--|
| <b>Virus-based vectors</b><br>Retroviruses<br>Lentiviruses<br>Adenoviruses<br>Adeno-associated virus<br>Baculoviruses<br>                                                                                                                                                              |  | <b>Advantages</b><br><ul style="list-style-type: none"> <li>· Inherent susceptibility and high infected efficiency</li> <li>· Stable long-term expression of integrated genes</li> <li>· Carrying large fragments of exogenous DNA</li> <li>· Transcription to express overlapping open reading frames</li> </ul> <b>Disadvantages</b><br><ul style="list-style-type: none"> <li>· Low transduction efficiency (AV and AAV)</li> <li>· Inherent immunogenicity</li> <li>· Risks of gene integration and insertion mutations</li> <li>· Uncontrolled exogenous gene expression</li> <li>· Lethal and carcinogenic risks</li> </ul> |  |
| <b>Physical methods</b><br>Sonotransfection<br><br>Electroporation<br>                                                                                                                              |  | <b>Chemical synthetic vectors</b><br>  <br>Liposome-based   Polymers   Inorganic                                                                                                                                                                                                                                                                                      |  |
| <b>Advantages</b><br><ul style="list-style-type: none"> <li>· High transfection efficiency</li> <li>· Controlled gene expression</li> </ul> <b>Disadvantages</b><br><ul style="list-style-type: none"> <li>· High cytotoxicity</li> <li>· Lack of targeting</li> <li>· Restricted DNA protected effect from DNase</li> <li>· Difficult to apply <i>in vivo</i></li> </ul> |  | <b>Advantages</b><br><ul style="list-style-type: none"> <li>· Greater versatility and controllability</li> <li>· Low cytotoxicity and good bio-safety</li> <li>· Can be used <i>in vivo</i></li> </ul> <b>Disadvantages</b><br><ul style="list-style-type: none"> <li>· Variable transfection levels across cell types</li> <li>· Low transfection efficiency</li> <li>· Induce only transient gene expression</li> </ul>                                                                                                                                                                                                         |  |

**Fig. 3.** Advantages and Disadvantages of various gene transferring methods.

carcinogenic risks. (v) Uncontrolled prolonged exogenous gene expression. (vi) Some viruses, such as AAV, have insert size limits.

#### 2.1.1. Retroviruses

Double-stranded RNA retroviruses have a lipid envelope with receptor-binding proteins for cell targeting and express reverse transcriptase for the integration of viral genes into the host cell genome. After receptor binding, the viral envelope merges with the cell membrane and the virus particles are internalized. This is followed by the release of viral contents into the cytoplasm. Subsequently, the viral RNA is reverse transcribed into DNA using cellular reverse transcriptase, which is then integrated into the host genome. Retroviruses exhibit high infection efficiency only in proliferating cells. Cells not undergoing mitosis are not susceptible to retroviral infection, which limits their application [25]. Retroviral vectors have been used to transfer several genes into BMSCs, including *Foxa1* and *Hnf4a* to induce differentiation into hepatocyte-like cells [9]. One recent study found that the expression of stemness transcription factors NANOG, OCT-4, and SOX2 by MSCs was strongly associated with the expression of DNA polymerase (pol) genes by human endogenous retrovirus (HERV)-K and -H, and that expression levels were higher in early-passage MSCs than in later-passage MSCs [10].

#### 2.1.2. Lentiviruses

Lentiviruses are double-stranded RNA viruses with capacities for integration into the host genome and long-term expression of exogenous genes. In contrast to retroviruses, lentiviral vectors are capable of infecting dividing, slowly dividing, and quiescent cells without attenuating host cell viability, growth, and differentiation potential [26]. In fact, the transfection efficiency of lentiviral vectors was reported to reach 50% in human MSCs (hMSCs) and 85% in mouse MSCs (mMSCs). Transfection efficiency can be suppressed by the proteasome inhibitor MG132, suggesting that proteasomes regulate the infection process [27].

Given the high efficiency and stability of transfection, lentiviruses are among the most widely applied vectors for gene transfer into MSCs. For instance, a lentiviral vector was effectively used to induce the overexpression of N-acetyltransferase 10 (NAT10) in MSCs and enhance osteogenic differentiation [11]. Also, the silencing of prolyl hydroxylase 2 (PHD2) using a lentiviral vector enhanced the paracrine effect of BMSCs via an NF- $\kappa$ B-dependent mechanism in a rat model of necrotizing enterocolitis [12].

#### 2.1.3. Adenoviruses

Adenoviruses are unenveloped 70–90 nm particles with a protein capsid consisting of 252 capsomeres arranged in icosahedrons surrounding a linear double-stranded DNA molecule. The major advantages of adenoviral vectors for gene transfer include low risk of pathogenicity or insertional mutagenesis and high DNA carrying capacity.

Various strategies have been explored to improve the viability and differentiation potential of MSCs using adenoviruses vectors. BMSCs infected with adenoviruses carrying shRNA against mammalian Ste20-like kinase 1 (Mst1) demonstrated enhanced resistance against HO-induced oxidative stress injury concomitant with Mst1 inhibition [28]. Further, MSCs overexpressing fibroblast growth factor 2 (FGF2) and platelet-derived growth factor BB (PDGF-BB) from adenovirus vector infection induced collateral vessel formation and angiogenesis in a hind limb ischemia model [13]. Human endometrial MSCs infected with an adenoviral vector encoding soluble fms related receptor tyrosine kinase 1 (Flt-1) also mitigated endometrial lesions in nonobese diabetic/severe combined immunodeficiency (NOD/SCID) mice [14].

Although adenoviral vectors have several inherent advantages for gene transfer into MSCs, their transduction efficiency is considerably lower than that of lentiviral vectors at the same particle concentration [29]. In addition, adenoviral vectors possess greater immunogenicity than some other viral vectors as transfection can result in the low-level expression of viral genes, resulting in the activation of CD4<sup>+</sup>, CD8<sup>+</sup>, and

antigen-presenting cells. The viral capsid proteins and the expression of the transgene activating the host's immune response sometimes cause the silencing of the transgene in host cells and the elimination of viral particles [22]. Another drawback is that adenoviral vectors may induce only transient expression of the transgene [23].

#### 2.1.4. Adeno-associated virus

AAVs are single-stranded DNA viruses that depend on adenovirus genes for replication [30]. After binding to the integrin FGF4 and  $\alpha$ V $\beta$ 5 receptor, AAVs enter host cells and the viral genome integrates into a specific host genome site termed AAVS1. This site-specific integration eliminates the risks of unpredictable insertional oncogenesis and other genetic disruptions. Further, AAVs have low pathogenicity, low immunogenicity, and the capacity to infect many different cell types [31,32].

Several preclinical studies have demonstrated the beneficial effects of MSCs carrying genes transfected using AAVs. BMSCs overexpressing Scavenger receptor class A, member 3 (Scara3) facilitated osteogenesis and suppressed adipogenesis [33]. Also, BMSCs induced to overexpress interleukin (IL)-10 via AAV vector infection demonstrated increased immunomodulatory activities, including T cell suppression, in an osteoarthritis model [34]. The overexpression of Sirtuin-3 (Sirt3) by the intravenous injection of recombinant AAV-9 significantly retarded BMSC senescence and senile osteoporosis [35].

AAVs can be used to infect a broad range of cells, many show serotype specificity toward certain cell types, thereby influencing gene delivery efficiency. For instance, AAV-DJ infects mouse BMSCs 5 times more efficiently than AAV-2 [36]. Most people in the population possess antibodies against AAVs, thereby reducing vector efficiency for clinical use, especially in the case of AAV-2 [37]. However, AAV-DJ demonstrated an infection efficacy approaching 100% for both human and mouse adipose-derived MSCs (AD-MSCs) [38]. These results suggest that AAV-DJ is more suitable for the genetic modification of MSCs in clinical settings.

#### 2.1.5. Baculoviruses

Baculoviruses are enveloped circular double-stranded DNA viruses with two distinct phenotypes, the budded virus and polyhedron-derived virus. The viral genome within the nucleocapsid can accommodate large exogenous DNA fragments, making baculovirus-based vectors ideal for expressing large genes and proteins [39]. Baculoviruses can infect a wide spectrum of mammalian cells and express the transgene under the control of a special promoter that can be selectively activated in mammalian cells [40]. In addition, baculovirus-based vectors show high efficiency for gene delivery into many types of stem cells, including MSCs. The virus also does not replicate in mammalian cells and is non-pathogenic, making it ideal for clinical applications [41].

The transfection of rat AD-MSCs with a hybrid baculovirus vector enabled the robust, simultaneous, and prolonged overexpression of three neurotrophic factors (NTFs), brain derived neurotrophic factor (BDNF), glial cell derived neurotrophic factor (GDNF), and nerve growth factor (NGF), for at least 21 days and CRISPRa-engineered rat AD-MSC sheets overexpressing these NTFs promoted Schwann cell migration, neuronal proliferation, and neurite outgrowth *in vitro* as well as peripheral nerve injury (PNI) repair *in vivo* [15]. In another study, BMSCs overexpressing the sodium iodide symporter (NIS) by infection with a hybrid baculovirus vector improved the therapeutic effect of radioiodine injection on hypopharyngeal cancer [16]. A major drawback of baculovirus vectors, however, is that they may activate the human complement system, leading to viral inactivation [42]. Other disadvantages include probable immunogenicity, insolubility, and poor secretion of some target proteins from infected cells [42].

### 2.2. Nonviral gene delivery methods

Nonviral gene delivery broadly refers to the transfer of plasmid DNA (pDNA) or non-coding RNA (ncRNA) into cell nuclei using chemicals or

physical stimuli [43]. For pDNA to reach the nucleus efficiently and be transcribed, several obstacles must be overcome: (i) it must be protected from degradation by host cell DNase; (ii) it must pass through the plasma membrane, cytoplasm, and nuclear membrane; (iii) complexes of pDNA and nonviral vector molecules entering the cell via endocytosis must be released from the endosome and decomplexed (in case of chemical methods) [44,45].

Nonviral gene delivery methods can be divided into two major categories: chemical and physical. Chemical methods use natural or synthetic materials that can compress and transfer genetic material into cells [46,47]. Chemical methods are generally less destructive than physical methods and show greater versatility, but their transfection efficiency is usually lower. Alternatively, physical methods, such as nano-microinjection, molecular vibration, particle bombardment, sonoporation, electroporation, and laser irradiation, allow naked plasmid DNA to enter into cells. Compared with chemical methods, physical transfer is easier and often more efficient for difficult-to-transfect cells [48,49].

Nonviral gene delivery methods offer several advantages over viral vector transfection, including no or low immunogenicity, allowing for multiple applications, no risk of infectious disease transmission, flexibility in the molecular size of loaded DNA, and low cost. Importantly, some chemically synthesized nanomaterials have unique biological functions for MSC, such as promoting growth, differentiation, eliminating oxidative stress and resisting apoptosis. Unfortunately, they also present some drawbacks. Physical methods are difficult to apply *in vivo* and can cause tissue or cell damage, whereas chemical methods may (i) demonstrate lower transfection efficiencies compared with viral vectors, both *in vitro* and *in vivo*, (ii) induce only transient gene expression, (iii) potentially exhibit high toxicity, and (iv) induced highly variable transfection levels across cell types.

## 2.2.1. Chemical synthetic vectors

### 2.2.1.1. Cationic lipid-based vectors.

Often, primary cells, such as MSCs, are refractory to transfection using nonviral vectors. Currently, most commercial transfection reagents used for the genetic engineering of MSCs are cationic lipids, including Lipofectin®, Lipofectamine™, Effectene™, FuGENE™ 6, and METAFECTEN®. All of these agents form cationic liposomes with internal nanoscale polar heads that interact with anionic DNA and external nonpolar tails that can integrate with the cell membrane [50,51].

Lipofectamine™ is the most commonly used transfection reagent for many cells, including MSCs. Subcutaneous implantation of osteochondral biopsy tissues containing fibrin/hyaluronan (FB/HA) hydrogel loaded with miR-221/lipofectamine significantly enhanced cartilage repair by endogenous cells [52]. Knockdown of platelet-derived growth factor-β (PDNF-β/CD140b) expression by Lipofectamine transfection of a targeted siRNA suppressed the proliferation, laminin adhesion, migration and proangiogenic effect of AD-MSCs on human retinal endothelial cells [53]. One study reported a maximum transfection efficiency of 50% for hMSCs using 0.9 μL of Lipofectamine 2000 with 0.6 mg of DNA [54], but others have reported transfection efficiencies ranging from 15% to 35% for various tissues. A study systematically investigating the efficiencies of various vectors and two commercially available cationic lipid carriers found marked differences among human MSCs depending on donor and source tissue, with the highest levels of transgene expression using human AD-MSCs and mini-intronic plasmids, delivered using Lipofectamine 3000, containing the CMV promoter. Interestingly, different donors and tissue sources affected the transfection efficiency [55]. Another study also reported that different passages of MSCs yielded varied transfection efficiencies. The senescent MSCs were less efficiently transfected than younger cells [56].

FuGENE 6 is a nonliposomal reagent used to transfect DNA into a wide variety of cell lines with high efficiency and low toxicity. Using

FuGENE 6, almost 20% of hBMSCs and 16% of human meniscal fibrochondrocytes were transfected with the cDNA encoding bone morphogenic protein 2 (BMP2) [57]. However, a study directly comparing common transfection reagents for gene transfer into rat BMSCs found that Lipofectamine 2000 and Polyfect were significantly more efficient than either FuGENE HD or Effecte [58]. In human dental pulp stem cells (DPSCs), Lipofectamine 2000 showed higher transfection efficiency than FuGENE 6 (19.2% vs. 13.4%, respectively). Although possibly less efficient, FuGENE 6 demonstrated significantly lower cytotoxicity than Lipofectamine 2000 [59]. Hence, there may be a trade-off between residual viability and the proportion of transfected cells.

### 2.2.1.2. Cationic polymer-based vectors.

Polymers are versatile molecules that can also be designed to act as gene carriers for transfection. Polyethylenimine (PEI) has superior gene transfection efficiency compared with many other agents and is often used as a reference in gene delivery experiments. Commercial PEI-based reagents such as ExGen500 and jetPEI™ have been used for gene transfer into MSCs [60, 61]. A PEI-plasmid polyplex system demonstrated up to 10% transfection efficacy in rat BMSCs [62]. Another study reported highest efficiency at a molar number of polymer to molar number of plasmid ratio (N/P ratio) of 8 [63]. Functionalization of PEI has also been used to improve the physicochemical and biological properties of PEI as a gene carrier. Lyophilized mesoporous silica nanoparticles (MSNs) with a core-cone structure and PEI coating (MSN-CC-PEI) carrying miRNA-26a-5p efficiently promoted the osteogenic differentiation of rat BMSCs *in vitro* [64]. In another study, cross-linked PEI on graphene oxide (GO) as a gene transport compound (GO-PEI) efficiently carried pDNA encoding basic fibroblast growth factor 2 (bFGF2) into rat BMSCs and stimulated neural-like differentiation both *in vivo* and *in vitro* [65]. In addition to the common commercial transfected reagents, chemicals such as chitosan, pullulan, poly(lactic-co-glycolic acid) (PLGA), poly(amidoamine) (PAMAM), and some nanoparticles have been used for gene delivery into MSCs [66–69]. For instance, nanocomplexes of chitosan with pDNA-BMP2 immobilized on a nanofibrous polycaprolactone scaffold functionalized with metalloprotease-sensitive peptide promoted the osteogenic differentiation of MSCs *in vivo* and *in vitro* [70]. Also, AD-MSCs transfected with pDNA-IL12 using alkyl peptide-modified PAMAM demonstrated enhanced migration toward cancer cells over normal cells, suggesting the potential applications of these MSCs as carriers for targeted cancer immunotherapy. In addition, alkyl peptide-modified PAMAM demonstrated better transfection efficiency and lower toxicity than PAMAM and other synthetic derivatives (PAMAM-alkyl or PAMAM-peptide) [71].

### 2.2.1.3. Silica-based nanoparticles.

Silicon-based nanoparticles (SNPs) have the characteristics of biocompatibility and easy biodegradation, making them an ideal choice for MSC-targeted gene delivery vectors. Additionally, SNPs can achieve fine nanostructure tailoring through a variety of surface chemistry [72,73]. Based on the known contributions of various physicochemical parameters, such as particle size, shape, porosity, and surface function, SNPs have been designed to facilitate transfection efficiency for plasmid DNA (pDNA) delivery. In serum-free media, SNPs are mainly internalized by cells through clathrin-dependent endocytosis [74]. Different sizes of SNPs have different interactions. Larger SNPs significantly promote the cellular uptake of smaller SNPs, while smaller SNPs inhibit the internalization of larger SNPs, and the total number of absorbed particles of intracellular SNPs increases [75].

In addition to the nanosize of SNPs, which play an important role in the successful delivery of pDNA. SNPs with controllable nanoscale surface morphologies including rambutan, raspberry and flower morphologies were constructed by panicle, hemisphere and bowl subunits. Compared with SNPs with other surface structures, rambutan nanoparticles with spiky nanotopologies enhanced pDNA loading capacity and binding affinity, showed the highest transfection efficiency of

plasmids compared with reported silicon-based nanocarriers, and better protected DNA molecules from nuclease degradation than standard commercial products [76].

Mesoporous Silica Nanoparticles (MSNs) is the famous vector among known SNPs for their textural properties, their physical-chemical stability and their ease of chemical modification. Some works have reported to use normal MSNs as vector to transfect micro-RNAs to enhance the cell differentiation capacities of BMSCs [69,77]. Yan et al. reported the delivery of miR-26a using surface modified MSNs for BMSCs. However, the small pores of MSNs limited the loading efficiency. To solve this problem, Sepanta et al. developed an efficient miRNA carrier based on a recently developed MSN (core-cone mesoporous silica nanoparticles, MSN-CC) with large radial pores and evaluated its miRNA delivery performance and stability [78]. After functionalizing MSNs with polyethylenimine (PEI), the loading rate of RNO-miRNA-26A-5P was significantly improved. The loaded nanoparticles significantly promoted the osteogenic differentiation ability of BMSCs. Interestingly, the nanoparticle complex remained efficient and functional at  $-30^{\circ}\text{C}$  for at least 6 months [64]. Although the loading capacity was increased by enlarging the pore size, the transfection efficiency of MSNs for MSC was not very high. Naveen et al. the virus mimetic magnetic silica nanoclusters (VMSNCs) for pDNA transfection to MSCs. The transfection level of magnetic field VMSNCs (1.0 T) was the highest (40.01%), much higher than that of commercial transfection reagents (Lipofectamine 2000, less than 10%) [79]. Another effort is to successively anchor PEI-PLL-25K copolymer and arginine-glycine-aspartate (RGD) peptide on the surface of MSNs to construct a two-factor drug loading system, which enables DNA surface adsorption and dexamethasone loading in the MSNs. Through this MSN nanocomplex, the transfection efficiency was improved, and the osteogenic ability of BMSCs was finally promoted [80].

Another advantage of SNPs is that MSCs well tolerate SNPs uptake in a long time, without inducing cytotoxicity or genotoxicity, and without altering the proliferation and differentiation potential [81,82]. More importantly, internalized SNPs accumulate in lysosomal compartments, leading to a transient blockade of autophagic flux, resulting in increased adhesion phenotype and connexin-43 expression and promoting adhesion properties of hMSCs, both processes regulated by autophagic activity [83]. The internalization of SNPs promotes CXCR4 expression and hMSCs migration in response to SDF1 $\alpha$ , revealing a high potential to improve hMSCs homing to the lesion site [84].

**2.2.1.4. Gold-based nanoparticles.** Gold-based nanoparticles (AuNPs) exhibit good high stability, biocompatibility, and non-reactivity with other molecules [85]. The surface of the AuNPs can be easily functionalized for application purposes [86]. AuNPs showed well transfected capacity and has been widely used in transfection studies of cancer cell [87]. However, naked AuNPs are difficult to transfect into stem cells and primary cells. By binding to molecules targeting cell membranes and genetic material, the transfection efficiency of AuNPs can be improved [88].

Zhao et al. designed a gold nanorod (AuNR) modified to negatively charge by sodium poly4-styrene sulfonate (PSS) and positively charge by polyacrylamine salt base (PAH), which presented a positive surface charge under electrostatic action, enabling efficient siRNA binding. Compared with AuNR alone, AuNR-PSS-PAH complex carrying lysine specific demethylase 1 (LSD1) siRNA had an absorption efficiency of 70%. Compared with control cells, si-LSD1-transfected hMSCs down-regulated LSD1 transcription by more than 50%, which promoted the differentiation of hMSCs into hepatocyte lineage [89]. Recently, Yi et al. reported the design and fabrication of a gene delivery system based on multiple-clustered gold particles (mCGNPs) and singly-clustered gold-based nanoparticles (sCGNPs) that could achieve prolonged gene delivery in MSCs. Compared with sCGNPs, the mCGNPs showed higher transfection efficiency in MSCs and lower cytotoxicity and longer

expression of pDNAs (Fig. 5) [90].

The enhancement of neural differentiation ability of stem cells by biocompatible gold modified nanocomposites is a major function in the gene editing of MSCs. AuNPs can promote the differentiation of ESCs into dopaminergic (DA) neurons by activating mTOR/p70S6K signaling pathway [91]. The neural differentiation induced by AuNPs may also be related to FGF2-mediated and RA (retinoic acid) -mediated differentiation pathways [92]. A chitosan modified AuNPs (Chi-Au) catheter can stimulate the expression of BDNF and GDNF in NSCs and promote neural tissue regeneration, which suggested that AuNPs have the functions of activating neural differentiation associated paracrine system [93]. Another study found that Chi-Au 50 ppm shows better biocompatibility and significantly enhances MSC colony formation. The expression of matrix metalloproteinase 9 (MMP-9) and vinculin was also upregulated by Chi-Au 50 ppm treatment, as well as some neural marker expression ( $\beta$ -III tubulin, GFAP and nestin), which promoting the neural differentiation of MSCs [94].

**2.2.1.5. Magnetic nanoparticles.** Magnetic nanoparticles (MNPs) have been extensively explored and used for non-invasive cell tracking and magnetic manipulation [95]. Several groups have investigated the potential of superparamagnetic iron oxide nanoparticles (SPIONs) for gene delivery, as they can provide unique capabilities for magnetic resonance imaging (MRI) [96,97]. Magnetofection methods, which use static or oscillating magnetic fields to promote the absorption of SPIONs conjugated with the genetic payload into cells, can significantly improve the transfection effect [98].

MNPs and their different derivatives are then used to deliver genes into stem cells. siRNA was successfully delivered into BMSCs using PEI-coated spherical  $\text{Fe}_3\text{O}_4$  nanoparticles with an average diameter of 12 nm and a surface charge of  $+44.0$  mV. When the NP ratio was 4:1, MNPs combined with siRNA could achieve high transfection efficiency, low cytotoxicity and up to 60% silencing efficiency. Endocytosis is the mechanism by which these nanoparticles are internalized [99]. In addition to transfect siRNA, the MNPs were also explored for the delivery of miRNA and pDNAs into MSCs. PEI-modified MNPs were used to deliver miR-335 to hMSCs, which promoted the proliferation, differentiation and migration of hMSCs. Compared with the control group, the expression of miR-335 in the transfected cells of miR-PEI-MNP group was increased by 1000 times. After 72 h, the expression of miR-335 in miR-PEI-MNP group was 4 times higher than that in miR-PEI group. In addition, in contrast to the miR-PEI complex, the miR-PEI-MNP complex is retained in the cytoplasm, resulting in sustained expression of miR and enhancing the efficiency of miR-mediated downregulation of target gene expression [100]. For DNA transfection, a new iron oxide nanoparticle named SAMN (surfactant maghemite nanoparticle) was used to create a new SAMN@DNA complex in which an intimate chemical contact occurs between SAMN and pDNA by covalent binding. SAMN@pDNA was used for transgene expression in PB-MSCs, and the fluorescence intensity increased with increasing nanoparticle concentration, reaching an optimal transfection efficiency (up to 70%) at 300  $\mu\text{g}/\text{mL}$  SAMN@pDNA, corresponding to 6  $\mu\text{g}/\text{mL}$  pDNA. In addition, SAMN, as a pDNA vector, showed higher transfection efficiency than commercial liposomes [101]. By reducing the N/P ratio and polyclonal method, the transfection efficiency of Magnetofection method for MSCs can be improved. In addition, the increase of culture time and the use of Opti-MEM instead of serum-free DMEM also contributed to better transfection efficiency and lower cytotoxicity. The Nanog protein expression of MSCs transfected with the optimized method was about 6 times higher than that of the control group, which promoted the proliferation rate of adult MSCs and fully restored the weakened myogenic differentiation potential [102]. Because these nanoparticles also have imaging properties via MRI, this gene delivery system could be well suited for stem cell therapeutic applications.

**2.2.1.6. Cell-penetrating peptides.** Cell-penetrating peptides (CPPs) are relatively short peptides, 4–40 aa, with the ability to gain access to the cell interior by means of different mechanisms, mainly including endocytosis, and/or with the capacity to promote the intracellular effects by these peptides themselves, or by the delivered covalently or non-covalently conjugated bioactive cargoes [103]. The CPPs studied so far include Tat, oligo-ArG, Transportan, and pentatin, which bind to biological groups via covalent bonds and are used for cross-barrier transport of a variety of bioactive cargoes. These discoveries led to breakthroughs in cellular biochemistry, breaking the conventional dogma that proteins and peptides cannot penetrate cell membranes. CPPs have been widely used to transport cargo molecules *in vitro* and *in vivo*. The transport and absorption mechanisms of CPPs and their particles consist of two main pathways: direct osmosis and endocytosis [104,105].

In recent years, the optimization of intracellular pDNA delivery by different CPPs has been an important research goal. However, there are few reports on primary cell transport plasmids. Liu et al. constructed a novel CPP nanocomplex of R9-LK15 for AD-MSC transfection. The transfection efficiency of positive cells in the samples treated with R9-LK15/miR-29b nanocomplex reached 78.33%, which was much higher than that of the samples treated with Lipo2000 nanocomplex (36.43%). In addition, the fluorescence intensity in cells treated with R9-LK15/miR-29b nanocomplex was 10-fold higher than that in cells treated with Lipo2000 group [106]. An  $\alpha$ -helical cyclic LK peptide (cLK) based on the initial LK peptide was developed as an effective carrier of p-Runx2 into MSCs. The combined effect of p-Runx2 and cLK may have a positive effect on the proliferation and osteogenic differentiation of MSCs [107]. Similar results were found by using a glycosaminoglycan-binding enhanced transduction (GET) delivery system containing 8R and P21 peptides. GET-RUNX2 effectively transduced MSCs and induced osteogenesis by directly increasing the expression of target genes [108].

In order to compare the difference of MSC transfection between different CPPs, 5 different CPPs (H4G, Mpg-H4G, Pep1-H4G, VAGO-H4G, VANTA-H4G) with various transfected strategies were included. H4G and VANTA-H4G vectors carrying 0.4  $\mu$ g and 0.5  $\mu$ g pEGFP were the most efficient CPPs. The transfection efficiency of VANTA-H4G was more than 50%. In addition, AD-MSC is not a suitable cell model for Pep1 and MPG transfection. Vanta-H4G targeting VEGFR-1 was not only the most effective vector for AD-MSC transfection, but also did not have any significant negative effects on physical integrity, metabolic activity, genetic composition or cell differentiation [109].

**2.2.1.7. Carbon nanotubes.** Carbon nanotubes (CNTs) are formed with six-membered rings by covalently binding sp<sup>2</sup> carbon atoms [110]. They are elongated tubular nanostructures that can be used as scaffolds to promote the proliferation and differentiation of stem cells [111]. Carbon nanotubes can be divided into single-walled (SWCNT) or multi-walled (MWCNT). SWCNTs are smaller in diameter, more flexible, and provide photoluminescence for imaging purposes, while MWCNTs have a wider surface area that can be used for cargo attachment and functionalization, supporting more efficient internal packaging. The original CNTs lack solubility and form thick and heterogeneous nerve bundles with short cycling half-lives, immunogenicity, and low biocompatibility. Surface modifications of CNTs, such as functionalization by polyethylene glycol, enhance the biodistribution of CNTs and ultimately eliminate CNTs upon uptake into cells [112].

Several studies have suggested SWCNTs [113,114] and MWCNTs [115,116] can be used as scaffold materials to promote neural differentiation and proliferation various MSCs. In addition, a study has indicated CNTs significantly promoted neurotrophic factors, especially nerve growth factor and brain-derived neurotrophic factor. MSCs cultured in CNT-collagen hydrogels released neurotrophic factors to affect the neural differentiation of PC12 cells, which showed that the

neurite growth behavior was significantly enhanced [117].

These results indicate that CNTs as a carrier can promote the neural differentiation of MSCs and paracrine effect of neurotrophs, which is an ideal gene delivery vectors for neural tissue repair. However, there are few examples of CNTs being used as gene delivery vectors in MSCs or potential useful for MSC-based therapy. Hanieh et al. constructed a nanovector system of PEI-grafted MWCNT combined with chitosan substrate as gene delivery was developed, and the transfection efficiency was evaluated in bone marrow mesenchymal stem cells (BMSCs) using plasmid DNA encoding enhanced green fluorescent protein (pEGFP) as a reporter gene [118].

**2.2.1.8. Quantum dots.** Fluorescent molecular probes present challenges for *in vivo* and *in vitro* biomolecular imaging. Unlike conventional fluorescent probes induced *in vivo* scattering, absorption, and inhibition, quantum dots (QDs) exhibit a broad spectrum of excitation spectra and a narrow, clear emission peak with a large Stokes shift that is stable and shows reduced photobleaching. Some studies have pointed out that quantum dots can also be used as therapeutic nanocarriers to achieve high sensitivity and resolution for a long time at a low cost [119,120].

The QDs-si-PPAR $\gamma$ -Dex nanocomplex co-transfected Runx2 with higher expression (over 90% expression) than the QD-conjugated si-PPAR $\gamma$  or Dex delivery of a single molecule alone. In addition, the QD can fluoresce for up to 14 days. In their subsequent studies, it was confirmed that QD-nanocarriers could co-deliver small molecules and siRNA into stem cells, and this common cargo transport made MSCs significantly more effective in osteogenesis than Lipofectamine 2000 gene transport based on oligonucleotide [121]. Compared with traditional CdSe/ZnS QDs, ZNXS-Agyin1-YS2 (ZAIS) QDs synthesized by acoustic chemical synthesis method showed lower cytotoxicity and phototoxicity when transfected into hMSCs and human brain tumor cells by changing the concentration of organomaterial precursor elements and retaining strong fluorescence in the cytoplasm. When ZAIS was used as a gene carrier for siRNA, the mRNA knockdown efficiency was up to 80% [122].

However, despite their potential gene carrier capacity, the significant cytotoxicity of QDs is a hindrance to their further widespread use. Some necessary modifications or covalent binding can reduce the significant toxicity. The cytotoxicity of QDs was reduced by adding ZnS shell and PEG coating. Isobifunctional cross-linkers sulfo-succinyl-4-(N-maleimide methyl) and/or cyclohexane-1-carboxylate (sulfo-SMCC) can be bio-coupled at selective sites in proteins by binding stable thioethers to a sulfhydryl-exposed antibody (QD-SMCC). The cytotoxic effect of QD-SMCC in the concentration range of 20–120 pmol/L was lower than that of PEI [123]. The transfection and knockdown efficiency of QD-SMCC bound SOX9 siRNA were significantly better than that of PEI-SISOX9. However, the inhibition of SOX9 down-regulated the expression of type II collagen and poly aggrecan, and accelerated the differentiation of human MSCs into chondrocytes. Another novel QDs designed showed less cytotoxicity as a nanocomplex bound to positively charged peptides such as RGD and  $\beta$ -cyclodextrin than QDs alone. The positively charged RGD binds easily to siRNA and further promotes transfection to MSCs by ligating to integrin receptors on the cell surface [124].

## 2.2.2. Physical methods

Several physical methods such as electroporation, nucleofection, and sonotransfection are used for the delivery of beneficial genes into MSCs. The electroporation technique has demonstrated high transfection efficiency for various MSCs but exhibits significant cell toxicity. In one study, the optimal transfection conditions for human AD-MSCs were a pulse magnitude of 1500 V for 20 ms. Under these conditions, the transfection efficiency and viability were 50% and 78%, respectively [125]. Even greater transfection efficiencies were obtained in human DPSCs [59] and human BMSCs [126].

Nucleofection™ is a commercially available electroporation-based method that facilitates the transfer of pDNA directly into the cell nucleus with the aid of various cell type-specific reagents. Nucleofection™ demonstrated higher transfection efficiency for MSCs than traditional lipid-based transfection methods [127]. In one study, the transfection efficiency of hMSCs using Nucleofection™ reached 45%, albeit with low cell viability [128]. Cell origin significantly affected the transfection efficiency obtained using Nucleofection™ as the efficiency was 3-fold higher in rat MSCs than in human MSCs [129].

Transient ultrasound can be used to enhance cell membrane permeability and thereby increase the efficiency of gene delivery. Otani et al. combined ultrasound with microbubbles to deliver small-interfering RNA (siRNA) into MSCs. However, cell viability was significantly decreased with the increase in acoustic intensity [130]. Other physical methods, such as electric field-induced molecular vibration [131] and nano-microinjection [132,133], have also been used for transient gene delivery into MSCs.

### 2.2.3. Reverse transfection

The traditional gene delivery method is to combine the gene with the vector, and then add the gene to the culture medium and co-culture with the cells. This transfection method is limited by the fact that the cells are in 2D culture environment, and the transfection efficiency is often low for MSCs that are difficult to be transiently transfected. Recently, a different method of gene delivery called reverse transfection has been developed. The difference between the two methods is that the DNA and cells are added in different order. Different from traditional transfection methods, reverse transfection involves adding DNA or DNA transfection complexes to the substrate material and then seeding the cells for transfection [134]. DNA can interact with the substrate scaffold material in a specific or nonspecific manner. The cells were cultured in 3D environment under the action of substrate scaffold material. This culture protocol greatly increases the transfection efficiency and reduces the steps of using MSCs to treat diseases which need scaffold materials implanted together with MSCs into the tissue, such as bone defect or large peripheral nerve defect. It is a potential gene editing protocol suitable for MSCs.

Uchimura reported transfection complexes of pEGFP-N1/Jet-PEI on glass surfaces using negatively charged gold colloid (GC) using reverse staining. Compared with the transfection group without GC, GC/Jet-PEI increased the fluorescence intensity of enhanced green fluorescent protein (EGFP) of hMSCs by more than 2.5 times [135]. A set of cross-sectional contrast experiments used a series of silica nanosheet networks to facilitate the delivery of bare plasmid DNA into MSCs. By adding different chemicals to the reaction mixture to corrode silica glass, the inorganic/organic hybrid nanosheet networks with different physicochemical properties were prepared without cytotoxicity, and the survival rate of transfected MSCs was high (90%). The transfection efficiency mainly depends on the aspect ratio (wall height/wall thickness) of the nanosheet. The effect of chemical or other surface properties was not significant. The transfection efficiency of MSCs could reach 70% in large aspect ratio nanosheet [136]. This transfection system, by holding the DNA complex for a long time, has the ability to express genes more persistently than conventional transient transfection. When polylactic acid co-glycolic acid (PLGA) sponges containing DNA complexes and hMSCs were implanted into the subcutaneous tissue of Severe Combined SCID (SCID) mice, it was found that transgene expression could be detected for up to 15 weeks, indicating long-term effective transfection of the system [137]. Sustained release of DNA complexes via scaffold materials may be less cytotoxic than direct ingestion of most DNA/nanomaterial complexes. Finally, reverse transfection is an ideal method to implant cell scaffold composites. This is probably the closest transfection method to electrotransfection reported so far.

### 2.2.4. Combined strategies to enhance transfection efficiency

Given the low transfection rates and (or) transgene expression levels

induced by individual methods, several recent studies have examined the use of combined physical methods, chemical methods, or both to enhance transfection efficiency in some difficult-to-transfected cells, including MSCs. Ishiguro et al. found that the combined use of Lipofectamine LTX and FuGENE HD increased the efficiency of gene transfer into primary fibroblasts and hepatoblasts compared with either reagent alone (a "1 + 1 > 2" effect) [138]. These findings suggest the potential utility of combined methods for enhancing the transfection efficacy of MSCs as well. Indeed, Li et al. reported that combining ultrasound with PEI-loaded microbubbles increased the pEGFP- hepatocyte growth factor (HGF) transfection rate to 57.43% at 7 days post transfection with little reduction in cell viability [139]. Poly-L-lysine conjugated with palmitic acid (PLL-PA) also increased cellular uptake as well as the expression of the cell marker enhanced green fluorescent protein by nearly 10-fold in MSCs compared with either method alone. Further, the transfection efficiency of PLL-PA was approximately 2-fold higher than that achieved using Lipofectamine 2000, and the combination of PLL-PA with Lipofectamine 2000 further increased the transfection efficiency [140].

Transfection methods in general are stressful to cells, and may induce protective responses that reduce gene transfer, gene expression, or cell viability. A promising approach to promote gene transfer is through pretreatment with pharmacological agents that can temporarily overcome barriers to gene transfer (priming). Kelly et al. first reported that pretreatment with dexamethasone (DEX) enhanced human MSC transfection efficiency by 3-fold and transgene expression by 4–15-fold. In addition, priming with DEX significantly prolonged transgene expression compared with transfection without priming. This priming effect may stem from the binding of glucocorticoid receptor (GR) and partly by the maintenance of normal cellular metabolism [78]. A follow-up study found that the enhancement is mediated by the transcriptional activation of endogenous MSC genes by cytosolic GR, which alleviated transfection-induced endoplasmic reticulum and oxidative stresses, inflammatory responses, and apoptosis pathway activation, thereby preventing metabolic failure and protein synthesis inhibition. These effects may be enhanced using a GR transcription-activation synergist [141]. Similarly, a high-throughput analysis of 707 FDA-approved drugs revealed that pretreatment with glucocorticoids, including clobetasol propionate, DEX, triamcinolone acetonide, fluorometholone, and triamcinolone acetonide, increased the transfection efficiency of human AD-MSCs [142].

Another way to improve transfection efficiency is to control the concentration of  $\text{Ca}^{2+}$  in the medium. Increased  $\text{Ca}^{2+}$  concentrations have been shown to further enhance transfection with a variety of nonviral gene delivery methods, including polycationic and lipid-based systems [143–145]. Interestingly, the change of the concentration of other metal ions did not promote the improvement of transfection efficiency. At the same time, the improvement of transfection efficiency has obvious reagent dependence [145]. A study indicated that  $\text{Ca}^{2+}$  at 4–6 mM promoted the transfection efficiency of some reagents, such as CPP, but had no significant effect on the transfection efficiency of PEI or Lipofectamine [143]. However, another study pointed out that with the increase of  $\text{Ca}^{2+}$  concentration, the transfection efficiency of PEI on BMSCs gradually improved, reaching the peak when the concentration of  $\text{Ca}^{2+}$  reached 10 mM, and the increase of  $\text{Ca}^{2+}$  concentration promoted the osteogenic effect of BMSCs [146]. This result indicates that different cell types have different sensitivity to  $\text{Ca}^{2+}$  concentration.

In most cases, the levels of gene expression obtained by nonviral gene delivery methods are lower than those obtained by viral vectors, suggesting that the treatment is ineffective using nonviral gene delivery methods. With the development of non-viral transfection technology, the transfection efficiency of some new composite nanomaterials can reach more than 70%, which is close to viral transfection (Table 1). In addition, both *in vitro* and *in vivo* studies have shown promising results using various nonviral gene delivery methods for tissue engineering and regeneration applications, implying that transfection efficiency alone is

**Table 1**

Advantages and disadvantages of different transfection methods for application of MSC in neural injury.

| Methods                           | Carrier                                                       | Cell           | Reported transfection efficiency                             | Advantages                                                                                                                                                                                                                                                                                   | Limitations/Risks                                                                                                                                                                                          |
|-----------------------------------|---------------------------------------------------------------|----------------|--------------------------------------------------------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| <b>Viral-based vectors</b>        |                                                               |                |                                                              |                                                                                                                                                                                                                                                                                              |                                                                                                                                                                                                            |
| Lentivirus                        | –                                                             | MSCs           | The MOI can reach up to 90% with the increase of virus titer | <ul style="list-style-type: none"> <li>● The inherent susceptibility of rapidly proliferating MSCs to viral infection</li> </ul>                                                                                                                                                             | <ul style="list-style-type: none"> <li>● Lacking specificity</li> <li>● Raising safety concerns</li> </ul>                                                                                                 |
| Retroviruses                      |                                                               |                |                                                              | <ul style="list-style-type: none"> <li>● Stable long-term expression of integrated genes</li> </ul>                                                                                                                                                                                          | <ul style="list-style-type: none"> <li>● Only infect proliferating cells</li> <li>● Silencing of viral transgenes</li> <li>● Oncogenous risk</li> </ul>                                                    |
| Adenoviruses                      |                                                               |                |                                                              | <ul style="list-style-type: none"> <li>● The capacity to carry large exogenous DNA fragments</li> </ul>                                                                                                                                                                                      | <ul style="list-style-type: none"> <li>● Potential immunogenicity</li> <li>● The transient expression of the transgene</li> </ul>                                                                          |
| Adeno-associated virus            |                                                               |                |                                                              | <ul style="list-style-type: none"> <li>● Intra-nuclear replication and transcription to express overlapping open reading frames.</li> </ul>                                                                                                                                                  | <ul style="list-style-type: none"> <li>● Potential neuroinflammatory risk</li> <li>● Maximum gene size</li> </ul>                                                                                          |
| Baculoviruses                     |                                                               |                |                                                              |                                                                                                                                                                                                                                                                                              | <ul style="list-style-type: none"> <li>● Inactivation by the human complement system</li> <li>● Glycosylation</li> </ul>                                                                                   |
| <b>Chemical synthetic vectors</b> |                                                               |                |                                                              |                                                                                                                                                                                                                                                                                              |                                                                                                                                                                                                            |
| Cationic lipid-based vectors      | Lipofectamine™ FUGENE6                                        | human MSCs     | Routine for 15–35%, up to 50% (pDNA)                         | <ul style="list-style-type: none"> <li>● Easy to prepare</li> <li>● Low cost</li> </ul>                                                                                                                                                                                                      | <ul style="list-style-type: none"> <li>● Cytotoxicity</li> <li>● Low efficiency at the presence of serum component</li> </ul>                                                                              |
| Cationic Polymer-based Vectors    | PEI<br>PAMAM<br>PLGA<br>Chitosan pullulan                     | rat BMSCs      | <sup>a</sup> PEI:10% (pDNA)                                  | <ul style="list-style-type: none"> <li>● Effective</li> <li>● Easy to prepare</li> </ul>                                                                                                                                                                                                     | <ul style="list-style-type: none"> <li>● Cytotoxicity, safety concern for undegradable polymers</li> </ul>                                                                                                 |
| Silica-based Nanoparticles        | MSN, VMSNC                                                    | MSCs           | VMSNC:40.01% (pDNA)                                          | <ul style="list-style-type: none"> <li>● Low cost</li> <li>● Can be used as a modification material for modification</li> </ul>                                                                                                                                                              | <ul style="list-style-type: none"> <li>● Easy degradation</li> <li>● The transfection ability of common MSN was insufficient</li> </ul>                                                                    |
| Gold-based Nanoparticles          | AuNR, mCGNPs                                                  | human BMSCs    | mCGNPs:20% (pDNA)                                            | <ul style="list-style-type: none"> <li>● High efficiency</li> <li>● Biocompatibility</li> <li>● Easy biodegradation</li> <li>● Specific surface area</li> <li>● Tunable size</li> <li>● High stability</li> <li>● Biocompatibility</li> <li>● Non-reactivity with other molecules</li> </ul> | <ul style="list-style-type: none"> <li>● The transfection effect of AuNPs alone was poor</li> </ul>                                                                                                        |
| Magnetic Nanoparticles            | MNP, SAMN                                                     | PB-MSCs        | SAMN: 70% (pDNA)                                             | <ul style="list-style-type: none"> <li>● Prolonged expression time</li> <li>● High efficiency</li> <li>● Non-invasive cell tracking</li> <li>● Magnetic manipulation</li> </ul>                                                                                                              | <ul style="list-style-type: none"> <li>● The operation and manufacturing process are complex</li> </ul>                                                                                                    |
| Cell-penetrating Peptides         | R9-LK15, cLK, 8R, H4G, Mpg-H4G, Pep1-H4G, VAGO-H4G, VANTA-H4G | BMSCs<br>AMSCs | R9-LK15: 78.33% (miRNA)<br>VANTA-H4G: 50% (pDNA)             | <ul style="list-style-type: none"> <li>● No irritation</li> <li>● Little cytotoxicity</li> <li>● No dependence on endocytosis</li> </ul>                                                                                                                                                     | <ul style="list-style-type: none"> <li>● Biological activities are easily affected</li> </ul>                                                                                                              |
| Carbon Nanotubes                  | PEI-MWCNT                                                     | rat BMSC       | –                                                            | <ul style="list-style-type: none"> <li>● Promote neural differentiation and proliferation various MSC</li> </ul>                                                                                                                                                                             | –                                                                                                                                                                                                          |
| Quantum Dots                      | –                                                             | human BMSCs    | 80–90% (siRNA)                                               | <ul style="list-style-type: none"> <li>● In vivo imaging</li> <li>● Biomarker</li> <li>● Low cost</li> <li>● High efficiency</li> </ul>                                                                                                                                                      | <ul style="list-style-type: none"> <li>● Cytotoxicity</li> </ul>                                                                                                                                           |
| <b>Physical methods</b>           |                                                               |                |                                                              |                                                                                                                                                                                                                                                                                              |                                                                                                                                                                                                            |
| Electroporation                   | Electroporation/<br>Nucleofection                             | human BMSCs    | Nucleofection: 45% (pDNA)                                    | <ul style="list-style-type: none"> <li>● Effective</li> <li>● Able to transfer large size DNA</li> <li>● Good repeatability</li> <li>● No carrier required</li> <li>● No restrictions on cell types and conditions</li> </ul>                                                                | <ul style="list-style-type: none"> <li>● Cytotoxicity</li> <li>● Need special equipment</li> <li>● Need to optimize the electrical pulse and voltage parameters</li> </ul>                                 |
| Sonotransfection                  | –                                                             | –              | –                                                            | <ul style="list-style-type: none"> <li>● Low cytotoxicity</li> <li>● Easy to operate</li> </ul>                                                                                                                                                                                              | <ul style="list-style-type: none"> <li>● Need special equipment</li> <li>● Need to optimize related condition parameters</li> </ul>                                                                        |
| Nano-microinjection               | Nanoneedle                                                    | human BMSCs    | Nanoneedle:74% (pDNA)                                        | <ul style="list-style-type: none"> <li>● Effective</li> <li>● Simple, Effective, Ee reproducible, non-toxic</li> <li>● Able to transfer large size DNA</li> <li>● Single cell transfection is possible</li> <li>● No vector is required</li> </ul>                                           | <ul style="list-style-type: none"> <li>● Cytotoxicity</li> <li>● Need special equipment</li> <li>● It is technically demanding and labor intensive (only one cell can be transfected at a time)</li> </ul> |

Abbreviation: BMSC: bone marrow mesenchymal stem cell; mCGNPs: multiply-clustered gold-based nanoparticles; MOI: multiplicity of infection; MWCNT: multi-walled carbon nanotubes; PEI: Polyethylenimine; PLGA: poly(lactic-co-glycolic acid), PAMAM: poly(amidoamine), VMSNC: virus mimetic magnetic silica nano-clusters; SAMN: surfactant maghemite nanoparticle.

<sup>a</sup> Other compounds modified with PEI are excluded.

not always the main factor determining the therapeutic outcome. Paracrine and autocrine signaling as well as intercellular contact can trigger the wider expression of gene cascades that in turn enhance the ultimate treatment effect. Further, low-efficiency transfection or transient gene expression may be sufficient for tissue formation [147,148]. Thus, nonviral gene transfer methods have substantial potential for MSC-based treatment of various diseases. Nonetheless, the development of MSC-specific transfection strategies with improved efficiency and reduced cytotoxicity are critical for the future clinical applications of MSC-based therapies.

### 3. Selecting appropriate genes for transduction

#### 3.1. Neural differentiation-associated genes

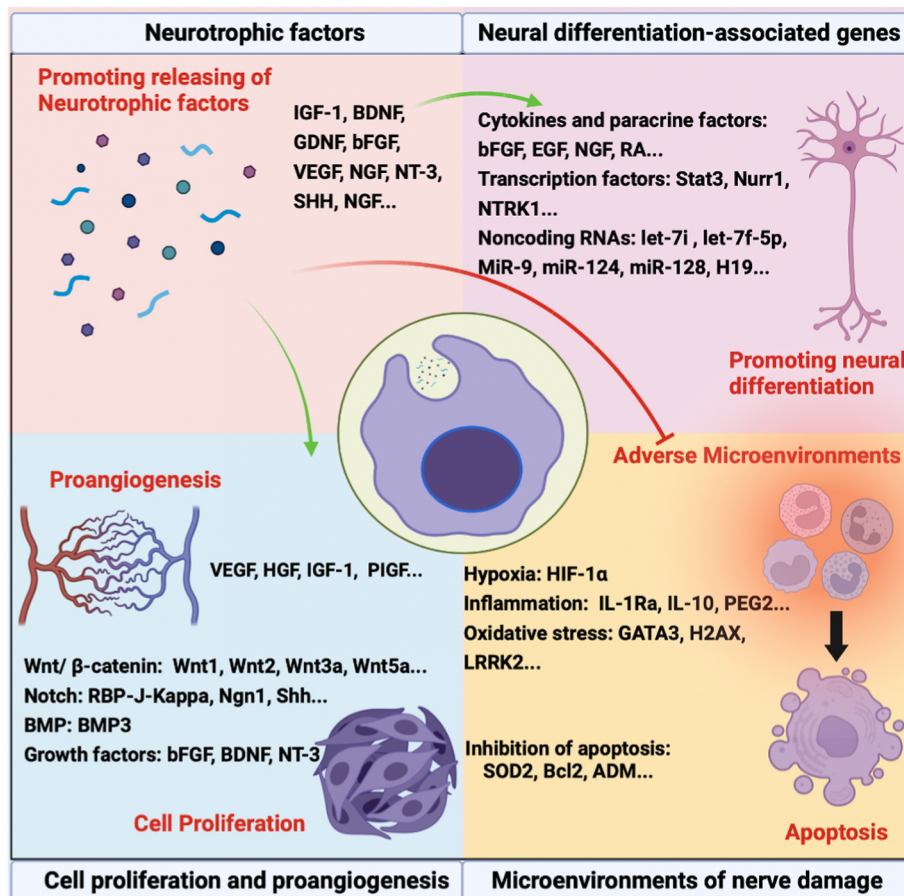
##### 3.1.1. Cytokines and paracrine factors

Although MSCs originate from the mesoderm, they can differentiate horizontally into cells of nonmesodermal origin, such as neurons and glia, under certain conditions. Hence, a potential therapeutic strategy for neurodegenerative disorders is to induce MSC differentiation into neural precursors for transplantation (Fig. 4). Growth factors demonstrated to induce MSC differentiation into neurons or neuron-like cells include bFGF (Fig. 6) [149], epidermal growth factor (EGF) [150], NGF [151], BDNF [152], sonic hedgehog (SHH) [153], neurotrophin 3 (NT-3) [154], and retinoic acid (RA) [155]. Numerous studies have investigated the potential therapeutic efficacy of overexpressing growth factors in MSCs or adding extrinsic recombinant growth factors to cell substrates (such as biocompatible scaffolds) to enhance neuronal differentiation or neurotrophic signaling by transplanted MSCs. Loading a PCL nanofibrous nerve guide conduit with NGF was found to promote neurite extension *in vitro*, whereas nerve guide conduits implanted with MSCs

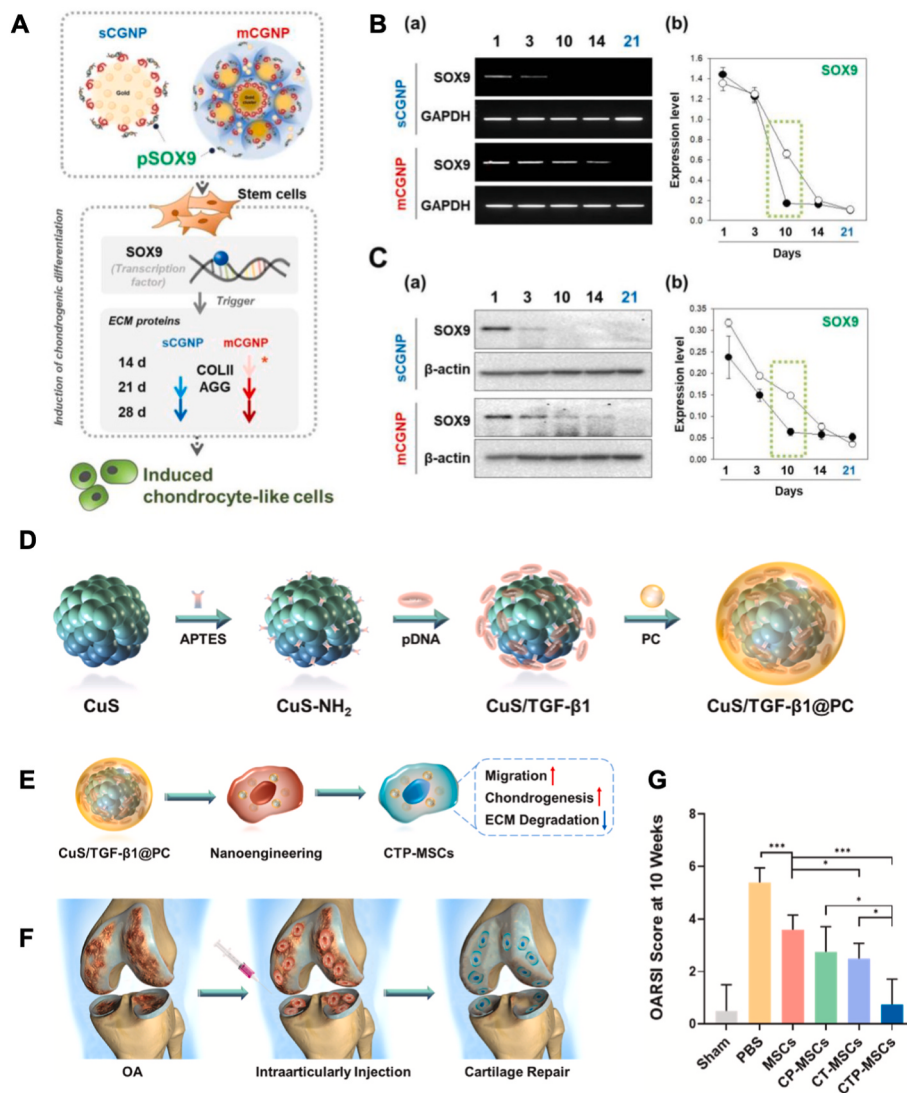
were reported to repair 15-mm segmental defects in a rat sciatic nerve injury model *in vivo* [156]. Similarly, human UC-MSCs cocultured with activated astrocytes and then seeded on RADA16-BDNF-loaded self-assembling peptide hydrogel (R-B-SPH) differentiated into neuron-like cells with neurites extending in three-dimensional directions, which may benefit both the neural differentiation of ectogenic UC-MSCs and endogenous neurogenesis in a rat TBI model [157], suggesting potential therapeutic utility for reconstructing damaged neuronal circuits. Also, the overexpression of NT-3 promoted the neuronal differentiation of BMSCs and improved cognitive function in Alzheimer's disease (AD) model rats [158]. BMSCs overexpressing SHH increased the expression and secretion of SHH, bFGF and VEGF, that benefit the survival and nervous behavioral function of BMSCs and significantly enhanced the recovery of neurological function after SCI in rats [159].

##### 3.1.2. Transcription factors

In addition to signaling factors that are released into the microenvironment, overexpression or knockdown of intercellular signaling molecules such as transcription factors has demonstrated potential for enhancing the therapeutic response to MSC therapy. By overexpressing or knocking down genes associated with neural differentiation, MSCs can be induced to differentiate into neurons. The transcription factor signal transducer and activator of transcription 3 (STAT3) engages in a variety of physiological events involved in nervous system development and governs nerve cell survival and repair via the production of neuroregulatory cytokines [160]. Recent studies have reported that neural repair following TBI [161], stroke [162], SCI [163], and sciatic nerve injury [164] depends on the activation of STAT3 signaling following the transplantation of MSCs. Hence, STAT3 overexpression in MSCs is a promising strategy for cell-based therapy. Consistent with this notion, transplanted BMSCs overexpressing STAT3 via recombinant lentivirus



**Fig. 4. Different gene modification strategies for MSCs to treat neural injury.** Nerve injury diseases are often accompanied by the loss of large numbers of neurons and other neural stromal cells. Hence, the strategies targeting neural differentiation-associated genes are helpful to increase the differentiation of efficiency of MSCs into neural cells including neuron, microglial cell, Schwann cell et al. However, the transplanted MSCs from local injection or I.V. all face harsh survival conditions, such as hypoxia, inflammation and oxidative stress that induce apoptosis of MSCs. Genetic modification in response to adverse environments or prevent apoptosis can improve the survival rate of MSCs. Another strategy to increase the number of transplanted MSCs is to enhance capacity of proangiogenesis and directly promote MSC proliferation. Some classical signaling pathways associated with cell proliferation of MSCs include Wnt/β-catenin, Notch and BMP. Transfection of neurotrophic factor can promote the release of more these factors to extracellular of MSC, which can better activate the paracrine signaling pathways. Neurotrophic factors can not only play neurotrophic and protective roles against damaged and newly formed neurons, but also promote neuronal differentiation, cell proliferation or angiogenesis, as well as regulating the local microenvironment and inhibiting apoptosis.



**Fig. 5.** Novel nanoparticles with high gene delivery capacity are applied in gene modification of MSCs. A complex gene delivery system based on multiply-clustered gold particles (mCGNPs) were designed and fabricated that could achieve prolonged gene expression in MSCs, promoting to improved induction of differentiation. (A) Flowchart presenting efficient gene delivery of pDNA-SOX9 by mCGNPs triggered induction of chondrogenic differentiation of MSCs by enhancing the expression of extracellular matrix (ECM) proteins, including aggrecan and COLII, earlier and more effectively than singly-clustered gold-based nanoparticles (sCGNPs). The RNA and protein levels of pDNA-SOX9 expression delivering with sCGNPs or mCGNPs after 21 days after transfection were evaluated by RT-PCR (B) and western blotting analysis (C), respectively. (D–F) Flowchart presents biomimetic copper sulfide@phosphatidylcholine (CuS@PC) nanoparticles (NPs) loaded with plasmid of transforming growth factor-beta 1 (TGF-β1) to engineer MSCs for enhanced osteoarthritis therapy via cartilage regeneration. (G) OARSI scores were determined according to the IHC assay of the samples collected at 10 weeks post-surgery.

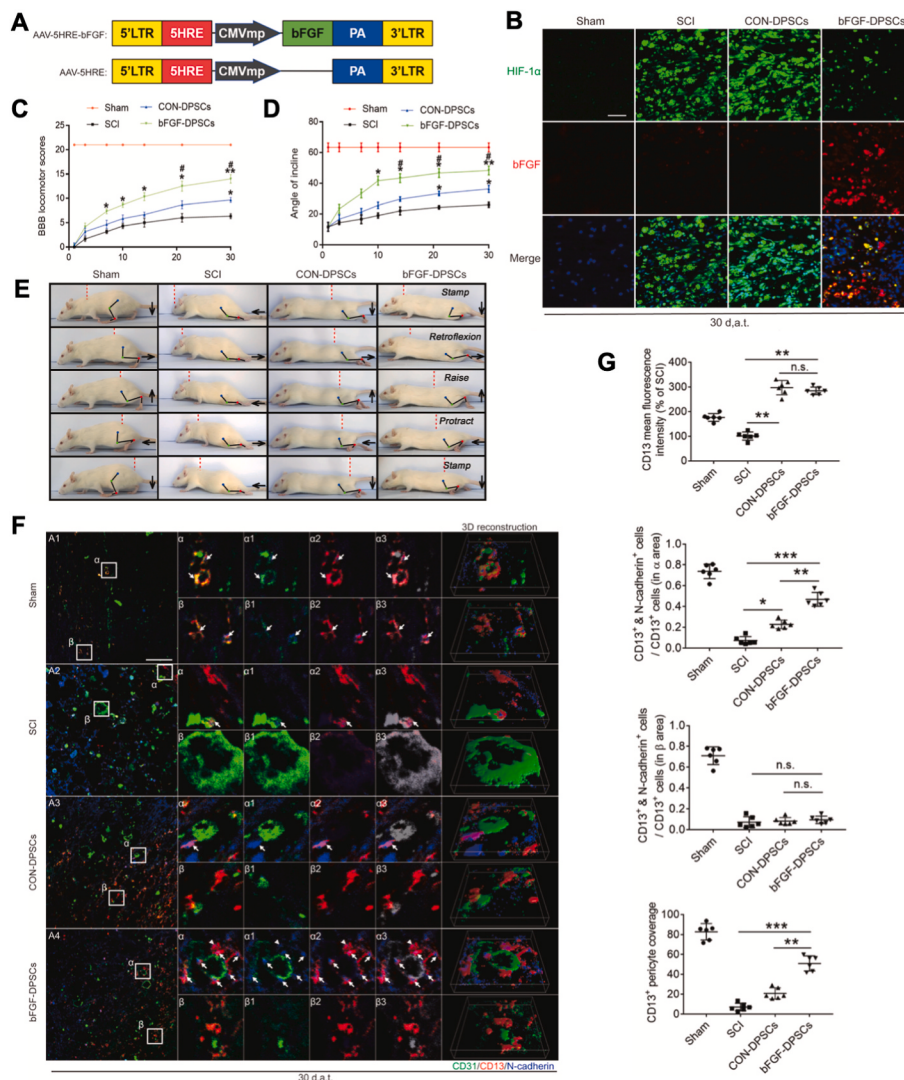
infection promoted the survival of fetal rats with spina bifida aperta (SBA), and this effect was associated with reduced BMSC apoptosis, enhanced BMSC survival, and increased neural marker expression [165]. Overexpression of the transcription factor nuclear receptor-related factor 1 (Nurr1), a member of the orphan nuclear receptor family implicated in dopaminergic neuron development and maturation, accelerated the dopaminergic differentiation of UC-MSCs [166] and BMSCs [167] and promoted the recovery of a rat model of Parkinson's disease (PD). Other transcription factor- or cytokine receptor-associated cell signaling pathways that promote MSC differentiation into dopaminergic neurons include neurotrophic tyrosine receptor kinase 1 (NTRK1), achaete-scute family bHLH transcription factor 1 (ASCL1), conserved dopamine neurotrophic factor (CDNF) [168], and neurogenin 1 [169]. Khan et al. identified 3252 differentially expressed genes (DEGs) ( $\text{adj-P} < 0.05$ ,  $\log_2 \text{FC} > 1$ ) between control rBMSCs and rBMSCs induced to differentiate into neural progenitor-like cells (NPCs) by bFGF and EGF treatment. The top 5 DEGs associated with neural differentiation were *Txnip*, *Vcam1*, *AABR07054614.1*, *Aldh1a3*, and *Cox4i2*. Although the biological statuses of most of these genes in the process of BMSC differentiation requires validation, these genes can have potential applications in combination with MSCs in the treatment of neurological diseases [170].

### 3.1.3. Noncoding RNAs

Altering the expression levels of noncoding RNAs (ncRNAs) has drawn considerable attention as ncRNAs can modulate the post-transcriptional control of target genes in a biological manner to promote the neurorestorative effects of stem cell transplantation.

**3.1.3.1. MicroRNAs.** Huat et al. identified 46 microRNAs (miRNAs) differentially expressed by BMSCs at 1, 3, and 5 days following two different neurogenic induction protocols. Members of the Let-7 family of miRNAs were downregulated, suggesting their essential functions in neurogenesis [171]. The downregulation of let-7i promoted the expression of the proneural gene achaete-scute complex-like 1 [172]. In addition, let-7f-5p was downregulated during the neurogenesis of BMSCs, and the inhibition of let-7f-5p facilitated the differentiation of BMSCs into neuron-like cells. Mechanistic analysis revealed that let-7f-5p prevented neurogenesis by directly interacting with partitioning defective 6 homologue  $\alpha$  (Par6 $\alpha$ ), which is essential for axon specification during neuronal development [173]. MiR-9 [174,175], miR-124 [176,177], and miR-128 [178] have also been implicated in the differentiation of MSCs into NPCs.

**3.1.3.2. Long noncoding RNAs.** H19 is a conserved long noncoding RNA (lncRNA) abundantly expressed in mammalian cells. The inhibition of



**Fig. 6.** Adeno-associated virus drive hypoxia-response element (HRE) to mediate the expression of human bFGF in dental pulp stem cells (AAV-5HRE-bFGF-DPSCs) have significant therapeutic effect on SCI rats by recovering damage caused from hypoxic microenvironment. (A) Schematic diagrams showing the vector construction: AAV-5HRE-bFGF and AAV-5HRE. (B) Immunofluorescence staining showing HIF-1α (green) and bFGF (red) in the Sham, SCI group, AAV-5HRE-DPSCs (CON-DPSCs) group and AAV-5HRE-bFGF-DPSCs (bFGF-DPSCs) group in 30 d.a.t. (C) The BBB scores and (D) the inclined plane test scores of the rats in the Sham, SCI group, AAV-5HRE-DPSCs (CON-DPSCs) group and AAV-5HRE-bFGF-DPSCs (bFGF-DPSCs) group. (E) Video sequences showing rats walk in 30 d. a.t. Accordingly, the weight support, leg extensor spasms, slow steps and foot placement were analyzed. Foot movement is highlighted by arrows. The initial position of the move is highlighted by red dots. Scale bar: 20 mm. CD13<sup>+</sup> pericytes rely on N-cadherin to attach to vascular endothelial cells. (F) Immunofluorescence staining showing the expression of CD31 (green), CD13 (red) and N-cadherin (blue) in the Sham, SCI group, AAV-5HRE-DPSCs (CON-DPSCs) group and AAV-5HRE-bFGF-DPSCs (bFGF-DPSCs) group. (G) The fluorescence intensity of CD13, ratio of N-cadherin<sup>+</sup> & CD13<sup>+</sup> pericytes in α and β areas and coverage of CD13<sup>+</sup> pericyte. \*P < 0.05. \*\*P < 0.01. \*\*\*P < 0.001. n.s. no statistical significance.

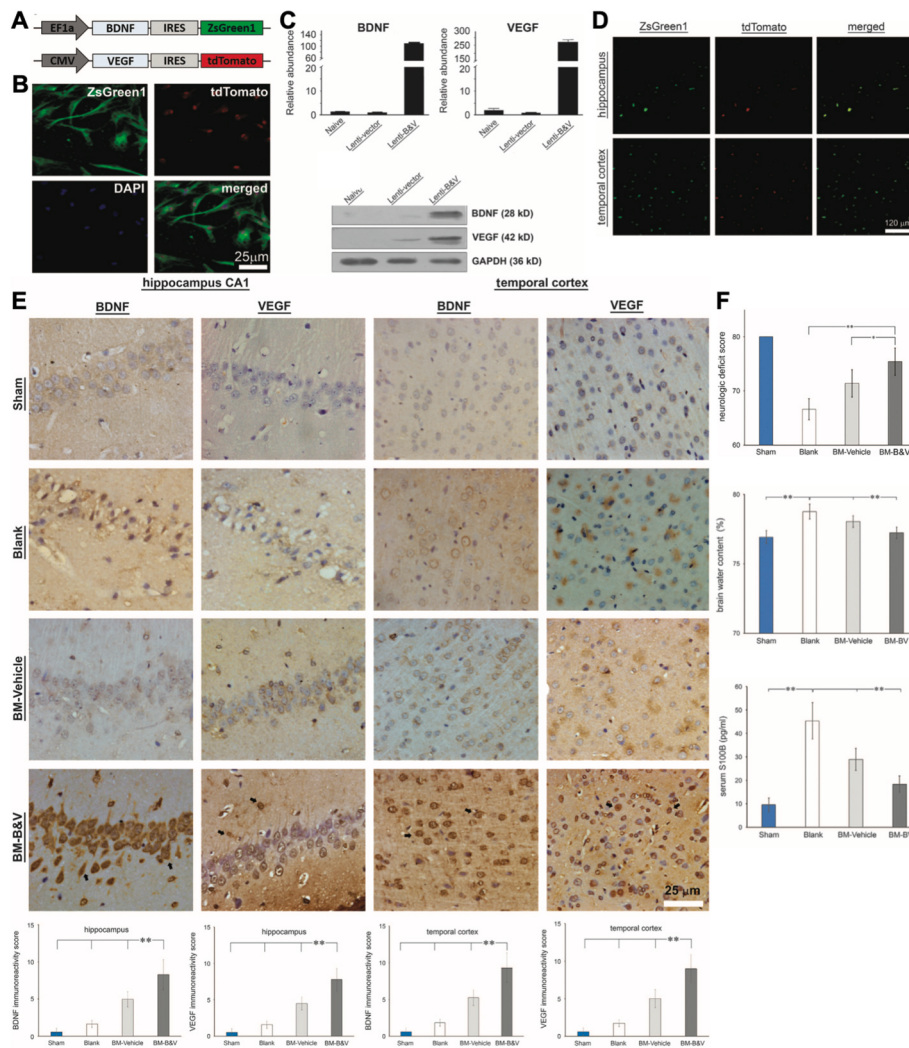
H19 or its derivative miR-675 promoted the upregulation of insulin-like growth factor receptor type 1 (IGF-1R), which in turn contributed to the neural differentiation of BMSCs [179]. During the early stages of mammalian CNS development, H19 controls IGF-1R signaling by acting as a reservoir for miR-675 [180]. The knockout of differentiation antagonizing nonprotein coding RNA (DANCR) in progenitor cell populations promoted rapid differentiation and gene induction in the absence of any other stimulus [181]. Jia et al. also found that the downregulation of DANCR promoted the neural regeneration of DPSCs [182]. Collectively, these findings suggest that the manipulation of lncRNAs may be an important strategy for neurological disease treatment using MSCs.

### 3.2. Neurotrophic factors

NTFs provide an optimal environment during development and maturation by participating in cellular and molecular mechanisms and regulating cellular biological activity and tissue organization [183]. In nervous system diseases, endogenous NTFs are produced in response to internal and external damage and restore the structural and functional plasticity of damaged neurons. However, NTF secretion is often insufficient to repair prolonged and severe nerve damage [184]. MSCs also secrete NTFs in response to the pathological process of neural injuries and neurodegenerative diseases. In addition, the efficacy of stem cell therapies for nervous diseases is strongly influenced by trophic factors,

such as BDNF [185], GDNF [186], NGF [187], NT-3 [188], HGF [189], and vascular endothelial growth factor (VEGF) [190].

Several studies have investigated one or more trophic factors in combination with MSCs in the treatment of neurodegenerative diseases. Zhou et al. demonstrated the neuroprotective efficacy of engineered BMSCs co-overexpressing BDNF and VEGF for the treatment of cardiac arrest-induced global cerebral ischemic injury (CA-GCII) in rats, thereby providing proof of concept for further translational validation before clinical testing (Fig. 7) [191]. The upregulation of GDNF expression in MSCs by either coculture or genetic overexpression has also been shown to enhance recovery in rat models of PD [192,193] and SCI [194,195]. Further, the simultaneous upregulation of BDNF, GDNF, and NGF in rat adipose-derived stem cell sheets using a hybrid baculovirus CRISPRa system improved Schwann cell migration, neuron regeneration, and neurite outgrowth *in vitro*, whereas the implantation of these ASC sheets into sciatic nerve injury sites enhanced reinnervation, axon regeneration, and remyelination [15]. In addition, Bai et al. identified a central role of the HGF/MET proto-oncogene, receptor tyrosine kinase (cMet) signaling pathway in mediating functional recovery and remyelination stimulated by MSCs [196]. These results suggest the critical role and potential application of combining neurotrophic and neuroprotective factors with MSCs in a treatment strategy in the future.



**Fig. 7.** BMSC co-expressing BDNF and VEGF showed Enhanced neuroprotective efficacy in a rat model of cardiac arrest-induced global cerebral ischemia. (A) Schematics show the expression cassette with either rat brain BDNF exon IV or VEGF-A open reading frame coding sequence followed by an internal ribosome entry site (IRES)-directed ZsGreen1 and tdTomato fluorescent protein coding sequences in the lentivirus constructs, respectively; (B) fluorescence micrographs (four panels in the bottom) show that transduced BMSCs co-express both ZsGreen1 (green) and tdTomato (red) fluorescent proteins; (C) RT-PCR (Top) and Western blots (Bottom) detect significantly increased expression of BDNF and VEGF in cells co-transduced with both BDNF- and VEGF-lentiviruses; (D) Fluorescence detect the distribution of lentivirus-transduced BMSCs transplanted in rat brain (hippocampus and temporal cortex areas); (E) Transplantation of lentivirus-transduced BMSCs upregulated the expression of BDNF and VEGF proteins in the rat brain; (F) Neurofunctional assessment, brain edema and serum S100B levels are quantified. (n = 5) Error bars = SD. \*\*P < 0.01.

### 3.3. Cell proliferation and survival factors

#### 3.3.1. Cell proliferation and proangiogenic factors

Although MSC therapy is a promising approach for the treatment of a wide range of neurological diseases, its low efficacy warrants further attention. This may be attributed to not only the significant loss of transplanted cells due to blood circulation, myocardial contraction, and leakage from the injection site but also to the low survival of resident cells owing to a harsh environment resulting from ischemia, hypoxia, oxidative stress, or inflammatory response [197]. A majority of MSCs often die within the first few hours of *in vivo* delivery.

Notch signaling, Wnt/ $\beta$ -catenin signaling, BMP signaling and some growth factors were tightly associated with proliferation of MSC. For example, TCF1-4 recruit  $\beta$ -catenin transferring into nucleus and interacting with the promoter regions of the target genes, which include *cycling D1*, *CD44* and *c-myc* that related to cell proliferation. In addition, Wnt1, Wnt2, Wnt-3a, and Wnt-5a played important roles in regulation of dopaminergic (DA) cell differentiation [198]. Most BMP families are associated with osteoblastic and chondrogenic differentiation of MSCs. However, BMP3 is an exception, which promotes MSCs proliferation through activating TGF- $\beta$ /activin signaling pathway [199]. Over-expression of NT-3 can simulate MSCs proliferation through upregulating Ngn1 expression and activating Notch signaling pathway [200]. However, due to the multi-lineage differentiation, the activation of these signaling pathways has a complex cascade reaction, which may lead simultaneous occurring of proliferation and nonneuronal differentiation

of MSCs. The transports of these genes need to be treated carefully.

To circumvent this issue, genetic engineering has been used to increase the survival of MSCs *in vivo*. A common strategy is to supply growth-promoting genes to increase the number of MSCs to compensate for the loss of MSCs. For instance, Luo et al. used MSCs in combination with bFGF and GelMA hydrogel to treat PNI [201] and SCI [202] in rats and reported better effect of repair and functional recovery in comparison with MSCs alone. Viral vectors can also include a switching element to control the expression of target genes under specific conditions. For example, Zhu et al. found that the severity of hypoxia varied regionally in SCI model rats. Hence, they constructed an AAV vector encoding bFGF under the control of a hypoxia-responsive element. As a result, increased numbers of red blood cells carried more oxygen to the damaged area and local hypoxic microenvironment, thereby ameliorating SCI [203].

Some acute mechanical injuries to the CNS or spinal cord inevitably also damage the blood–brain barrier (BBB) or blood–spinal cord barrier (BSCB), which may result in chronic ischemia in the injured tissue. Therefore, improving angiogenesis in the injured tissue could restore tissue homeostasis and facilitate endogenous repair activity. The prototypic angiogenic factor VEGF was employed as a therapeutic gene in several neural injury models, such as SCI [204], stroke [205], and PNI [206]. Other nonclassical angiogenic factors, including HGF [207], IGF-I [208], and placental growth factor (PIGF) [209], have also shown excellent angiogenic activity in transplanted MSCs.

### 3.3.2. Microenvironments of nerve damage

The lesion microenvironment can be complex, exhibiting harsh living conditions for local neurons, glia cells, vascular endothelial cells, and transplanted stem cells, which may limit cell survival and last treatment effects. The most severe factors include hypoxia, inflammation, and oxidative stress. Some pretreatment methods increase the resistant ability of MSCs to cope with the extreme conditions of the transplant site. Hence, analyzing the main molecular mechanism and performing direct gene delivery may yield superior results for MSC survival *in vivo*.

**3.3.2.1. Hypoxia.** Pathological conditions that can be treated using MSC transplantation are normally linked to the death of specialized cells in a particular tissue as a result of toxic agents or autoimmune processes. The microenvironment around this damaged tissue is severely ischemic ( $\leq 1\%$  oxygen), which may also trigger cell death. *In vitro* MSC cultures are usually maintained under normoxia (21% oxygen), whereas the natural niche of MSCs has constant moderate hypoxia (1%–7% oxygen) [210]. Hypoxia-inducible factor 1- $\alpha$  (HIF-1 $\alpha$ ) is the master regulator of cellular adaptation to hypoxia and strongly influences the survival, proliferation, migration, and differentiation of MSCs [211,212]. As therapeutic MSCs are likely to be transplanted into a hypoxic environment, culturing these cells under mild hypoxic conditions prior to transplant may improve their survival *in vivo*. This preconditioning effect is associated with HIF-1 $\alpha$  upregulation, which enables cells to survive under oxygen deprivation by enhancing oxygen-independent adenosine triphosphate (ATP) production and (or) inhibiting hypoxia-induced apoptosis [213,214]. Even in aged animals, MSCs showed a greater regenerative and osteogenic ability after being cultured in those conditions [215,216]. Importantly, HIF-1 $\alpha$  overexpression in DPSCs induces their immunosuppressive ability on different immune cell populations, including dendritic cells (DCs), natural killer (NK) cells, and monocytes [217].

**3.3.2.2. Inflammation.** Inflammatory activity is another key cascade following nerve injury. The inflammatory response is induced to help clear damaged tissue and promote synaptic reconstruction under physiological conditions through cytokines released by immune cells, but sustained inflammatory activity after nerve injury can exacerbate tissue damage and impede repair [218].

The immunomodulatory role of MSCs has been extensively studied in peripheral regions. MSCs exert immunomodulatory functions by secreting a variety of cytokines, such as IL-1Ra, IL-10, prostaglandin E2 (PGE2), tumor necrosis factor-inducible gene 6 protein (TSG-6), monocyte chemoattractant protein-1 (MCP-1/CCL2), and TGF- $\beta$ , or by inducing the expression of immunoregulators, such as indoleamine-pyrrrole 2,3-dioxygenase (IDO) (in humans) and human leukocyte antigen (HLA)-G5 [219]. In general, these factors inhibit the proliferation, differentiation, and activation of several immune cell subtypes, such as neutrophils, macrophages, B cells, T cells, NK cells, mast cells, and DCs, and promote the production of regulatory T cells [220]. Importantly, MSCs also promote the polarization of immune cells, such as macrophages, T cells, DCs, and NK cells, toward anti-inflammatory phenotypes [221].

Pretreatment with IL-1 $\alpha$  can significantly improve the anti-inflammatory activities of MSCs, similar to the effect of hypoxia priming. Researchers have used anti-inflammatory molecules to render even stronger anti-inflammatory activities to MSCs. Compared with BMSCs, BMSCs overexpressing PGE2 further increase IL-10 levels in the serum and supernatant [222]. In a model of ischemic stroke, human UC-MSCs overexpressing CCL2 showed stronger anti-inflammatory activity than naïve MSCs [223]. Activating MSCs with interferon gamma (IFN- $\gamma$ ) also upregulated neuron-glia antigen 2 and induced a potent pro-regenerative, promyelinating, and anti-inflammatory phenotype of MSCs [224].

**3.3.2.3. Oxidative stress.** Oxidative stress is a key link in the cascade of several neurological diseases, such as stroke, SCI, PD, and AD [225,226]. It is caused by the failure to control the cellular balance of anti- vs. pro-oxidant production, which may disrupt the normal function of proteins and lipids and lead to DNA damage. The most well-known pro-oxidant reactive oxygen species (ROS) and reactive nitrogen species (RNS) play crucial biological roles in normal physiological processes, including immune defense, cell signaling, cell senescence, apoptosis, and toxic compound degradation [227]. However, in CNS disorders, superfluous RNS and ROS generation may lead to detrimental processes, such as lipid peroxidation, swelling and necrosis of organelles, protein denaturation, and DNA modification and fragmentation, which impair the functions of neurons, neuroglia cells, and vascular endothelial cells, thereby leading to apoptosis, autophagy, and consequently tissue injury [228–230]. Therefore, therapeutic strategies that inhibit pro-oxidants or promote antioxidant production may contribute to clinically functional recovery.

RNS-mediated caveolin-1 and matrix metalloproteinase (MMP) signaling pathways participate in the progression of neuroinflammation and disruption of BBB. Collectively, targeting the overproduction of ROS and RNS might provide satisfactory outcomes in the treatment of ischemic stroke. Li et al. found that oxygen-glucose deprivation/reoxygenation (OGD) decreased the expression of GATA3 in rat BMSCs, whereas GATA3 overexpression in rat BMSCs alleviated OGD-induced cell injury and enhanced autophagy. GATA3 overexpression also improved the therapeutic effects of BMSC transplantation in an ischemic stroke model [231]. MSCs overexpressing mitochondrial rho GTPase 1 significantly improved the recovery of neurological functions in an experimentally induced stroke model. Mitochondrial impairment in differentiated cells can be compensated by receiving healthy mitochondria from mouse MSCs [232]. The H2A.X variant histone (H2AX) is crucial for maintaining nuclear DNA integrity and dictates the cell's decision to undergo DNA repair or apoptosis, and phosphorylation at tyrosine 142 (Y142) is an important determinant of the switch between DNA repair and apoptosis. Jiang et al. reported that BMSC-derived dopaminergic neurons expressing H2AX (Y142F) were resistant to DNA damage induced by oxidative toxicity, suggesting that H2AX (Y142F) upregulation may improve the success of stem cell transplantation therapy for PD [233]. Also, in a PD model, it was reported that inhibiting the expression of the protein kinase leucine rich repeat kinase 2 (LRRK2) by pharmacological or genetic methods increased the resistance to ROS in MSCs [234].

### 3.3.3. Inhibition of apoptosis

Local injection of MSCs into lesion sites shields cells from attack by pulmonary phagocytes, which seems to improve the likelihood of survival of most locally transplanted MSCs. However, several recently published studies have reported that >90% of locally transplanted MSCs undergo apoptosis within 3–5 days after transplantation, similar to the results obtained after the intravenous administration of MSCs [235]. Locally transplanted MSCs were found in almost all tissue-specific phagocytes within 1 week after injection [236]. *In vitro* evidence suggests that MSCs activate the complement system and cause damage after serum exposure, suggesting that local injection of MSCs are involved in complement recognition and attack *in vivo* [237]. Apoptosis of injected MSCs can also be induced by proinflammatory T cells via IFN- $\gamma$  and TNF- $\alpha$  [235]. Hence, another strategy to promote MSC survival is to prevent apoptosis.

Many studies have reported on developing MSCs expressing anti-apoptotic genes to promote cell survival and inhibit apoptosis induced by hypoxia or ROS, including superoxide dismutase 2 (SOD2) [238], Bcl-2 [239], and adrenomedullin (ADM) [240]. In addition, the co-overexpression of VEGF and Bcl-2 genes not only reduced apoptosis and decreased autophagy but also promoted more rapid proliferation *in vitro* under OGD [241]. The concomitant overexpression of SOD-1, SOD-3, and glutathione peroxidase-1 (GPx) or host treatment with

N-acetylcysteine significantly enhanced the survival of transplanted BMSCs and improved severe limb ischemia in diabetic mice [242]. The knockdown of the proapoptotic gene ubiquitin C-terminal hydrolase 1 (UCHL1) suppressed cytokine-induced MSC apoptosis via the upregulation of Bcl-2 [243]. Given the importance of apoptosis in MSC survival, preventing apoptotic signaling by different genes involved in MSC survival is an intriguing and important aspect for MSC transplantation, which may provide new insights for the effective application of MSCs.

#### 4. Gene transfer into MSCs for neurological disease treatment

At present, a large number of *in vivo* experiments of gene-edited MSC for the treatment of nerve injuries, including central nerve injuries,

peripheral nerves and neurodegenerative diseases, have been reported clinically. According to the existing reports, no matter which gene editing strategy can effectively improve the repair effect of MSC on nerve injury, which confirms the effectiveness of the gene modification strategy we summarized. In order to comprehensively compare and understand the effect of gene-modified MSC on nerve injury repair, all relevant *in vivo* experimental reports are summarized in Tables 2–5. It is worth mentioning that the current reports all show the effectiveness of gene-edited MSC, and some possible negative results are selectively ignored, which may pose challenges for future clinical trials.

**Table 2**

Recent studies about application of gene manipulated MSCs in spinal cord injury.

| Study                 | Types of MSC       | Animal       | Treatment Model        | Genes              | Delivery Method               | Injection | Result                                                                                                                                                                                                                                      |
|-----------------------|--------------------|--------------|------------------------|--------------------|-------------------------------|-----------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Qiu et al., 2015      | Rat/BMSC           | SD rats      | Spinal cord truncation | NT-3+TrkC          | Adenovirus                    | L.I.      | NT-3+TrkC group were able to move three joints of the hindlimbs, which showed significantly higher locomotion performance than MSC group and control group.                                                                                 |
| Yin et al., 2019      | BMSC               | SD rats      | Spinal cord injury     | Snail              | Lipofectamine 2000            | L.I.      | Snail group had better functional recovery. The synergistic effects of NRG1 on Snail can promote changes of the BMSC cytoskeleton and migration, and further promote functional recovery.                                                   |
| Jia et al., 2014      | Rat/BMSC           | SD rats      | Spinal cord injury     | Shh                | Lentivirus                    | L.I.      | Shh-BMSCs group showed higher motor improvement than BMSCs group and PBS group. Shh significantly improve BMSC survival <i>in vivo</i> .                                                                                                    |
| Lin et al., 2018      | Rat/BMSC           | SD rats      | Hemi-section Model     | Nice4              | Lentivirus                    | L.I.      | The hind limbs activity was significantly greater in Nice4 treatment group than in BMSC treatment control group and model group.                                                                                                            |
| Wei et al., 2017      | Human/BMSC         | Nude rats    | Spinal cord injury     | as-miR-383         | adeno- associated virus       | L.I.      | MiR-383-depletion group enhanced the recovery of locomotor activity in nude rats that underwent SCI, compared to null-transduced MSCs. MiR-383-depletion in MSCs on SCI recovery was at least as effective as additional injection of GDNF. |
| Jia et al., 2021      | Rat/MSC Exos       | SD rats      | Spinal cord injury     | miR-381/BRD4/WNT5A | Lipofectamine 2000/Lentivirus | I.V.      | MiR-381 derived from Exos from MSCs promoted the recovery of SCI by mediating the BRD4/WNT5A axis.                                                                                                                                          |
| Hodgetts et al., 2018 | Rat/MPCs           | F344 rats    | Spinal cord injury     | CNTF               | adeno- associated virus       | L.I.      | Treatment with CNTF-rMPCs, yielded functional improvements over rMPCs alone. Cyst size was significantly reduced in the CNTF-rMPCs treatment group.                                                                                         |
| Zou et al., 2014      | Rat/BMSC           | SD rats      | Spinal cord injury     | miR-214            | Lentivirus                    | L.I.      | Compared to the control group, recovery was significantly greater in the miR-124 group from day 14 after injury.                                                                                                                            |
| Li et al., 2020       | Rat/BMSC           | SD rats      | Spinal cord injury     | BDNF               | Adenovirus                    | L.I.      | BDNF-EPO group has significant better functional recovery than BDNF, EPO and control groups.                                                                                                                                                |
| Ji et al., 2019       | Obese rat/BMSC EVs | SD rats      | Spinal cord injury     | miR-21             | Lipofectamine 3000            | I.V.      | Obe-exo showed decreased miR-21 and little protective effect against SCI. Overexpression of miR-21 restored the protective effect of Obe-exo against SCI.                                                                                   |
| Jiang et al., 2021    | Rat/BMSC Exos      | SD rats      | Spinal cord injury     | miR-145-5p         | Lipofectamine 2000            | I.V.      | MSC-EXs containing miR-145-5p improved functional recovery and reduced histopathological injury and inflammation in SCI rats.                                                                                                               |
| Luo et al., 2020      | Rat/BMSC Exos      | SD rats      | Spinal cord injury     | GIT1               | Adenovirus                    | I.V.      | GIT1-BMSCs-Exos had better functional behavioral recovery than those treated with PBS or BMSCs-Exos only.                                                                                                                                   |
| Günther et al., 2015  | Rat/BMSC           | F344 rats    | Hemi-section Model     | BDNF               | Lentivirus                    | L.I.      | When used in combination with BMSCs expressing BDNF, the number of regenerating axons is further increased.                                                                                                                                 |
| Li et al., 2014       | Rat/BMSC           | Fetus rats   | Spina bifida aperta    | EGF                | Lentivirus                    | L.I.      | <i>In vivo</i> EGF introduction together with MSC transplantation affect the fate of transplanted MSCs in a fetal rat spina bifida model.                                                                                                   |
| Zhu et al., 2021      | Human/DPSC         | SD rats      | Spinal cord injury     | bFGF               | Adeno- associated virus       | L.I.      | AAV-5HRE-bFGF-DPSCs can improve SCI recovery via promoting vascular development and neuron differentiation.                                                                                                                                 |
| Yin et al., 2022      | Rat/BMSC Exos      | C57BL/6 mice | Spinal cord injury     | KCC2               | Lentivirus                    | –         | Exo <sub>KCC2</sub> can better promote the differentiation of neural stem cells into neurons, improve the functional recovery of SCI mice, and accelerate the nerve regeneration in the injured site.                                       |
| Lai et al., 2022      | Human/UCMSC Exos   | SD rats      | Spinal cord injury     | miR-146a-5p        | Lipofectamine 3000            | I.V.      | Exosomes modified by miR-146a-5p promote neural function recovery in rats with spinal cord injury by targeting neurotoxic astrocytes.                                                                                                       |
| Lai et al., 2022      | eGFP mice/BMSC     | FVB mice     | Spinal cord injury     | miR-31             | Polyplus                      | L.I.      | miR-31 can promote the migration of bone marrow mesenchymal stem cells and repair and improve the function of SCI tissue.                                                                                                                   |

Abbreviation: BMSC: bone marrow mesenchymal stem cell; BDNF: brain-derived neurotrophic factor; bFGF: basic fibroblast growth factor; CNTF: ciliary neurotrophic factor; DPSC: dental pulp stem cell; EVs: Extracellular vesicles; Exo: exosome; EGF: epidermal growth factor; F344: Adult female Fischer; GIT1: GIT ArfGAP 1; KCC: K<sup>+</sup>–Cl<sup>–</sup> co-transporter; L.I.: local injection; MPC: mesenchymal progenitor cell; NT-3: neurotrophin-3; I.V.: intravenous injection; SD rats: Sprague-Dawley rats; SCI: Spinal cord injury.

**Table 3**

Recent studies about application of gene manipulated MSCs in stroke.

| Study                  | Types of MSC     | Animal             | Treatment Model               | Gene           | Delivery Method        | Injection              | Result                                                                                                                                                                                                                                                                         |
|------------------------|------------------|--------------------|-------------------------------|----------------|------------------------|------------------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Bang et al., 2012      | Human/BMSC       | SD rats            | tMCAo                         | CXCR4          | Adenovirus             | I.V.                   | CXCR4-overexpressed MSCs elicited functional improvement compared with the control group and the naive MSC transfected group.                                                                                                                                                  |
| Sun et al., 2019       | Rat/BMSC         | SD rats            | cerebral ischemia reperfusion | SOD3           | Lentivirus             | I.V.                   | ECSOD-MSCs significantly reduced the infarct volume of ischemic stroke rats, and significantly improved neurological function in rats.                                                                                                                                         |
| Deng et al., 2019      | BMSC EVs         | C57BL/6 mice       | MCAO                          | miR-138-5p     | Lentivirus             | I.V.                   | BMSCs delivered miR-138-5p to the astrocytes via exosomes and BMSCs-derived exosomal miR-138-5p alleviated neuron injury in IS mice.                                                                                                                                           |
| Kim et al., 2020       | Human/BMSC       | SD rats            | MCAO                          | Ngn1           | Lentivirus             | I.V.                   | Ngn1 increases the homing ability of MSCs, enhancing their engraftment efficiency in the ischemic rat brain. Intra-arterial delivery of neurally induced MSCs/Ngn1 3 days after ischemic injury blocks neuronal cell death and inflammation, and improves functional recovery. |
| Gao et al., 2019       | Rat/BMSC         | Wistar rats        | MCAO                          | ERK1/2         | Lentivirus             | L.I.                   | After transplantation, more numbers of grafted cells and improved functional recovery were observed in ERK/ MSCs-treated rats compared with MSCs-treated rats.                                                                                                                 |
| Liu et al., 2010       | Human/UCMSC      | SD rats            | ICH                           | HGF            | Lentivirus             | L.I.                   | Animals receiving transplanted HGF-transduced MSCs exhibited significantly better motor function recovery than animals treated with MSCs alone (group II), which in turn performed better than the controls at 2 weeks after transplantation.                                  |
| Ryu et al., 2019       | Human/ADMSC      | SD rats            | MCAo                          | NGR1           | Adenovirus             | L.I.                   | Administration of NRG1-AdMSCs resulted in significant improvement of functional outcome following stroke compared to AdMSCs- or adenoviral NRG1-treated group, in addition to the reduction in the infarct size evaluated by hematoxylin and eosin staining                    |
| Wei et al., 2021       | Rat/BMSC Exos    | SD rats            | MCAo                          | Zeb2/Axin2     | Lentivirus             | L.I.                   | After stimulation with Zeb2/Axin2-enriched BMSC exosomes, the spatial memory and nerve function of MCAo rats showed marked recovery and the increased number of neurons.                                                                                                       |
| Zhang et al., 2019     | Rat/BMSC         | SD rats            | MCAo                          | TSP4           | Lentivirus             | I.V.                   | In vivo, the TSP4-BMSC infusion improved the neurological function score of MCAo rats and expanded the expression of the von Willebrand factor (vWF), Ang-1, MMP2 and MMP9 proteins in cerebral ischemic penumbra.                                                             |
| Cui et al., 2017       | Rat/BMSC         | RccHan Wistar rats | MCAo                          | ITGA4          | Lentivirus             | Intracarotid Injection | ITGA4 overexpression on MSCs enhances transendothelial migration <i>in vitro</i> , but not <i>in vivo</i> , although it improves safety after intracarotid transplantation into stroke rats.                                                                                   |
| Nakajima et al., 2017  | Human/BMSC       | SD rats            | MCAo                          | IL-10          | Adeno-associated virus | I.V.                   | Transplantation of MSC/IL-10, but not MSCs alone, significantly reduced infarct volumes and improved motor function compared with vehicle groups at 72 h and 7 days after MCAo.                                                                                                |
| Cheng et al., 2014     | Rat/BMSC         | SD rats            | MCAo                          | Ngn2           | Lentivirus             | L.I.                   | Transfection with Ngn2 convert the mesodermal cell fate of MSCs into a neural stem one, and significantly improved functional recovery and reduced ischemic damage in a rat model of MCAo.                                                                                     |
| Lv et al., 2017        | Rat/BMSC         | SD rats            | MCAo                          | HIF-1 $\alpha$ | Lentivirus             | L.I.                   | Hif-1 $\alpha$ might be contributed in the survival of BMSCs by regulating the activation of AMPK and mTOR, as well as by promoting autophagy                                                                                                                                  |
| Yu et al., 2012        | Rat/BMSC         | SD rats            | MCAo                          | CXCR4          | Lentivirus             | I.V.                   | Overexpression of CXCR4 in MSCs engrafted into the ischemic brain was effective for post-infarction recovery of neural function.                                                                                                                                               |
| Yang et al., 2020      | Human/UCMSC Exos | SD rats            | MCAo                          | CCR2           | Lentivirus             | I.V.                   | CCR2 over-expression enhanced the therapeutic effects of exosomes on the experimental PSCI by promoting M2 microglia/macrophage polarization, enhancing oligodendrogenesis and remyelination.                                                                                  |
| Lee et al., 2020       | Human/UCMSC      | SD rats            | MCAo                          | CCL2           | Lipofectamine 2000     | I.V.                   | CCL2-overexpressing UCMSCs effectively restored functional deficits in an animal stroke model by promoting continuous increases in CCL2 levels in the brain, enhancing angiogenesis and neurogenesis, and decreasing neuro-inflammation                                        |
| Velthoven et al., 2012 | Rat/BMSC         | SD rats            | MCAo                          | BDNF           | Adenovirus             | Intranasally Injection | Intranasal application of BDNF-secreting MSC is equally effective in reducing gray and white matter loss, and motor deficits, and inducing cell proliferation after neonatal MCAO as use of MS                                                                                 |
| Zhou et al., 2017      | Rat/BMSC         | SD rats            | CA-GCII                       | BDNF/VEGF      | Lentivirus             | I.V.                   | BMSCs co-overexpressed both BDNF and VEGF in the hippocampus and temporal cortex along with significantly ameliorated brain pathology and improved neurofunctional performance in CA-GCII rats after transplantation.                                                          |
|                        |                  |                    |                               |                | Adenovirus             |                        |                                                                                                                                                                                                                                                                                |

(continued on next page)

Table 3 (continued)

| Study                        | Types of MSC    | Animal        | Treatment Model  | Gene               | Delivery Method    | Injection              | Result                                                                                                                                |
|------------------------------|-----------------|---------------|------------------|--------------------|--------------------|------------------------|---------------------------------------------------------------------------------------------------------------------------------------|
| Velthoven et al., 2014 [255] | Mouse/BMSC      | C57Bl/6J mice | Hypoxia-ischemia | BDNF/EGFL7/PSP/SHH |                    | Intranasally Injection | BDNF or EGFL7 modification has significant positive effect to hypoxia-ischemia.                                                       |
| Zhou et al., 2022            | Rat/BMSC Exos   | SD rats       | MCAo             | miR-145            | Lipofectamine 2000 | I.V.                   | Exosomal miR-145 from MSCs was able to attenuate cerebral I/R injury through downregulation of FOXO1.                                 |
| Huaitao et al., 2022         | Mouse/BMSC Exos | C57Bl/6J mice | MCAo             | ZFAS1              | Lipofectamine 2000 | I.V.                   | BMSC-released exosome ZFAS1 exerts antioxidant and anti-inflammatory potential in ischemic stroke by inhibiting miR-15a-5p.           |
| Liu et al., 2022             | Rat/BMSC Exos   | SD rats       | SCIR             | miR-455-5p         | Lentivirus         | I.V.                   | Exosomes containing miR-455-5p have a neuroprotective effect on SCIR injury by promoting autophagy and inhibiting neuronal apoptosis. |

Abbreviation: ADMSC: adipose tissue-derived mesenchymal stem cell; BMSC: bone marrow mesenchymal stem cell; BDNF: brain-derived neurotrophic factor; CA-GCII: Cardiac arrest-induced global cerebral ischemia injury; CCL2: C-C motif chemokine ligand 2; CCR2: chemokine receptor type 2; CCAs: bilateral common carotids; CXCR4: C-X-C motif chemokine receptor 4; ERK: Extracellular signal-regulated kinase; Ngn: neurogenin; tMCAo: transient middle cerebral artery occlusion; SCIR: spinal cord ischemia reperfusion; TSP4: tumor suppressor region 4; ITGA4: integrin subunit alpha 4; ICH: intracerebral hemorrhage; HGF: hepatocyte growth factor; HIF-1 $\alpha$ : Hypoxia-inducible factor 1 $\alpha$ ; L.I.: local injection; I.V.: intravenous injection; SD rats: Sprague-Dawley rats; VEGF: vascular endothelial growth factor; EGFL7: epidermal growth factor-like 7, PSP: persephin, SHH: sonic hedgehog; UCMSC: umbilical cord mesenchymal stem cells.

Table 4

Recent studies about application of gene manipulated MSCs in peripheral nerve injuries.

| Study               | Type of MSC | Animal           | Treatment Model                      | Gene      | Delivery Method        | Conduit                                                                           | Result                                                                                                                                                                                                                                                        |
|---------------------|-------------|------------------|--------------------------------------|-----------|------------------------|-----------------------------------------------------------------------------------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Cortés et al., 2014 | Mouse/AMSC  | Wistar male rats | 10 mm defect of sciatic nerve        | VIP       | Lentivirus             | DL-lactic- $\epsilon$ -caprolactone conduit                                       | The rats that received a single injection of VIP-expressing ASCs showed a significant functional recovery in the ankle stance angle and a higher number of myelinated fibers in the middle and distal segments of the operated nerve versus the other groups. |
| Man et al., 2016    | Human/BMSC  | C57BL/6 mice     | Sciatic nerve transection            | VEGF-A    | Lentivirus             | Fabrication of Poly-L-Lactide Acid Conduits                                       | Genetically engineered MSCs in treating peripheral nerve injury as demonstrated by increased VEGF presentation and prolonged transient survival                                                                                                               |
| Tian et al., 2020   | Human/MSC   | SD rats          | CCI of Sciatic nervous               | Sirtuin 1 | Adenovirus             | None                                                                              | Transplantation of Sirtuin 1-overexpressed hMSCs achieved substantially improved effects on amelioration of neuropathic pain when compared to MSCs injection alone                                                                                            |
| Ke et al., 2015     | Rat/BMSC    | SD rats          | 3-mm crushed region of sciatic nerve | Netrin 1  | Adenovirus             | None                                                                              | Transplantation of BMSCs that produce netrin-1 improved the function of the sciatic nerve after injury                                                                                                                                                        |
| Li et al., 2019     | Mouse/BMSC  | C57BL/6 mice     | 10 mm defect of sciatic nerve        | KLF7      | Adeno-associated virus | Acellular nerve allografts                                                        | KLF7-BMSCs promoted peripheral nerve axon regeneration and myelination, and ultimately, motor functional recovery.                                                                                                                                            |
| Liu et al., 2014    | Rat/BMSC    | Wistar rats      | 5 mm defect of sciatic nerve         | CDNF      | Lentivirus             | Type I collagen tube                                                              | CDNF gene therapy delivered by MSCs is capable of promoting nerve regeneration and functional recovery.                                                                                                                                                       |
| Zhang et al., 2020  | Rat/BMSC    | SD rats          | 10 mm defect of sciatic nerve        | BDNF/GDNF | Lentivirus             | Highly oriented poly (L-lactic acid)/soy protein isolate nanofiber nerve conduits | Conduits containing BMSCs overexpressing BDNF and GDNF double-factors group had better nerve repairing efficiency than blank BMSCs and single BDNF or GDNF factor groups, and superior to autografts group in some aspects.                                   |

Abbreviation: AMSC: amniotic mesenchymal stem cells; BMSC: bone marrow mesenchymal stem cell; BDNF: brain-derived neurotrophic factor; CDFN: cerebral dopamine neurotrophic factor; GDNF: glial cell line-derived neurotrophic factor; CCI: Chronic Constriction Injury; KLF7: Krüppel-like Factor 7; L.I.: local injection; I.V.: intravenous injection; SD rats: Sprague-Dawley rats; VEGF: vascular endothelial growth factor; VIP: vasoactive intestinal peptide.

#### 4.1. Central nervous system injury

##### 4.1.1. Spinal cord injury

Severe SCI may result in the complete or partial loss (or both) of motor and sensory functions below the level of the lesion due cell death, axonal damage, and demyelination owing to the primary mechanical insult and secondary processes, such as inflammation, oxidative stress, and excitotoxicity [244]. Considering the ability of MSCs to transdifferentiate into neurons and myelinating cells *ex vivo* and *in vivo*, they have become the stem cell of choice for SCI rehabilitation [245].

MSCs can secrete several NTFs known to promote the survival and growth of developing neurons and maintain the functions of mature neurons. To enhance the immunomodulatory functions of MSCs, Qiu et al. used an adenovirus vector to overexpress neurotrophin-3 (NT-3)

and its receptor TrkC in rat BMSCs and reported that implantation at the lesion site significantly enhanced the survival of both the therapeutic MSCs and injured host neurons at the injury site. In addition to neurons, MSC-derived neural-like cells implanted on a GS scaffold reportedly transdifferentiated into myelin-forming cells in completely transected rat spinal cord [246].

Other potential treatment strategies using implanted MSCs include promoting neural differentiation (EGF, Shh and miR-124) [159,176,247,248]; enhancing proliferation (bFGF, PTEN, IGF-1, and Nice4, Fig. 6) [203,249–251]; increasing migration ability (ChABC, Snail, miR-31 and SDF-1 $\alpha$ ) [250,252–254]; inhibiting apoptosis (GIT1 and miR-21) [255,256]; reducing oxidative stress (miR-200a) [256]; proangiogenesis (miR-17-5p) [257]; and neurotrophic effect (CNTF, BDNF, miR-146a-5p and miR-383) [258–262]. The details of these

**Table 5**

Recent studies about application of gene manipulated MSCs in neurodegenerative disorders.

| Study                      | Types of MSC          | Animal                                     | Treatment Model           | Gene           | Delivery Method        | Injection                 | Result                                                                                                                                                                                                     |
|----------------------------|-----------------------|--------------------------------------------|---------------------------|----------------|------------------------|---------------------------|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| <b>Parkinson's disease</b> |                       |                                            |                           |                |                        |                           |                                                                                                                                                                                                            |
| Yin et al., 2014           | Rat/BMSC              | SD rats                                    | 6-OHDA injection into MFB | Persephin      | Lentivirus             | L.I.                      | Persephin-MSCs showed higher survival rate in rat brain compared with MSCs, and showed the most significant improvement compared with those in other groups.                                               |
| Ko et al., 2015            | Human/UCMSC           | SD rats                                    | 6-OHDA injection into MFB | Nurr1/FGF8/Shh | Electroporation        | L.I.                      | Nurr1, FGF8, Shh, and NCM can synergistically enhance the differentiation of HUMSCs into dopaminergic cells and may pave the way for HUMSC-based treatments for PD.                                        |
| Liu et al., 2017           | Human/PBMS            | SD rats                                    | 6-OHDA injection into MFB | NTRK1          | Adenovirus             | Intraperitoneal injection | DA neuron repair was increased in lesion site, rotational performance was also improved in MSC transplanted PD rat, with most potent effect in NTRK1 overexpressed PBMS transplanted PD rat.               |
| Ren et al., 2014           | Monkey/BMSC           | cynomolgus Monkey                          | MPTP                      | GDNF           | Lentivirus             | L.I.                      | Autologous GDNF-MSCs themselves did not cause significant motor behavior changes before transplantation, but prevented MPTP-induced injury, suggesting that early intervention in PD is a viable strategy. |
| Shin et al., 2022          | Rat/BMSC              | SD rats                                    | –                         | $\alpha$ -syn  | Adeno-associated virus | I.V.                      | In the Parkinson model, MSC priming with $\alpha$ syn overexpression exerts neuroprotective effects by enhancing stem cell and possibly autophagy-mediated modulation of $\alpha$ syn.                     |
| Li et al., 2021            | C57BL/6 mice/AMSC Exo | C57BL/6 mice                               | MPTP                      | miR-188-3p     | Lipofectamine 3000     | I.V.                      | MIR-188-3p-enriched exosome treatment suppressed autophagy and pyroptosis, whereas increased proliferation via targeting CDK5 and NLRP3 in mice and MN9D cells.                                            |
| Hoban et al., 2015         | Rat/BMSC              | SD rats                                    | LPS into substantia nigra | GDNF           | Lentivirus             | L.I.                      | GDNF provided extra protective effect of MSC against LPS-induced inflammation of PD.                                                                                                                       |
| <b>Alzheimer's disease</b> |                       |                                            |                           |                |                        |                           |                                                                                                                                                                                                            |
| Garcia et al., 2014        | Mouse/BMSC            | 2xTg-AD male congenic mice                 |                           | VEGF           | Electroporation        | L.I.                      | MSC-VEGF treatment favored the neovascularization and diminished senile plaques in hippocampal specific layers, and was able to provide behavioral benefits and reduce cognitive deficits.                 |
| Liu et al., 2015           | Mouse/BMSC            | APP/PS1 mice                               |                           | as-miR-937     | Adeno-associated virus | L.I.                      | Transplantation of as-miR-937-expressing MSCs significantly reduced the deposition of A $\beta$ , increased the levels of BDNF, and significantly improved the appearance in SR and PM-DAT in AD mice.     |
| Zhang et al., 2018         | Human/UCMSC           | APP mice                                   |                           | FOXQ-1         | Lentivirus             | I.V.                      | FOXQ1 overexpression promotes anti-senescence and migration of hUC-MSCs <i>in vitro</i> and <i>in vivo</i>                                                                                                 |
| Hu et al., 2019            | Human/UCMSC           | APP/PS1 mice                               |                           | BDNF           | Lentivirus             | L.I.                      | Transplantation of BDNF modified UCMSCs-derived cholinergic-like neurons significantly improved spatial learning and memory abilities in the AD rats                                                       |
| Wu et al., 2019            | Mouse/BMSC            | APP/PS1 mice                               |                           | Lin28B         | Lipofectamine 2000     | L.I.                      | Lin28B promoted MSCs proliferation and migration, and protected MSCs against A $\beta$ 1–42-induced cell death by upregulating insulin-like growth factor-2 (IGF-2).                                       |
| Yan et al., 2021           | Rat/BMSC              | oligomeric A $\beta$ 1–42 injected SD rats |                           | NT-3           | Lentivirus             | L.I.                      | NT-3 promoted the differentiation of BMSCs into neurons <i>in vitro</i> and <i>in vivo</i> and improved cognitive function in rats with experimental AD.                                                   |

Abbreviation: AD: Alzheimer's disease; BMSC: bone marrow mesenchymal stem cell; MFB: medial forebrain bundle; BDNF: brain-derived neurotrophic factor; FGF8: fibroblast growth factor-8; Shh: sonic hedgehog; NTRK1: neurotrophic tyrosine receptor kinase 1; PB: peripheral blood; FOXQ-1: forkhead box Q1; I.V.: intravenous injection; L.I.: local injection; NT-3: neurotrophin-3; PD: Parkinson's disease; UCMSC: umbilical cord mesenchymal stem cells; VEGF: vascular endothelial growth factor.

transgenic MSCs for SCI treatment are summarized in Table 2.

#### 4.1.2. Stroke

Currently, the only therapies available for stroke are intervention to prevent inappropriate coagulation, surgical procedures to repair vascular abnormalities, and thrombolytic therapy to reestablish local perfusion. As there are currently no treatments that can reliably reverse neural damage or restore function, recent efforts have focused on the use of stem cell-based therapies to replace lost neurons and promote the survival and differentiation of both surviving and transplanted cells [141]. For instance, BMSCs have been shown to differentiate into neuronal cells, cross BBB, migrate to areas of damage, and secrete growth factors and cytokines [142]. Moreover, transplantation following stroke can result in improved functional recovery [143]. To further improve the efficacy of stem cell therapies for stroke, thereby

increasing their clinical potential, recent efforts have focused on engineering stem cells with neuroprotective factors as well as factors that promote neurite outgrowth.

Transplantation of MSCs several hours to days after the induction of stroke can reduce infarct size and improve functional outcomes in rodent stroke models [7–9]. However, in most studies, the proportion of neurons differentiated from MSCs was limited. Neural stem cells (NSCs) represent an excellent cell therapy choice and have been widely employed in preclinical stroke studies with encouraging results [10,11]. A recent meta-analysis of preclinical studies employing intravenous cell delivery indicated that NSCs yielded the greatest behavioral recovery compared with MSCs and other cell types [12]. Hence, an important step in the MSC engineering process is inducing the differentiation of MSCs into NSCs, NPCs, or neurons. Cheng et al. reprogrammed MSCs into NSCs by overexpressing neurogenin 2 (Ngn2), a proneural gene that directs

neuronal differentiation of progenitor cells. Transfection with Ngn2 switched the mesodermal cell fate of MSCs to a neural stem cell fate, promoted MSC survival by reducing apoptosis, significantly reduced ischemic damage, and improved functional recovery in a rat model of middle cerebral artery occlusion [263].

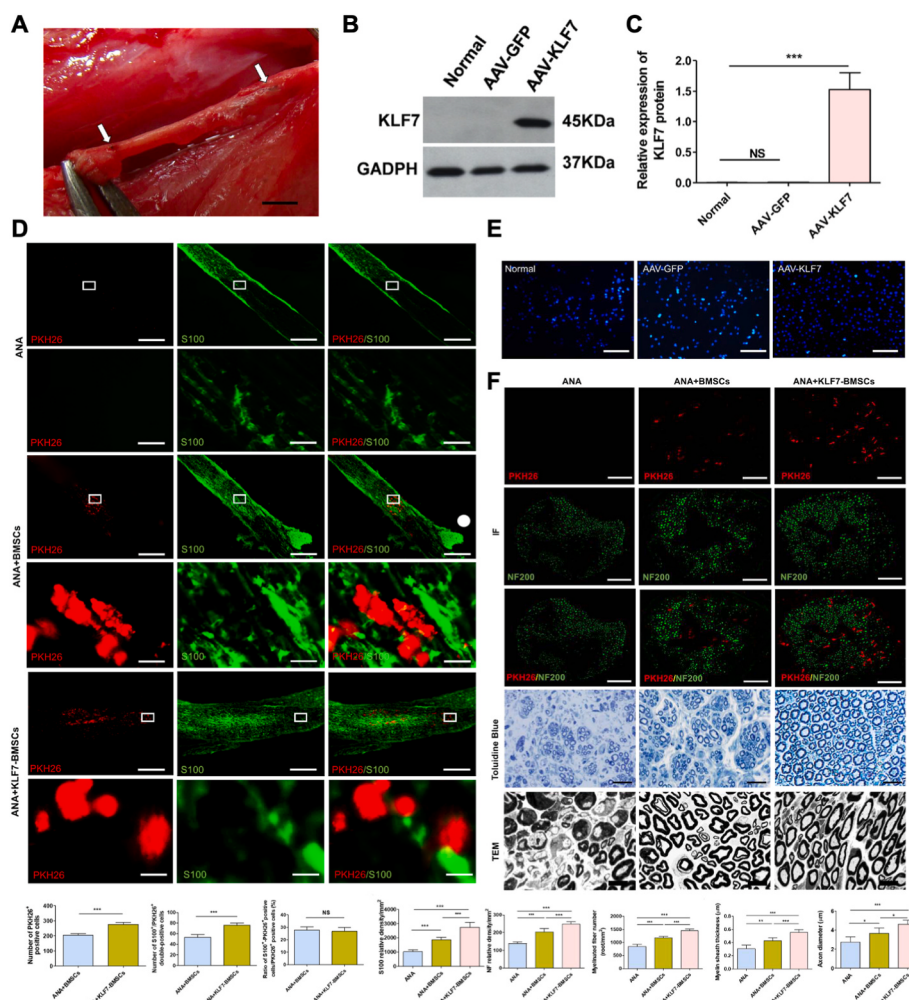
To enhance MSC survival in the microenvironment of ischemic stroke, BMSCs were transfected with a lentiviral vector to overexpress the transcription factor Hif-1, which subsequently demonstrated improved recovery in an OGD rat stroke model. Hif-1 $\alpha$  may contribute to BMSC survival by regulating the activation of AMPK and mTOR as well as by promoting autophagy [264]. In another study, BMSCs were transfected with ERK1/2, a mitogen-activated protein kinase known to promote cell survival and exhibit antiapoptotic effects against a variety of stressors, including oxidative and ischemic shock. As speculated, the transplantation of MSCs overexpressing ERK1/2 resulted in superior functional recovery in a rat stroke model compared with naive MSCs, likely by enhancing MSC survival and possibly by modulating proliferation, apoptosis, and neuroinflammation. Notably, more neurons were differentiated from ERK1/2-overexpressing MSCs than from unedited MSCs [265]. Additionally, the overexpression of chemokine (C-X-C motif) receptor 4 (CXCR4) by transient transfection [266] or stable transfection [267] in MSCs promoted MSC mobilization and enhanced neuroprotection in a rat cerebral ischemia model [266,267]. Other studies have shown that the overexpression of SOD3 [268], HGF [269], IL-10 [270], CCL2 [223], NGN1 [271], NGR1 [272], TSP4 [273], ITGA4 [274], BDNF [275] and EGFL7 [276] as well as miR-138-5p [277] can enhance the immunomodulatory and neuroprotective effects of MSCs

and significantly improve the recovery of motor functions when transplanted into a rat stroke model.

#### 4.2. Peripheral nerve injury

PNI is a common clinical nervous system disease characterized by motor, sensory, and autonomic nerve dysfunction of the trunk or limbs. After PNI, nerves begin to progressively degrade, including the infiltration of monocytes, neuroedema, and neurodegenerative demyelination [278]. Different from the CNS, peripheral nerves can regenerate spontaneously after injury due to the activation of the intrinsic growth capacity of neurons. For PNIs with a nerve gap of >1 cm, autologous or allograft nerve transplantation results in better therapeutic effects [279]. However, donor shortage and immune rejection are the main problems in the clinical application of this technique [280,281]. In the past decades, tissue engineer with the application of stem cells has been commonly used and examined. Except for NSCs, the combination of conduit materials seeded with induced pluripotent stem cells, muscle-derived stem cells, or MSCs has been used for PNI repair [282]. However, currently approved therapies remain unsatisfactory.

Neurons are the fundamental structures and functional units of the neural system. Axons of neurons are supported by glia cells and Schwann cells that produce myelin. Myelin sheaths and associated proteins forming the membrane of Schwann cells repeatedly wrap around axons, acting as insulators and increasing both the speed and efficiency of axonal action potential propagation [283]. The transcription factor Kruppel Like Factor 7 (KLF7) was reported to enhance the proliferation



**Fig. 8. Overexpression of KLF7 in BMSCs graft transplantation repairs gaps of sciatic nerve.** (A) Acellular nerve allografts (ANAs) were stitched to bridge both stumps of the sciatic nerve defect. (B) Western blot shows KLF7 were overexpressed in AAV-KLF7 group. (C) The quantitative analysis of gray value to the Western blot were performed. (D) Representative immunofluorescence images of S100 (green) and a fluorescent tracking dye PKH26-labeled BMSCs (red) in ANA in longitudinal sections from all three ANA transplant groups 28 days post-surgery. The high magnification inserts of boxed area of interest show red labeled BMSC do not colocalize much with green S100 staining.  $n = 6$  mice/group. The histograms showed the number of PKH26 positive cells, the number of double-labeled PKH26 and S100<sup>+</sup> cells, PKH26 and S100 colocalization ratio of the total PKH26 positive cells, and S100 protein expression. (E) Apoptotic cells were detected by a TUNEL assay. (F) Representative immunofluorescent images for a fluorescent tracking dye PKH26-labeled BMSCs (red) and neurofilament (NF200, green) in transverse sections, toluidine blue staining, and transmission electron microscope (TEM) analysis of myelinated fibers and axon in regenerating ANAs of the three treatment groups ( $n = 6$ ). Quantification of NF200 protein expression, myelinated fiber number, myelin sheath thickness and axon diameter in the center of ANAs are shown. Error bars = SD. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ .

of transplanted and native Schwann cells and promote axonal regeneration [284]. Transplantation of BMSCs overexpressing KLF7 within acellular nerve allografts was previously examined for the repair of nerve gap defects and found to increase BMSC survival and promote axonal regeneration by enhancing the expression of NGF, BDNF, GFNF, and CTNF (Fig. 8) [285]. To promote the proliferation of native Schwann cells, Ke et al. transplanted BMSCs overexpressing netrin-1, a secreted protein that directs the migration of neuronal cells and axon growth cones during neural development, into a sciatic nerve crush injury site. Some transplanted cells differentiated into myelin-producing Schwann cells, whereas others were incorporated into the perineural vasculature, resulting in improved neural function recovery [286]. Similarly, Chen et al. reported that human amniotic MSCs overexpressing miRNA-214 promoted the myelination of Schwann-like cells by targeting c-Jun and improved peripheral nerve regeneration [287].

Among the most common clinical problems arising from injury to peripheral sensory neurons is chronic pain. Intrathecal injection of human MSCs overexpressing Sirtuin 1 (SIRT1) into the cisterna magna was reported to mitigate neuropathic pain in a chronic constriction injury rat model. Compared with human MSCs-control implantation, MSCs-SIRT1 (MSCs overexpressing SIRT1) implantation exhibited superior effects on reducing proinflammatory cytokine levels in the serum and spinal dorsal horn [288]. Further, MSCs overexpressing BDNF, CDNF, GDNF, or VEGF have also demonstrated efficacy for PNI treatment in rat models [168,190,289]. Combinations of these factors may be even more effective. A mixture of BMSCs overexpressing BDNF or GDNF as seed cells greatly improved sciatic nerve repair compared with separate populations overexpressing BDNF or GDNF alone and the effect was similar to autograft treatment [289]. In addition to NTFs, overexpression of a vasoactive intestinal peptide (VIP), which elicits a broad spectrum of biologic functions, including vasodilatation, immunomodulation, neuroprotection and hormone regulation, also showed significant functional promotion to the AD-MSC in the repairment of 10 mm defect of rat sciatic nerve [290].

#### 4.3. Neurodegenerative disease

##### 4.3.1. Parkinson's disease

The loss of dopaminergic neurons in the substantia nigra pars compacta (SNpc) is one of the major pathologic features of PD, which causes a significant reduction in the concentration of dopamine (DA) in the striatum as well as in the Lewy corpuscular body and the major components of  $\alpha$ -nucleoprotein surviving neurons [291]. Neuronal loss in PD leads to progressive motor impairments, such as rigidity, bradykinesia, static tremor, and postural gait disorders as well cognitive deficits [292].

As the first preclinical and clinical studies reporting that MSC transplantation can replace dopaminergic neurons or protect surviving host dopaminergic neurons in PD, many studies have been conducted aiming to improve the efficacy of neuroprotection and repair using genetically modified MSCs. To promote more efficient MSC differentiation into dopaminergic neurons, peripheral blood MSCs (PB-MSCs) were transfected with an adenovirus vector encoding neurotrophic receptor tyrosine kinase 1 (NTRK1) and injected directly into the SNpc of hemiparkinsonian rats. These modified MSCs increased dopaminergic neuron survival at the lesion site and reduced amphetamine-induced rotation [293]. MSCs overexpressing Lmx1 $\alpha$ /NTN, which promotes mesencephalic dopaminergic differentiation during embryogenesis, also ameliorated disease progression in hemiparkinsonian rhesus monkeys [294]. Similarly, transplantation of human UMSCs overexpressing Nurr1, a transcription factor essential not only for the generation of mesencephalic dopaminergic neurons but also a regulator dopamine synthesis and storage, significantly improved lesion-induced amphetamine-evoked rotation in hemiparkinsonian rats [166]. This effect is noteworthy because Nurr1 gene expression is significantly reduced in PD patients, suggesting that implantation of Nurr1-overexpressing MSC compensates directly for one pathogenic characteristic of human PD.

MiR-7-5p, derived from primed MSCs, induces autophagy in  $\alpha$ -syn overexpressing neuronal cells, dependent on another key endpoint of BECN1 and AMBRA1. Specifically, upregulation of Akt phosphorylation was associated with reversal of the action of  $\alpha$ -syn-primed MSCs by upregulation of miR-7-5p, thereby increasing AMBRA1 levels in  $\alpha$ -syn-overexpressing neuronal cells [295].

PD is also characterized by enhanced ROS generation and oxidative stress within the SNpc as well as accumulation of DNA damage compared to age-matched controls [296,297]. As oxidative DNA damage can disrupt neuronal energy homeostasis and induce apoptosis, transplantation of MSCs overexpressing antioxidant and antiapoptotic factors may also slow PD progression. Indeed, BMSCs overexpressing phosphorylated H2AX, which serves to stabilize genomic DNA under oxidative stress, enhanced the apoptotic resistance of BMSC-derived dopaminergic neurons to neurotoxic insult [233]. In PD that targets the degeneration of dopaminergic neurons, researchers have considered the introduction of NTFs to nourish the remaining neurons to slow or prevent further degeneration of dopaminergic neurons during PD. Compared with recombinant cytokines, using mesenchymal stem cells as vectors and transfecting continuous expression of cytokines may be a better strategy. Of the NTFs tested, GDNF showed the most potent beneficial effects on dopaminergic neurons in rodent [298] and primate models [299] of PD. In addition, persephin can also promotes prognosis of PD rats [300].

##### 4.3.2. Alzheimer's disease

The major pathological features of AD include intracellular neurofibrillary tangles composed of hyperphosphorylated Tau protein, extracellular senile plaques composed of amyloid peptide aggregates (A $\beta$ ), synaptic dysfunction, and progressive focal loss of cortical neurons, particularly in temporal and frontal lobes. These pathological changes eventually lead to progressive cognitive dysfunction and early death [301].

Recent studies have reported that MSC transplantation can stimulate neuronal regeneration and repair in an adult murine model of AD by enhancing the proliferation of endogenous NSCs in the subgranular region of the dentate gyrus, promoting neurogenesis, and by increasing the proliferation and neural differentiation of newly formed cells in the subventricular region [302,303]. However, the therapeutic effects of MSCs on neurogenesis are still insufficient for substantial restoration of function.

The transcription factor Brn-4 plays an essential role in neuronal development and regeneration [304,305]. While Brn-4 mRNA is highly expressed in MSCs, protein expression is relatively low due to high expression levels of miR-937, which blocks Brn-4 mRNA translation through binding to the 3'-UTR. Consistent with a major potential therapeutic effect of Brn-4 upregulation, transplantation of MSCs overexpressing miR-937 significantly downregulated A $\beta$  deposition, increased the expression of BDNF, and improved or preserved the cognitive functions of AD model mice [306]. Interestingly, directly overexpressed BDNF [307], Lin28B [308] or VEGF [309] in MSC can also promote repairment of cognitive functions in AD mice. BMSCs overexpressing NT-3 significantly activate the local Wnt/ $\beta$ -catenin signaling pathway in the hippocampus, promote the neural differentiation of BMSCs, and effectively improve the cognitive impairment of AD rats [158].

Several studies have shown that MSCs become senescent over multiple passages *in vitro*, resulting in slower proliferation [310] and reduced potential therapeutic efficacy. Therefore, it is essential to identify genes able to slow senescence in long-term culture and enhance homing for the treatment of nerve injury *in vivo*. Using whole genome CRISPR Cas9 screening technology, Wang et al. identified more than 100 senescence-promoting genes in morbid mesenchymal precursor cells, of which the histone acetyltransferase KAT7 was the most strongly associated with senescence. Knockout of KAT7 significantly delayed aging and extend the life span of aged mice [311]. In addition, microarray

analysis identified 13 DEGs between normal aging MSCs growing in fetal bovine serum versus those cultured in pooled human platelet lysate (pHPL)-based medium. Hyaluronan and proteoglycan link protein 1, keratin 18, BDNF, ribosomal protein S6 kinase, PTPL1-associated Rho-GAP 1 m, and renal tumor antigen were all significantly upregulated in pHPL-treated MSCs, whereas pleiotrophin was downregulated [310]. Zhang et al. found that FOXQ1 overexpression reduced senescence and enhanced the migration of hUC-MSCs *in vitro*, and more effectively ameliorated cognitive dysfunction in AD model rats compared to MSCs grown in serum (Fig. 9) [312].

#### 4.4. Gene-edited extracellular vesicles (EVs) or exosomes of MSCs for the treatment of nerve injury

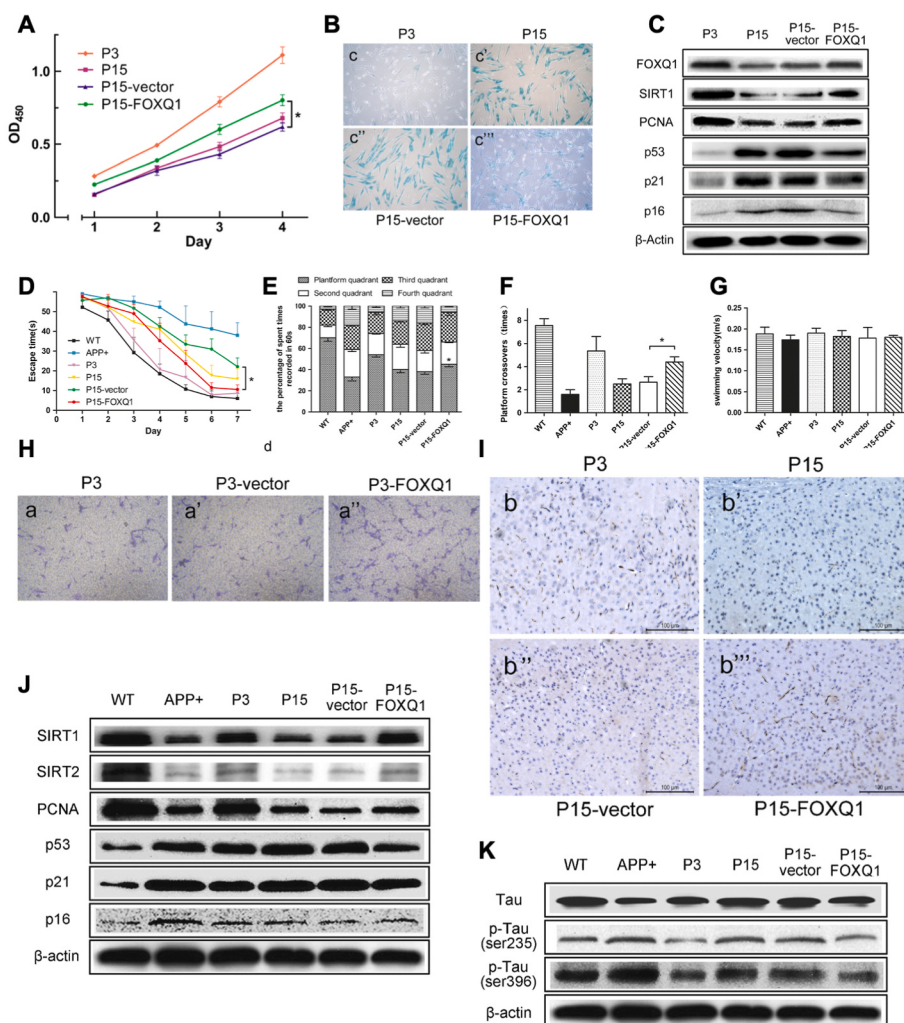
EVs are nanovesicles with diameters ranging from 30 to 1000 nm that can be isolated from culture supernatants of cells. Many cells release EVs, such as stem cells, T cells, B cells, dendritic cells, platelets, adipocytes, and others. Proteins, lipids, mRNAs, and non-coding RNAs are contained in EVs. Small EVs with diameters between 30 and 150 nm are called exosomes, which are derived from cytoplasmic membrane by endocytosis to form multivesicular bodies and then fuse with the plasma membrane and exit the cell. MSCs are rapidly cleared by the body after transplantation, but their actual therapeutic effect can be maintained for a long time, which indicates that paracrine, EVs and exosomes play an important role in them.

Many studies have shown therapeutic effects of MSC-EVs in animal models of neurological diseases such as stroke, AD, TBI, multiple

sclerosis (MS), and subarachnoid hemorrhage (SAH). Through powerful neuroprotection, inducing neurogenesis, immunomodulatory, anti-inflammatory and apoptosis control effects, MSC-EVs play a great role in nerve injury. Especially for nerve damage in the brain, because exosomes can cross the BBB and are known mediators of cell-to-cell communication. Different from MSCs that can only be administered intravenously or locally, a natural clinical advantage is the intranasal administration of exosomes, which enables them to quickly enter the brain without being blocked by the lungs through intravenous administration.

##### 4.4.1. Engineered MSC-EVs to become the next-generation nanovector

Similar to gene-edited MSC, the current MSC-EVs cannot fully meet the clinical needs. More and more studies have begun to view MSC-EVs as an effective nanocarrier to enhance its neurotherapeutic effect by encapsulating different cargo, including small molecule drugs, proteins and nucleic acids, such as mRNA, miRNAs, and siRNA. There are two main strategies to genetically modify MSC-EVs, including pre-isolation and post-isolation. The pre-isolation method is the genetic modification of MSCs by expression vector transfection or transduction, leading to the endogenous synthesis and secretion of the desired product. The advantage of the pre-isolation method is that it allows one to collect EVs already loaded with cargo, eliminating interference and damage to the EVs structure later. However, the reproducibility of the results was challenged due to the uncontrolled expression of target genes. Another concern is that gene-edited MSCs alter gene expression profiles, resulting in uncontrolled changes in the effective content of MSC-EVs. Loading



**Fig. 9.** FOXQ1 overexpression promotes anti-senescence and migration effects of human UC-MSCs in AD. (A) CCK-8 assay was used to detect cell viabilities. (B) The SA-β-gal staining to detect senescent cells under an inverted light microscope ( $\times 100$ ). (C) Western blot was performed to evaluate the expression of senescence-associated proteins. (D) AD mice of escape time required for finding the platform. (E) The percentage of time consumed in the four quadrants during 60 s. (F) Frequency of platform crossover. (G) Swimming velocity. (H) Transwell assay was used to detect cell migration capacity. (I) In vivo experiment. Tissue sections of AD mice brain were stained with MAB1281. (J) Western blot was used to detect expression of senescence-associated proteins in the hippocampus. (K) Western blot was used to detect expression of Tau and p-Tau. Error bars = SD. \* $P < 0.05$ .

EVs after they have been isolated from cell cultures is known as post-isolation. The loading capacity of EVs can be improved by direct co-incubation of EVs and cargo with additional interventions, including electroporation, saponin penetration, and ultrasound. However, these techniques can lead to damage and aggregation of EVs, complicating the isolation and purification process.

#### 4.4.2. Application of engineered MSC-EVs in neurological diseases

After gene modified, MSC-EVs can enhance its neurotherapeutic effect by obtaining additional targeted genes. The logic of these gene-editing strategies is similar to that of modified MSCs, including promotion of neural differentiation, neuroprotection, anti-inflammatory, and anti-apoptosis. Notably, not only MSCs can be engineered with various genes but also their extracellular vesicles (EVs) or exosomes can be functionally modified with certain genes or miRNAs. BMSCs over-expressed exosomal  $K^+-Cl^-$  cotransporter (KCC2), which is highly expressed in neurons, is a key factor in maintaining neuronal chloride homeostasis. BMSCs can significantly promote neuronal differentiation of NSC, promote nerve regeneration at SCI injury, and restore motor function in mice [313]. In addition to being a carrier of proteins, miRNAs such as miR-544 [314], miR-381 [315], and miR-145-5p [316] delivered by EVs from MSCs facilitate the recovery of SCI in rat models, which provides a novel perspective for improving the treatment of SCI.

Because of the ability of exosomes to penetrate the blood-brain barrier, some gene-edited exosomes have been used to carry out nerve damage in the brain. In stroke animal models, the overexpression of miR-145 exosomes isolated from BMSCs can significantly reduce the infarct size of MCAO rats, transform microglia polarization to anti-inflammatory M2 phenotype in OGD/R-stimulated BV2 cells, and significantly inhibit apoptosis, cell cycle arrest and oxidative stress in OGD/R-treated BV2 cells. The inhibitory effect of miR-145 on apoptosis and inflammation depends on the inhibition of FOXO1 expression in the brain of MCAO rats [317]. Wei et al., using prefractionation, made BMSCs co-overexpress zinc finger E-box binding homeobox 2 protein (Zeb2) and Axin2, and collected BMSC-Exos. Compared with control, the overexpressed Zeb2/Axin2 further stimulated endogenous neurogenesis and promoted synaptic remodeling in the ischemic boundary zone by activation of wnt3a/ $\beta$ -catenin signaling pathway [318]. Others use EVs as carriers to transport proteins or miRNAs, such as ZFAS1 [319], miR-455-5p [320] and CCR2 [321] also can reduce the ischemic area of brain area, promoted nerve recovery and inhibited local neuronal apoptosis in stroke mice. Using exosomes as vectors, overexpression of miR-188-3p [322] can help to locally regulate apoptosis and autophagy in Parkinson's disease.

#### 4.5. Challenges and limitations of gene-modified MSCs in a model of nerve injury disease

Although the synergies of gene modification and MSCs are powerful, there are inherent challenges in both clinical translation and application models. This includes specific issues before cell therapy can be used clinically.

Before the application of genetically modified MSCs in clinical practice, the biggest concern is whether their safety is reliable. Accumulated clinical trial activity over the past decade has significantly reduced concerns about serious or life-threatening adverse events associated with MSC administration, whether from autologous or allogeneic sources, intravenous or topical administration. However, the safety of natural MSCs does not guarantee the safety of genetically modified MSCs. Because the gene expression profile of MSCs modified by different genes will change dramatically, especially the activation of some uncontrolled signaling pathways brings concerns about their safety. For the gene modification strategies that promote cell proliferation or survival under stress, their safety is more worthy of attention, because in many tumor tissues, the expression of these genes is also abnormal, such as bFGF, Bcl2, etc [323,324]. Although the

overexpression of these genes promoted the survival rate of MSCs in local transplantation, it also brought a certain risk of tumor development. Unfortunately, current animal experiments on gene-edited MSCs are only at the stage of observing their effectiveness, and monitoring their safety is not involved. Another concern arises from the choice of transfection vector. At present, most studies still choose viral vectors as the preferred choice, which is due to their excellent transduction efficiency. However, more pre-clinical studies need to be done to prevent potential tumor risk or other more serious complications. For example, in X-SCID clinical trials, CD34<sup>+</sup> bone marrow cells were isolated and transfected *in vitro* with Moloney murine leukemia virus carrying a common gamma chain cytokine receptor, and the altered cells were transplanted back into the patient. This approach treated most of the patients' immune deficiencies, but four of them developed leukemia over the next nine years due to the inserted tumor [325]. Another solution is to shift the research focus to efficient new transient transfection technology, which can naturally avoid most of the biosafety risks by using long-term effective transient transfection technology. The last concern is that the current reports are basically based on small sample size studies. Although similar results have been obtained in multiple laboratories for the transduction of some genes, such as Shh, BDNF and VEGF, the credibility of the conclusions is still worthy of attention. In addition, the comparison of efficacy between different genes may also require higher attention, especially in the pre-clinical research phase, it is important to select more effective and safe genes for transduction. In conclusion, it will be difficult for genetically modified MSCs to enter clinical trials until some specific issues are resolved:

- Are the transduced genes effective enough? How to select more effective and safer genes?
- Do genetically modified MSCs have a potential risk of tumor or other diseases?
- Are there specific risks to humans by the nature of certain viruses?

#### 5. Conclusion

Most existing therapies for neurological disorders address symptoms but do not reverse the underlying neural tissue damage. Many animal model experiments and even some clinical trials suggest that transplanted MSCs can replace lost neural cells and restore function. Although the therapeutic effects are still insufficient, gene delivery techniques can improve the therapeutic efficacy of MSCs.

Importantly, the bias in research focus is already clear for the future use of gene-edited MSCs to treat neurological diseases. First of all, for a variety of gene delivery tools, although viral vectors show better gene delivery efficiency, non-viral vectors have innate safety advantages, and with the development of nanotechnology, the defect of transfection efficiency has been further overcome. In addition, with the development of reverse transfection, non-viral vectors show unique advantages for nerve damage repair requiring scaffold materials in some scenarios. Second, the selection of different transfection scenarios is also particularly important. In this review, three different transfection strategies were summarized, including promoting MSC neural differentiation, promoting NTFs secretion, and promoting stem cell homing and angiogenesis. Although the three types of gene-editing strategies differ in their underlying logic, they are not independent of each other. The combined transfection strategy of multiple genes may have a very good application prospect, which depends on whether they can cooperate with each other to achieve the ideal cooperation effect. Third, some transfection materials have their own biological functions, which need to be considered in future applications. And when they appear unsuitable scene application logic, need to give up and choose other materials. For example, some materials have osteogenic or chondrogenic properties and are not suitable for use in diseases that require repair of nerve damage. Fourth, the application strategies of different MSCs are also very different. When MSC is used as the main repair tool, endodontic

mesenchymal stem cells derived from neural crest ectoderm may have better repair potential. However, when MSC is used as the expression vector, BMSC or AMSC may express more target genes because of their better transfection efficiency. Therefore, the practical application strategy should consider different application scenarios and make a comprehensive decision to give the best solution.

Nonetheless, many challenges remain before routine clinical application would be possible. First, viral vectors for gene transfer have inherent safety problems that preclude their use in clinical trials. In addition, MSCs are difficult to transfect using common nonviral methods. Hence, safe, highly efficient, and controllable transfection methods are needed. Second, although genetic manipulation of MSCs can improve regeneration, functional recovery remains relatively poor compared with normal or sham groups. The transfection of multiple genes conferring greater apoptosis resistance, antioxidant capacity, immunosuppressive activity, and neurotrophic factor release may be a better therapeutic strategy. Therefore, future studies should aim to identify the best gene combinations. Third, few studies have focused on the safety profiles of MSCs with gene transfection for transplantation. While naïve MSCs rarely cause teratoma and have low immunogenicity, the safety of modified MSCs, especially those overexpressing multiple genes, must be verified experimentally.

### Authors' contributions

Q.Y. conceived the concept of this project, H. Z., Y. H. and W. X. performed literature searching and wrote this manuscript. H. Z. and Y. Z. draw figures. S. J. and X. D. collated the data. Z. H. and W. S. finished the tables. M. L., Y. P. and Y. H. performed data curation. G. N. and Q. Y. provided language improvement, Q. Y. and Y. H. provided financial support. All authors read the final version of the manuscript and approved its submission for publication.

### Ethical approval and consent participate

No clinical or animal experiments are included in this study. Ethical consent is not required for this article.

### Declaration of competing interest

The authors declare that they have no competing interests regarding the publication of this paper.

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