PERSPECTIVE

Engineering mesenchymal stromal/stem cell-derived extracellular vesicles with improved targeting and therapeutic efficiency for the treatment of central nervous system disorders

Treatment for central nervous system (CNS) disorders is known to be limited by the low regenerative potential of neurons, and thus neurodegenerative insults became known as nearly irreversible ailments. Functional recovery for acquired CNS disorders, such as spinal cord injury (SCI), traumatic brain injury, ischemic stroke, Alzheimer's disease, Parkinson's disease, multiple sclerosis (MS), and for congenital CNS abnormalities, such as spina bifida, is not spontaneous and effective treatments are limited to non-existent.

Research in the past decades has proven the regenerative potential of stem cells, especially that of mesenchymal stromal/stem cells (MSCs) from various origins, such as bone marrow, placenta, and adipose tissue. Most notable MSC characteristics for their candidacy as CNS therapeutics include their immunomodulatory, angiogenic, and neuroprotective capabilities. For instance, in our previous studies using a fetal ovine model of spina bifida, we showed that placenta-derived MSCs (PMSCs) were able to improve neurological function by preserving spinal cord neurons (Wang et al., 2015). However, PMSCs did not persist following transplantation nor contributed to tissue regeneration by direct integration. Recently, using an *in vitro* neuronal injury/protection model, we showed that conditioned media or extracellular vesicles (EVs) derived from PMSC cultures, suppressed caspase activity and rescued the apoptotic neurons as efficiently as the stem cells themselves (Kumar et al., 2019). Most recently, we further showed that EVs derived from PMSCs reduced DNA damage in oligodendroglia populations, increased myelination and improved motor function outcomes in an experimental autoimmune encephalomyelitis rodent model of MS. Furthermore, we found that the high-dose PMSC-EV treatment exerted similar clinical outcomes to the stem cells in the experimental autoimmune encephalomyelitis model, proving their potential as cell-free alternative therapeutics (Clark et al., 2019). Results from these studies indicate that it is likely MSCs confer their therapeutic effects via a paracrine mechanism, consisting of secreted therapeutic components, including EVs.

Many CNS injuries are often caused by primary insults followed by secondary injuries, usually characterized as excess inflammation and cellular apoptosis. In a traumatic brain injury mouse model, application of EVs from bone marrow-derived MSCs (BM-MSCs) reduced neuroinflammation by polarizing microglia, from their pro-inflammatory to their anti-inflammatory phenotype (Ni et al., 2019). In a SCI rat model, EVs from BM-MSCs reduced inflammation, thus halting apoptosis, and even promoted angiogenesis (Huang et al., 2017). In a mouse model of ischemic stroke, BM-MSC-derived EVs suppressed inflammation and cellular apoptosis at the lesion site in the brain (Tian et al., 2018). In all of these studies, the reduction of inflammation ultimately served as a method of neuroprotection by reducing cellular apoptosis and improving functional recovery. Thus, animal-based studies are increasingly showing that EVs are capable of regulating immunomodulatory responses in the CNS post injury, and thus have potential as a cell-free alternative treatment for CNS disorders.

EVs are lipid bilayer membrane vesicles that mediate cell-cell communication and contain biological molecules, such as proteins, lipids, and nucleic acids. While systematically injected MSCs are typically trapped in the lungs or taken up by other organs, EVs are much smaller in size and thus may be better suited to avoid nonspecific delivery. Furthermore, native EVs are also shown to have the potential to home into areas of injury within the CNS (Guo et al., 2019). EVs are shown to be able to cross the blood-brain barrier (BBB) and in effect act as drug delivery vehicles for CNS disorders, an absent capability in their parent MSCs (Tian et al., 2018).

There are three subtypes of EVs: microvesicles, apoptotic bodies, and exosomes, classified by their mechanism of release from their parent cell. Exosomes are secreted by cells as a result of a dynamic endocytic pathway and are the EVs of most interest for therapeutic applications. Exosome biogenesis and release consist of several critical steps. First, a cell membrane inward budding event creates an early endosome, which subsequently experiences multiple inward budding events, culminating in the accumulation of intraluminal vesicles inside the early endosome. The intraluminal vesicles enclose proteins, lipids, and nucleic acids among other cellular components, via an endosomal sorting complex required for transport (ESCRT), within the maturing endosome. The sorting of molecules leads to morphological changes in the mature endosome, which is then referred to as a multivesicular body. Multivesicular body fusion with the cell plasma membrane then releases the intraluminal vesicles, which become known as "exosomes", into the extracellular space (Hessvik and Llorente, 2018). However, due to the consistent heterogeneity of isolated exosomes, these membranous vesicles are generally referred to as EVs.

While MSC-EVs have been promising in protecting and rescuing neural tissues in various animal models of CNS disorders, clinical translation remains challenging due to limited yield, targeting efficiency, and specific functions. High EV yield is crucial for obtaining the sizable quantities necessary for clinical applications. Furthermore, the properties of natural EVs can be augmented by enhancing their targeting abilities towards a specific tissue or cell type and improving their therapeutic efficiency. This can be achieved in a variety of ways including by engineering the parent cells or engineering the surface and/or content of EVs after they are isolated.

Cell engineering: EV yield, surface marker expression, and composition are all directly tied to the parent cell conditions and functions, which can be regulated by engineering certain extracellular and intracellular components, with the ultimate goal of improving yield, targeting and/or therapeutic efficiency of EVs (**Figure 1**).

The extracellular environment, including cell culture conditions and methods, can significantly change EV production. It has been shown that using culture media containing an agent that induces cell stress, such as tunicamycin, can increase EV secretion (Hessvik and Llorente, 2018). While the cargo of these stress induced EVs remains unknown, this does appear to be a promising approach to improve the yield of EVs. In addition, compared to conventional two-dimensional culture methods, high density three-dimensional culture systems such as biomaterial scaffolds, microcarriers and hallow-fiber bioreactors can lead to higher cell yields that will result in higher yield of EVs (Phan et al., 2018).

The intracellular components of the parent cells could also be engineered to improve the production yield and internal components of EVs, for improved targeting and therapeutic efficiency. Three different approaches could be taken to achieve this purpose. First, optimizing EV biogenesis pathways to improve EV production and yield. Many molecules are involved at various stages of the endolysosomal pathway, whose activation or suppression can influence the production and yield of EVs. For example, depleting TSG101 of the ESCRT protein complex decreased EV secretion, whereas knockdown of VPS4B, another ESCRT-protein increased EV secretion (Hessvik and Llorente, 2018). Second, loading therapeutic nucleic acids to EVs by manipulating the parent cells, to improve therapeutic efficiency. Various approaches have been established to pack therapeutic DNA or RNA components within donor cells, such that the donor cells will secrete EVs packed with these nucleic acids. Thus, certain DNA and RNA sequences involved in neuroprotection and angiogenesis can be applied in engineering MSCs to secrete modified EVs that confer the desired therapeutic effects for CNS disorders (Phan et al., 2018). Third, genetic manipulation of the parent cells to improve the therapeutic and targeting efficiency of EVs. Parent cells could be genetically engineered to produce EVs with desired internal cargos as well as specific targeting efficiency on the surface. For instance, Alvarez-Erviti et al. (2011) engineered dendritic cells to express Lamp2b, an exosomal membrane protein fused to the CNS specific rabies viral glycoprotein (RVG) peptide (YTIWMPENPRPGTPCDIFTNSRGKRASNG) that specifically binds to the acetylcholine receptor. It was shown that the Lamp2b-RVG protein was expressed on the parent cell surface as well as strongly expressed on the surface of RVG modified exosomes (RVG-exosomes). The RVG-exosomes were able to specifically target neurons, microglia, oligodendrocytes and their precursors in the mouse brain. It was also confirmed that engineering the parent cells using this approach did not appear to affect the physical properties of the modified exosomes (Alvarez-Erviti et al., 2011). Thus, engineering the parent cells via intracellular genetic manipulation could ultimately increase EV yield and improve EV therapeutic efficiency.

EV surface engineering: Natural MSC-EVs present particular surface proteins as a result of the inward budding from the plasma membrane during biogenesis. These surface components mediate EV-recipient cell interactions and confer important EV targeting functions. Modifying the EV surface could be achieved by engineering the parent cells. However, cell engineering techniques could not be applied to pre-isolated EVs. Thus, several other mechanisms have been explored in order to modify the surface of isolated EVs, and endow specific targeting potential to the EVs.

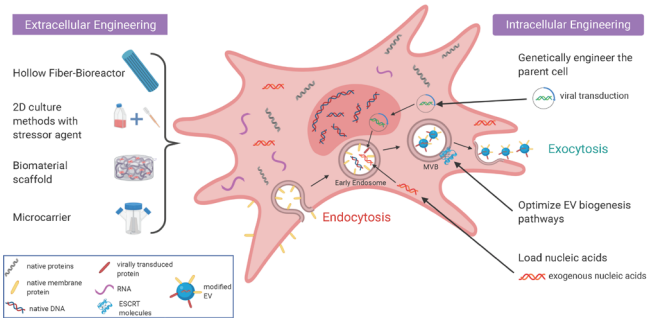

Figure 1 Cell engineering to improve EV production yield, targeting and therapeutic efficiency.
Cell engineering can be done by through (1) extracellular engineering, by using bioreactor, 2D culture with a stressor agent, and biomaterial scaffolds and/or microcarriers; (2) intracellular engineering by genetically engineering the parent cells, optimizing EV biogenesis pathways, and loading exogenous nucleic acids into the parent cell. 2D: Two-dimensional; EV: extracellular vesicle; MVB: multivesicular body.

Based on the membrane structure and components of EVs, a variety of chemical and biological approaches could be used to modify the EV surface. One of the most applicable methods is via Click chemistry. Click chemistry is an easy, rapid and efficient method to covalently conjugate functional ligands onto EV surfaces. In a recent animal study of ischemic stroke, BM-MSC-derived exosomes were modified with a cyclo peptide, c(RGDyK), which was conjugated to the EV surface, via click chemistry (Tian et al., 2018). The peptide c(RGDyK) has a high binding specificity to integrin αvβ3, which is expressed on reactive endothelial cells after the brain undergoes ischemic stroke. The peptide led the modified exosomes to the lesion site, where the modified exosomes targeted brain cells near the ruptured blood vessels (Tian et al., 2018). Furthermore, EV's surface is enriched in phosphatidylserine and transmembrane glycoproteins such as integrins, all of which can also be directly utilized for peptide binding. Since EVs are capable of crossing the BBB, with improved targeting efficiency, EVs can be more effectively delivered to sites of interest in the CNS.

EV content engineering: In order to further improve their therapeutic efficiency, EVs can also be engineered by loading various specialized cargo, in effect acting as drug delivery systems for specific diseases. Cell preconditioning, is a feasible method of engineering cargo, as the EVs will encapsulate bioactive molecules as a result of the endolysosomal pathway. However, cell engineering may limit the control researchers have over the specific cargo needed to be carried. Therefore, small molecules, proteins and nucleic acids that benefit CNS treatment and protection, can be preferentially encapsulated in the engineered EVs directly after they are isolated from culture supernatants.

Nucleic acids, such as siRNAs and miRNAs, hold great promise for the treatment of CNS diseases and can be loaded within engineered EVs. For instance, BACE1 is a protease responsible for N-terminal cleavage of the amyloid precursor protein that produces the aggregate-forming β-amyloid peptide, therefore, BACE1 is a potential therapeutic target in Alzheimer's disease. Alvarez-Erviti et al. (2011) loaded purified exosomes with exogenous siRNA targeting BACE1, by electroporation. The therapeutic potential of exosome-mediated siRNA delivery was demonstrated by the strong mRNA (60%) and protein (62%) knockdown of BACE1 in a mouse model (Alvarez-Erviti et al., 2011). In a recent study by Guo et al. (2019), the phosphatase and tensin homolog (PTEN) was targeted. PTEN is expressed in neurons and regenerating axons, and impedes regeneration by limiting axonal growth. BM-MSC-derived EVs were modified with PTEN-siRNA to reduce the expression of PTEN at the lesion site, in the spinal cord. It was shown that PTEN-siRNA modified MSC-EVs increased neurite length, branch points, and neurite count of dorsal root ganglia in culture to a much greater extent compared to controls, including unmodified MSC-exosomes and free PTEN-siRNA. Furthermore, the modified EVs homed to the SCI lesion in a rat model, and enabled functional recovery in addition to reducing neuroinflammation and increasing axonal regeneration and angiogenesis (Guo et al., 2019). Thus, therapeutic exogenous cargos can be loaded into isolated MSC-EVs, which could be simultaneously engineered with targeting ligands to further improve the therapeutic and targeting efficiency of EVs as drug delivery vehicles, and thus improve their therapeutic applications.

MSC-EVs are a promising alternative cell-free treatment of CNS disorders that can confer therapeutic effects and act as drug delivery vehicles, carrying therapeutic cargos across the BBB. In addition, therapeutic MSC-EVs could also be administered intrathecally, with potentially greater efficacy, to CNS injured or degenerative sites. Emerging approaches can be used to engineer MSC-EVs to further improve their production, targeting and therapeutic efficiency via cell bioengineering, surface modification, and cargo loading to offer more effective treatments for neurodegenerative diseases.

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