Article

The extracellular vesicles-derived from mesenchymal stromal cells: A new therapeutic option in regenerative medicine†

Running head: *extracellular vesicles in regenerative medicine*

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ABSTRACT

Mesenchymal stem cells (MSCs) are adult multipotent cells that due to their ability to homing to damaged tissues and differentiate into specialized cells, are remarkable cells in the field of regenerative medicine. It's suggested that the predominant mechanism of MSCs in tissue repair might be related to their paracrine activity. The utilization of MSCs for tissue repair is initially based on the differentiation ability of these cells; however now it has been revealed that only a small fraction of the transplanted MSCs actually fuse and survive in host tissues. Indeed, MSCs supply the microenvironment with the secretion of soluble trophic factors, survival signals and the release of extracellular vesicles (EVs) such as exosome. Also, the paracrine activity of EVs could mediate the cellular communication to induce cell-differentiation/self-renewal. Recent findings suggest that EVs released by MSCs may also be critical in the physiological function of these cells. This review provides an overview of MSC-derived extracellular vesicles as a hopeful opportunity to advance novel cell-free therapy strategies that might prevail over the obstacles and risks associated with the use of native or engineered stem cells. EVs are very stable; they can pass the biological barriers without rejection and can shuttle bioactive molecules from one cell to another, causing the exchange of genetic information and reprogramming of the recipient cells. Moreover, extracellular vesicles may provide therapeutic cargo for a wide range of diseases and cancer therapy.

Key Words: Mesenchymal Stem Cells, Extracellular vesicles, Exosome, Regenerative medicine.

1. Introduction

In the past few decades, regenerative medicine employed various types of human stem cells for treatment of injured, malfunctioning tissues and organs. emerging evidence have shown that stem cell therapies are a promising strategy to improve regeneration in damaged tissues such as heart, brain, spinal cord, liver (1) . In addition, stem cell therapies can be considered as a hopeful approach for irreversible diseases which there is not any treatment options at this moment. Regenerative medicine strategies include using stem cell differentiation ability to reconstruct missed or damaged tissues and organs by a safe, effective transfer methods (2). The regeneration potential of stem cells such as mesenchymal stromal cells (MSCs), induced pluripotent stem cells (iPSCs) and embryonic stem cells (ESCs) have established in order to restore human damaged tissues by means of secretion of growth factors, cytokines and extracellular vesicles. MSCs possess the particular advantages such as multi-lineage differentiation with immunomudulatory and immunosuppresion effects that make them as a safe effective stem cell-based therapy in clinical setting (3).

Moreover, the paracrine activity of could account the regenerative ability of MSCs and one of the predominant components is extracellular vesicles (EVs) that possess the crucial role in their therapeutic potentialMSC-derived EVs therapy display a desirable cell-free therapeutic option in regenerative medicine (4). EVs are naturally secreted membrane vesicles which referred to exosomes, microvesicles and apoptotic bodies. Mounting evidences show a great potential of EVs generated from MSCs as specific biomarkers for diagnosing, prognosis or prediction of diseases. Moreover, these vesicles can be used to treat many disorders through regenerative and immunoregulatory capacities or as a novel carrier in drug delivery systems to transfer drug agents or genetic materials (1, 5, 6). In this review, we aimed to discuss the beneficial therapeutic potentials of EVs for various diseases treatment.

2. Mesenchymal stem cells and its phenotyping

A group of adult stem cells are MSCs which extensively studied as therapeutic tools in regenerative medicine. In 1968, Friedenstein and colleagues found this adult stem cells in the stromal compartment of bone marrow (7).

The International Society for Cellular Therapy (ISCT) has considered the minimal criterion for multipotent human MSCs definition which include: (i) the adherent cells to plastic; (ii) the cells with fibroblast-like morphology which can differentiate under a certain stimulus into osteocytes, adipocytes and chondrocytes; (iii) positive expression of CD105, CD73 and CD90; negative expression of CD45, CD34, CD14 or CD11b, CD79alpha or CD19, and HLA-DR surface molecules (8-13). Human MSCs are also negative for CD4, CD8, CD11a, CD14, CD15, CD16, CD25, CD31, CD33, CD49b, CD49d, CD49f, CD50, CD62E, CD62L, CD62P, CD80, CD86, CD146 (vascular cell adhesion molecule [VCAM]-1), CD117, cadherin V, and glycophorin A, and positive for CD10, CD13, CD29 (b1-integrin), CD44, CD49e (a5-integrin), CD54 (intercellular adhesion molecule [ICAM]-1), CD58, CD71, CD146, CD166 (activated leukocyte cell adhesion molecule [ALCAM]), CD271, vimentin, cytokeratin (CK) 8, CK-18, nestin, and vonWillebrand factor (14) . Some surface markers are tissue specific for example; high levels of CD34 only express on adipose tissue-derived MSCs and CD27 could be found on bone marrow-derived MSCs. These criteria provide characteristics for MSC purification and their expansion by several-fold in-vitro, without

losing their differentiation capacity. Furthermore, colony-forming units of fibroblasts (CFU-f) is another characteristic of MSCs that consist of small colonies of low density plated cells which correspond to the progenitors that can differentiate into one of the mesenchymal cell lineages (15). MSCs have been used as the first kind of stem cells in clinical regenerative medicine after HSCs due to their easy isolation, rapidly ex vivo expansion, and also their autologous transplantation. These cells have other advantages including differentiation into multiple lineages, enhancing tissue repair, anti-inflammatory properties, and immunosuppression activities. Recently, the migration ability and incorporation of MSCs into specific tumors have been demonstrated in several different types of pre-clinical models which prove their potential as ideal anti-cancer agent's carriers (10, 16).

3. MSCs and immunomodulation

It's established that multipotent MSCs can differentiate into a wide variety of cells including mesodermal and non-mesodermal lineages. In addition to their differentiation potential, one of the best-explained functional characteristics of MSCs is their immunoregulatory abilities (7). Though, the underlying mechanisms of MSC immunomodulation properties must be clarified. Numerous researches have provided data that show MSCs immunosuppression abilities. They can suppress proliferation of pre-stimulated T cell, differentiation of naïve T cells to effector cells, secretion of pro-inflammatory cytokines and also their cytotoxicity. MSCs have an ability to affect the cytokine secretion profile of T-cells, dendritic cells and macrophages. Furthermore, MSCs can regulate functions of regulatory T cells (Tregs), the balance of Th1/Th2 whereas they can suppress the function of other cells of the immune system. (17). Interleukin-2 (IL-2)-induced natural killer (NK) cell activation and the maturation, activation and antigen presentation of dendritic cells are suppressed by their immunomodulatory effects (16). MSCs express low level of complex class II (MHCII) and co-stimulatory molecules (B7-1and B7-2). They get involve with different types of the immune responses by means of cell-to-cell contacts. Besides, transforming growth factor-β1 (TGF-β1), prostaglandin E2 (PGE2), hepatocyte growth factor (HGF), indoleamine-pyrrole 2, 3-dioxygenase (IDO), nitric oxide (NO), matrix metalloproteinases, and interleukin 6 and 10 have been considered as major modulator for MSCs immunosuppressive effects. Extensive proofs provide that interferon-γ (IFN-γ), tumor necrosis factor-α (TNF-α), IL-1α or IL-1β which called inflammatory cytokine can mediate MSCs immunosuppressive activity through cyclooxygenase 2 (COX-2), PGE2 and IDO (18, 19). ProstaglandinE2 (PGE2)-derived MSCs suppress proliferation of T-cell and induce the T regulatory cells. IDO secreted by MSCs has been shown to suppress allogeneic T-cell responses and can induce tolerance after kidney allograft transplantation (20).it's published that MSCs heme oxygenase-1 plays an immunosuppresion role for suppression of T-cell proliferation and Toll-like receptor activation. HO-1activity can be regulated by iNOS and IDO. NO is another soluble factors that secrete by MSCs which involve in the T cell proliferation and STAT5 phosphorylation inhibition. Also, the proliferation of IL2–induced NK cells and the expression of NKp30 and NKG2D are inhibited via IDO and PGE2. Cell to cell contact is a main factor in MSCs immunomodulatory impacts. It's reported by Han et al that contact-dependent mechanisms may decrease the survival and proliferation of T cells whereas make increase the proportion of Tregs . IFN-γ upregulate MSCs Cell adhesion molecules secretion including CD274 (also

known as Programmed death ligand 1), vascular cell adhesion molecule-1 and galectin-1 which are involved in cell to cell contact and the promotion of MSCs immunomodulation capacity. However, MSCs apply both direct cell to cell contact and soluble factors for their widely different regulation. These capacities make them as a promising candidate to control host-versus graft (HvG) and graft versus host disease (GVHD) in BM transplantation (16, 17, 21-23).

4. MSC in regenerative medicine

New and emerging terms in biotechnology and medicine are tissue engineering and regenerative medicine which employed to depict generation of complex tissues and organs from simpler pieces. Sever or chronic disease treatment by novel therapies is a gold main of this field (24). The "regenerative medicine" term is more concentrate on the utilizing stem cells as therapeutic tool and self-healing abilities. MSCs are the great opportunity in the field of stem cell therapy due to their appealing characteristics such as a: their wide ranging differentiation ability into tendons, bone, cartilage, ligaments, muscles, and neurons. B: their easy accessibility. C: The Straightforward isolation and their relatively fast large- scales expansion of MSCs in a short period of time through culture. d: human clinical trials by MSCs show a satisfactory results of both allogeneic and autologous MSC transplants due to their immunomodulatory effects (14, 25) . It's cleared that culture-expanded MSCs did not express MHC class II cell surface markers, but rather only MHC class I and no co-stimulator molecules (26) . Thus, human MSCs could not present antigen and would evade from host's immune system (27). These observations were used to proposed that MSCs could be used as a therapeutic tool in the regenerative medicine to repair damaged tissues because of different mechanisms, for example, differentiation into damaged cells, microenvironment repair with paracrine/juxtacrine effects of cytokines, soluble factors and growth factors or reorganization of extracellular matrix (28). Source of MSCs isolation may influence on therapeutic efficiency. For example, Human MSCs-derived adipose tissue (AT-MSCs) xeno-transplant improve angiogenesis and axonal regeneration and better functional recovery than BM-MSCs. AT-MSCs induced high levels of neurotropic factors such as BDNF, VEGF, and HGF (29).oseteogenesis potential of MSCs have been extensively studied and have been applied in bone repair and regeneration. They employ to treat bone disorders as osteogenesis imperfecta (30, 31) and engineering of bone tissue together "scaffolds" (32). The mesenchymal precursors osteogenic benefits depends on their differentiation potential into osteoblast but also on their ability to produce growth factors and cytokines to the damaged tissues thereby promote the regeneration process (33). Apart from MSCs advantages in bone repair, they are also used to involve in treatment of cardio- vascular diseases. MSC-based cell therapy can repair heart injuries by the production of new cardiomyocytes (34). However, there is currently no clear agreement if MSCs have differentiation ability into cardiomyocytes and, if so, by which signals. Some experiments haves been shown that small proportions of transplanted MSCs remain in the target tissues (35). It is believed that MSCs positive effects on heart disorders consist of differentiation capacity into cardiomyocytes, the release of trophic factors and inflammatory suppression properties. MSCs are also exploited for neuronal injury and neurodegenerative diseases treatment such as Alzheimer , Parkinson and Huntington diseases by means of MSCs ability to locally secrete high levels of brain-derived

neurotrophic factor (BDNF), nerve growth factor(NGF), vascular endothelial growth factor (VEGF) and hepatocyte growth factor (HGF), indeed the expression of these factors have been increased in vitro when MSCs are exposed to injured brain extracts (36). Moreover, the immune response modulation by MSCs is a key factor for neurodegenerative diseases treatment (37). Recently, there are two different opinions regarding the impact of MSCs on cancer which have no clear consequences. Some showed that MSCs have tumor tropism activity, immune suppression capabilities and could be considered as a novel candidate for cancer treatment. However, others believe that MSCs may promote tumor growth through promoting angiogenesis, creating a niche to support cancer stem cells survival, increasing formation of metastasis by modulation of the immune response against cancer cells (7).

4.1. MSCs in cancer therapy

Cancer, or malignant neoplasm (new tissue), has known as a global health problem of epidemic proportion. The conventional techniques of cancer therapies (surgery, chemotherapy or radiotherapy) do not handle their limitations such as cancer recurrence, metastasis following initial remission because of the lack of selectivity. Also, the final cancer therapy aim is to advance anticancer agents with precise specificity which strongly target tumor cells while sparing normal cells (38). Many adult stem cells (SCs) have been shown intrinsic tumour-tropic properties, proposing them as a considerable candidate for targeted cancer therapy strategies. These strategies divided into two separate parts which include their site-specific migration towards micro-metastatic lesions and another is their genetically modification to express or release different anti-cancer agents, thereby solve the short half-life of chemotherapeutic agents (39). Maximizing the therapeutic impact of SC therapy require which they escape through immune system towards malignant sites to make their therapeutic effects (40). While the advantages of MSCs in regenerative medicine is relatively well-found, MSCs using in anti-cancer therapy is getting developing attention (40). In 1993, Maestroni et al discovered the ability of MSCs tumor tropism which has led to a good agreement of MSCs role as a therapeutic agent in tumors. The cellular and molecular mechanisms that underlie MSC migrate across endothelium and home to the target tissues are far from being completely understood (41). To date, stromal cell-derived factor SDF-1 and its receptor CXC chemokine receptor-4 (CXCR4) are the key mediators of stem cell homing to tumor sites. The unmodified MSC have been noticed to have anti-tumor effects both in vitro and in different mouse models of cancer by means of releasing factors with anti-tumor properties that decrease glioma proliferation, melanoma, lung cancer, hepatoma and breast cancer cells. The migrations of Human MSCs to tumorigenesis sites suppress tumor growth in a mouse model of Kaposi's sarcoma. The anti-angiogenic effects of MSCs have been reported in melanoma models. Direct injection of MSC into mice bearing melanoma induced appotosis of tumor cells (38, 42). Moreover, MSCs properties such as tumor tropism and immunity granted nature make them a perfect anti-cancer drug delivery approach. The engineered MSCs effects which possess anti-cancer genes such as IFN-ß, IL-12, IFN-α, IFN-gamma, IL-2, NK4 and TRAIL have been explored in different cancer models(39). In other hand, MSCs tumor tropism can be employed for oncolytic viruses delivery to tumor

locations. Some studies described the positive anti-tumor effects of oncolytic viruses delivered by MSCs when they have been injected to tumor sites in animals (43, 44) .

4.2. MSCs in neurodegenerative disease

Stem cell-based therapy is considered a novel approach for neurodegenerative diseases. In spite of a small number of stem cells with limited regeneration capacity within some special areas such as subventricular zone (SVZ) of the lateral ventricle wall and the subgranular zone of the hippocampal dentate gyrus, neural stem cells are not so appropriate for neurodegenerative diseases treatment regarding their less accessibility.(45). However, neural stem cells are not easy accessible cells for neurodegenerative diseases treatment. Previously, several studies defined MSCs regeneration potential, differentiation abilities into all mature neural cell types and their easily isolation and expansion. MSCs have differentiation ability into all mature neural cell types. Especially, MSCs obtain new morphological properties, specific neural markers, and electrophysiological properties in neural progenitor maintenance medium which represent of neural differentiation (46). MSCs limit CNS tissue damages in animal models or have a mediator role in CNS tissue repair via replacement of damaged cell by means of differentiation or trans-differentiation through their paracrine functions. Many studies disclosed that secretion of protective factors from the injection site may provide therapeutic effect of MSCs by several classes of molecules including growth factors, anti-inflammatory and immunomodulatory chemokines of transplanted cells (47). Therefore, the stem cell replacement therapies investigations for neurodegenerative diseases treatment such as Alzheimer disease (AD), Parkinson disease (PD), amyotrophic lateral sclerosis (ALS), and multiple sclerosis (MS) became developing interests in regenerative medicine. In Alzheimer, as the most common reasons of dementia, losing various types of neuronal lineage cells occur in response to the production of amyloid-β peptide (Aβ) plaques and neurofibrilary tangles in different parts of the brain which finally result in cognitive impairment and loss of memory. Since available drugs do not have any significant effect on this disease, finding the new treatments is a crucial need. The authors found out that brain-derived neurotrophic factor increases synapse density and improving AD cognition through its important impact upon neuron outgrowth and synapse formation.(48). In addition, scientists have observed a neuroprotective effect in hemopoietic growth factors such as granulocyte colony-stimulating factor (G-CSF), erythropoietin (EPO), granulocyte-macrophage colony-stimulating factor (GM-CSF), stem cell factor (SCF), vascular endothelial growth factor (VEGF), and stromal cell-derived factor-1-alpha (SDF-1-alpha) in ischemic stroke (49, 50). Lack of data in humans is the main problem appraising growth factors effect on neurodegenerative disease treatments, even though there are some promising information earned from animal model. Some hopeful results of MSCs therapy in patients with ischemic stroke have been reported by clinical trials which may explain their effect on increasing growth factors in AD patients in the future.(51). MSCs might also be considered a new therapeutic method for ALS that can provide or return function of motor neurons. It has been published that transplantation of MSCs resulting in their survival and migration towards lumbar spinal cord of SOD1G93A mice, astrogliosis inhibition, suppression of microglial activation and ALS-related decrease in the number of motor neurons, so cause to an amelioration of motor performance (52).

Parkinson is the second most common neurodegenerative disease which accompanies with loss of dopaminergic neurons in substantia nigra, and motor functions. Although some palliative treatments are used as the current treatments for this disease, the MSCs have also been proposed for protecting neural cell types. These treatments were used as a new way for PD treatment in a PD mouse model and it was shown that dopaminergic neurons and tyrosine hydroxlase-positive cells will survive after MSCs transplantation. (53). The morphology and differentiation of MSCs derived from PD patients are similar to normal MSCs. In addition, PD-derived MSCs can differentiate with certain stimuli into dopamine cells. Also, PD-derived MSCs could suppress T cells proliferation which introduce MSCs as a promising cell for cell therapy (54). Pre-clinical experiments have been proposed that MSCs show also an effective therapy in animal models of myelin disease, such as multiple sclerosis, where MSCs might help to re-myelination and myelin recovery. Autologous naive BM-MSCs transplanted into PD in a pilot human study which followed for up to 36 months without any tumor formation evidences or side effects (45). Clinical studies of MSCs are a young field and there is a strong requirement to gather information related transplantation of MSCs and its efficacy.

4.3. MSCs in cardiovascular disease

Cardiovascular diseases (CVD) are considered as a serious problem which cause for one of the most leading mortality and morbidity worldwide. Despite the major improvement in cardiovascular diseases management it has failed to avoid myocardial scar formation and replacement of damaged cardiomyocyte mass with functional contractile cardiomyocyte. In the last decade, stem cell therapy for heart diseases has introduced as an alternative option for regeneration of lost cardiomyocytes and vascular endothelium in regenerative medicine. MSCs have been proven to be the most beneficial stem cells and their therapeutic applications for MI treatment is rising in comparison with other stem cells (55). Their outstanding characteristics including the ease of isolation from different tissues, possibility of their large-scale expansion, their differentiation ability into cardiomyocyte and vascular cells, their capability of homing towards the inflammation sites, their immunologically tolerance in recipient, the possibility of their systemically IV delivery without cardiac catheterization laboratories and the opportunity of using genetically modified MSCs to improve their engraftment or differentiation potentials make them a popular cell source (56) . It's revealed that MSCs express specific cardiac genes and show cardiomyocyte morphology and contractile activity on the exposure of 5-azacytidine. (24) . MSCs reduce the infract size and scar formation when implanted in an infracted heart and trigger myocyte regeneration. They exert their function via induction of vascular repair, angiogenesis, secretion of chemokines and ultimately stem cells homing. BM cells were administered for the first time for cardiomyoplasty in 1999 by Tomita et al. The autologous BM-MSCs injected intramyocardialy 3 weeks after cryoinjury. This cells home to injured sites and expressed muscle-specific proteins (57). Thereafter, various preclinical researches has been demonstrated left ventricular (LV) function improvement, reduction in infract size and mortality after MSCs transplantation in animal models such as mice, rats, swine, canine and sheep in acute or chronic MI (58). Schuleri et al designed a similar study through

intramyocardial MSC transplantation that showed LV function was increased. This result derived from improvement in myocardial blood flow and vessel size. The delivery methods and safety optimization are vital challenges of MSCs therapy in heart disorders. The majority of researchers have found that intramyocardial injection of progenitor cells in large animal models are safe and possible (59). On the other hand, dose relate investigations have found no directly results to date (60). furthermore, intramyocardial injections of high doses of allogenic MSCs have been displayed their safety in a swine (61) and in another study reported that intravenous injection of MSCs altered the myocardium electrophysiological properties (62). Although numerous studies have proven the beneficial effects of MSCs in heart regeneration, a number of publications have increased worries about tumor formation after BM-MSCs transplantation. The calcification and ossification is another serious concern of using MSCs and BM-MSCstem cells (63, 64). In contrast to these results, multi preclinical studies demonstrated their safety without any sign of tumor formation in large-animal models (65). According to this pre-clinical observation, some clinical trials have been done for heart injury treatment in patients. Some trials applied autologous bone marrow-derived mononuclear cells for MI treatment and reported that indicators of cardiac function have enhanced (66-68).

4.4. MSCs in Respiratory diseases

One of the serious health problems is lung diseases which lead to a high rate of mortality around the world. Recently, treatment of lung diseases including chronic lung diseases, acute respiratory distress syndrome (ARDS), idiopathic pulmonary fibrosis (IPF) and chronic obstructive pulmonary disorder (COPD) faced obstacles and that there is no treatment option except lung transplantation (69). However, the rejection and immunosuppressive side effects remained as major problems of transplantation (70). The obstructive diseases avoid normal breathing by the fibrosis formation, airways or parenchyma inflammation and increase the resistance of airflow and compliance reduction. Also, chronic injuries disrupt the healing mechanisms lead to scar formation, fibrosis and loss of lung function (71, 72). The present treatment strategies use current drugs, mechanical ventilation and transplantation for lung disease. In addition, scientists recently have focused on cell therapy as a new critical therapeutic option. Stem-cell based therapy is a promising substitution of transplantation for tissues or organ repair. MSCs have abilities to regulate immune systems, secrete some soluble factors such as growth factors and chemokines which allow them to play a key role in the decrease of inflammation, improving tissue repair and avoiding fibrosis through interactions with stromal and immune systems cells at in the damaged sites of the lung. The curative current treatment for acute lung diseases such as ARDS employs traditional fluid strategy (73) and ventilation protection methods but can't improve their mortality. Some investigations have been designed MSCs effectiveness on the endotoxin-induced ARDS animal models (74). These studies proved that MSCs transplantation increases the survival rates through suppressing secretion of inflammatory cytokines such as IL-1β, TNF-α, and IL-6 (75, 76) and anti-inflammatory cytokines rising. Moreover, clinical trial results display MSCs therapy could decrease the C-reactive protein level in COPD patients. Emphysema is a

hallmark of chronic pulmonary obstructive disease which induced by vascular endothelial growth factor (VEGF) inactivation and also proinflammatory cytokines growing up (77). Thus, MSCs injection can be a great approach to treat chronic lung disease. Efficacy of MSCs therapy in IPF patients advanced its clinical trial phase, with multi incomplete trials that registered in "www.clinicaltrials.gov" and one completed trials in the phase I. Numerous animal models experiments have shown the MSC beneficial effects in the various lung disease that suggest they may have a good effects on Brochiolitis obliterans (BO) treatment. Also, their positive effects have been revealed to avoid rejection in heterotopic tracheal transplantation models. The excellent effects of stem cell therapy in animal models develop their application in human clinical trials as a novel treatment in different lung diseases (78, 79).

5. Limitation of stem cell therapy

Regenerative medicine is using MSCs to treat a lot of disorders because of their great capabilities such as homing to injured and inflamed tissues, multi-lineage differentiation, and immunomodulatory effects on immune systems and even targeted-tumor therapy. There are some elusive problems which should be answered about the migration mechanisms of MSCs. It's understood that chemokines – chemokine receptors and MSCs adhesion molecules can improve the therapeutic strategies of ex-vivo expanded MSCs. Although, the safety and effectiveness of MSC therapy has been shown but still many aspects of their action mechanisms is not understood. However, there is an essential need to design studies of engraftment, homing and in vivo differentiation mechanisms and their long-term safety evaluation. MSC therapy makes some concerning challenges including their immune-related rejection, genetic instability, survival limitation and function restriction (82). Besides, these problems, tumor formation are a major issue of MSCs therapy in clinical applications. The standard methods for ex-vivo expansion of MSCs, large-scale production, storage and distribution should be validated. The clinical-grade expansion needs a consistent expansion in vitro, which can increase risks of genetic instability and production of transformed cells (83). It's investigated that upregulation of c-myc, expression of p-16 and telomerase activity may involve in MSCs transformation. Furthermore, genetic manipulation of MSCs raises their oncogenic potential because the transgene can disrupt the genome stability which has been demonstrated in some tumors such as gastric adenocarcinoma (84), lipoma (85) and osteosarcoma (86). In addition, novel-engineered tools for cell delivery to specific tissue should be produced such as cell-covered stents and catheter-based delivery in heart disease. In the light of these results, clinical MSCs should be exactly explored to make clear its cautionary challenges.

6. MSCs release extracellular vesicles as a novel approach of cell free therapeutics

MSCs are believed to exhibit therapeutic impact through a different mechanism of actions in response to occurring tissue damage. They could be activated and also recruited to the site of injury by signal sensing due to their widespread perivascular niche. New studies demonstrate that MSCs could be contributed in regeneration via exhibition of two crucial function

"replacing of diseased cells" by differentiation to resident target progenitor cells or more recently "cell empowerment" something beyond cell to cell contact (80). Despite of low cell retention time within a few hours and also poor engraftment in target organ, satisfactory therapeutic results have been observed after cell transplantation in cell-based therapies (81-84). Many investigators attribute therapeutic effect of MSCs mostly to their paracrine effect, particularly, through the secretion of extracellular vesicles (EVs) as medicinal cell-free products (85-96). Interestingly, uncovering the latest function of MSCs led to exploiting regenerative roles of MSC-derived EVs in regenerative medicine. Due to EV-carrying bioactive cargoes including nucleic acids, proteins, metabolites and lipids, by exchanging biological properties between cells, they could be involved in numerous normal physiological and also pathological conditions (97-100). Furthermore, EVs generated from MSCs have been reported as a targeted delivery vehicle through transferring of exogenous biological and chemical molecules in gene and cell-based therapeutics (85, 86). Recently, studies shows that EVs generated from MSCs could recapitulate their parent cell pleiotropic functions such as cell proliferation (101), anti-inflammatory and immunomudulatory (102, 103), anti-fibrosis (86) and also anti-apoptosis (89) in preclinical studies and also translation to EV-based clinical trials (92) in a wide range of diseases.

6.1. Extracellular vesicles: Exosomes and microvesicles

Extracellular vesicles were firstly discovered during maturation of sheep reticulocytes (104). These nano-sized bilayer membranous vesicles are produced and secreted from various cell types, in particular, MSCs (100, 105-107). Recently, the interest in EV research has been exponentially raised and most of the efforts have been paid on understanding the beneficial function of MSC-derived EVs of other cell types. In order to harmonization and clarification of vesicle nomenclature and terminology, the International Society for Extracellular Vesicles (ISEV) in 2011 suggested "Extracellular vesicle" (EV) as a general term for all membranous vesicles which are released to the surroundings. Extracellular vesicles are categorized to exosome (EX), microvesicle (MV) and apoptotic body based on their biogenesis pathways. Exosomes and microvesicles have been described two important subpopulation of EVs which most of researches have focused on their origin/biogenesis, size distribution, component and biological function. Exosomes comprise small membranous vesicles and they range 30 to 120 nanometers in size, whereas, microvesicles are partially larger vesicles and their size range between 50 nanometers to 1 micron.

Exosomes are regarded as homogenous population of vesicles generate by inward budding of the late endosome or multivesicular body (MVB) through the endosomal sorting complex required for transport (ESCRT)-dependent or independent mechanism. Some other proteins such as programmed cell death 6 interacting protein (PDCD6IP; also known as ALIX) and tumour susceptibility gene 101 protein (TSG101) are also involved in exosome biogenesis (108-110). They are subsequently secreted post-fusion to the cell membrane which mostly depends on activity of several Rab GTPase proteins (111).

Moreover, microvesicles and apoptotic bodies have been described heterogeneous EV population in size range. They are directly released via budding outside of the cellular membrane by regulatory proteins such as ADP-ribosylation factor 6 (ARF6) (112, 113).

6.2. EV Components and biological functions

Extracellular vesicles that are released in cellular microenvironment could influence on local adjacent cells or even distant cells systematically. EVs contain a complex set of molecules (proteins, mRNAs, miRNAs, DNAs and lipids) depending on their cell of origin, therefore, they contribute to cellular and biological processes such as self-renewal, differentiation, migration, extracellular remodeling, reduction of oxidative stress, angiogenesis and inflammation. Moreover, significant regenerative impact of MSC-derived EVs have been reported in myocardial ischemia (88), acute liver failure (ALF) (86, 114), acute kidney injury (AKI) (89), neurological disorders (115), wound healing (116) and graft versus host disease (GvHD) (92) owing to vesicle content. Therefore, investigation of critical molecules of EV cargos may provide new insight to EV-mediated beneficial mechanism. High-throughput mass spectrometry-based analysis of proteins revealed some surface receptors (CD105, CD73, CD29, CD81 and CD44), signaling molecules (which are involved in controlling of TGF-β, BMP, MAPK and PPAR recipient cell signaling pathways), adhesion molecules and MSC-associated markers which may account for therapeutic potential of MSC-derived EVs (117, 118). In addition, another study showed that MSC-derived MVs highly expressed angiogenesis and migration associated cytokines and ckemokines. For example, enhanced expression of Interleukine 6 (IL-6), vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF) as proangiogenic proteins and monocyte chemokine protein-1 (MCP-1) as migration-promoting chemokine to inflammation were determined in MSC-derived MVs content using immunoblot analysis (119). Recently, high-resolution analysis of MSC-derived EX/MV demonstrated that EX and MV could be distinguished in the context of proteomics. Most common pathways such as immune response, heparin binding and integrins were enriched in EX, however, mitochondrial, proteasomal and endosomal reticulum-associated proteins were enriched in MV (120). Interestingly, the result showed c MSCs, their restricted expansion has been shown avoiding their large-scale production for clinical application (121). Subsequently, multi investigation have been explored an alternative strategy to produce MSCs. it's reported that secretion of growth factors, miRNAs, and EVs effects on the biological properties of stem cells including stem cell regeneration, differentiation potential and immunomodulatory roles (122). Interestingly, the regulatory factors secreted from condition medium of MSCs improved the kidney and myocardial damages in animal model (123). Last several decades, MSCs immunoregulatory effects such as suppression of T and B cells proliferation, inducing tolerant dendritic cells (DCs), anti-inflamatory cytokines secretion have been demonstrated (124) .Moreover, MSC-derived EVs inhibit immune reactions through reduction of inflammatory cytokines and increasing of anti-inflammatory responses in same way to MSCs but the underlying mechanisms are still not cleared (125). Recently, Fattore et al., discovered that MSC-EVs display strong immunosuppression activity as MSCs by T cell apoptosis, increase T reg population and the

release of interleukin-10 (IL- 10). Also, MSCs-derived EVs down regulate TNF-a and IL-1b that are inhibitory factors for T cells maturation (126).

6.3. EV purification, storage condition and characterization

In order to get reproducible results of extracellular vesicles in preclinical and clinical studies, obviously, attention must be paid towards standardization of methods used for sample collection, isolation and also characterization of EVs (127, 128). Therefore, to minimize non-EV contamination, ISEV suggested minimal criteria to get a high yield and pure population of EVs. 24 to 48 hours serum free or EV-depleted serum containing conditioned media has been suggested for EV collection (129).

There are several methods such as ultracentrifugation, density gradient, ultrafiltration, immuno-affinity, polymeric precipitation and microfluidics have been reported for isolation of extracellular vesicles (129). Of above mentioned methods, differential ultracentrifugation is assumed to be a gold standard procedure displaying high amounts of yield and low/medium pure EVs. However, density gradient is considered as a second commonly applied protocol generating low/medium yield and high pure EV subpopulation (130).

Based upon the limited published studies have explored yet, EVs suspended in PBS buffer should be stored at -80°C until additional experiments (128). Nevertheless, disruption and loss of function have been observed after defreezing of EVs (128). Thus, it is not clearly determined that temperatures -80 or below that could have an impact on structure and functional properties of EVs. Besides, it cannot be claimed that whether fresh or freeze-thawed sample is preferred for clinical application. It seems that further research is needed to define optimum storage condition.

Among different quantification methods of EVs, Nanosight Tracking Analysis (NTA), Dynamic Light Scattering (DLS) and also BCA assay are reported to be mostly used for vesicle size and/or amount and protein concentration, respectively. Unlike DLS technique which is able to get only particle size, both size distribution and EV number are defined by NTA (131). Additionally, Electron microscopy is used to visualize the cup-shaped sphere morphology of EVs (132). Protein expression in EVs such as Alix, Tsg101 and tetraspanins including cluster of differentiation markers (CD9, CD81 and CD63) could be determined by western blotting and flow cytometry techniques. Based upon ISEV criteria it is suggested to examine at least three of mentioned proteins in order to ensure the purity of EVs (127).

7. Exosomes as gene delivery vehicles

Extracellular vesicles such as exosomes and microvesicles are harboring of DNA, mRNA, mirRNA, siRNA, proteins and showing a potential mode of intercellular communication(133). EVs are safe and biocompatible nanoparticle delivery tools from endogenous and exogenous nucleic acid sources. EV fraction extracted from HBV-infected cells contains abundant HBV DNA with the lack of HBsAg and could be transmitted to primary hepatocytes. This transmission by EVs is resistance to antibody neutralization and may be contributes occult hematite infection apart of HBV S gene mutants(134).

Communication between leukemic cell and normal blood cell with EV containg leukemic DNA increase malignancy and novel therapeutic targets to suppress tumor progressions(135). exoDNA is an important DNA appear in the circulation in early stage of cancer, and could be serve as a biomarkers. Intrestingly, circulating cell-free DNA is obtained from apoptosis and necrosis of tumor cells, which are belong to later stage malignancies(136). In glioblastoma multiform (GBM), EV secreted from tumor cells can cross from blood brain barrier,So they could be used to detect specific molecular mutation such as IDH1G395A(137). EVs derived from cardiomyocyte have DNA and RNA that induce 333 gene profile changes including suppressive and inductive effect, could change metabolic profile in target cells (138). Exogenous dsDNA loaded to EVs by electroporation method is limited to the size of DNA. The size limitation for this method is up to 1000 bp, however, for transfection methods is 4.5 -10 kbp. Transfection method has a lower efficacy than electroporation(139). Genetically modified cells that produce therapeutic EVs like prodrug-activating enzymes are assumed novel cancer vaccines for tumor cell. Mizrak et al. used MVs that express gene mRNA and protein–cytosine deaminase join to uracil phosphoribosyl transferase for the treatment of schwannomas in mouse models. This enzyme converts pro-drug (5-fluorocytosine (5-FC)) to 5-fluorouracil (5-FU) as an effective anti-cancer drug (140). Y RNA fragments as a non-coding RNA in high level in EVs, have an anti-inflammatory by inducing of IL-10 expression and cardioprotection effect(141). siRNA loaded in EVs by transfection methods can be used for the silencing of Huntingtin gene and protein. Glyceraldehyde 3-phosphate dehydrogenase(GAPDH) siRNA and (beta-secretase 1) BACE1 siRNA loaded into engineered dendritic cell-derived EVs delivery was exhibited the strong mRNA (60%) and protein (62%) knockdown of BACE1 and had therapeutic effect in Alzheimer ‘disease, in animal models(142, 143). MicroRNAs (miRNAs) are small conserved RNA molecules can decrease protein levels by degrading the mRNA or stopping the translation of the target gene. Exosomes deliver miRNAs to target cells, which reduces the levels of miRNA-target genes. MVs containing miR-29a/c significantly decreased the growth rate of the vasculature and tumors in gastric carcinoma(144). EVs derived from fibroblasts transfected with miR-195 led to decrease the size of cancer tumors, and improve survival of treated animals(145).

Modified and unmodified EVs have been exploited to achieve therapeutic effects. In modified cases, EVs are isolated from engineered cells and to be containing a specific mirRNA or siRNA that suppress specific mRNA, reduce inflammation or inhibit tumor growth. miR-124 and miR-145 have been transfected into MSCs, and these engineered MSCs released EVs containing miR-124 or miR-145, which induced the differentiation of neural cells in pathological conditions(146). EV- Let-7 targeted to EGFR-expressing cells provide a platform for clinical use of miRNA molecules to deliverantitumor miRNA to cancer tissues in vivo (147). In addition, promising results employing EVs as nucleic acid vectors to attenuating of HCV replication and expression of its receptor CD81, control obesity and immune responses have also been reported (148-150) (table 1).

8. MSC EVs as drug delivery vehicles

Drug delivery system (DDS) is a key technology to achieve effective and safe medication, since without DDS, therapeutic agents can easily distribute throughout the body and affect non-disease sites (151). An exciting and promising novel approach for efficient drug delivery is EVs. EVs can be used to deliver small pharmaceutical or biological molecules and pass major biological barriers such as the blood–brain barrier (BBB). At present, the well-known and ideal cell that has a scalable capacity to produce massive amounts of EVs is MSC. The use of EVs as new natural vesicles and ideal non-cell-based treatment strategy as delivery vehicles are being grown. Research has shown that MSC EVs as an ideal delivery vehicles are benign and non-immunogenic that unlike another cell sources of EVs such as dendritic cells (DCs) would not elicit immune rejection response or adverse effect when used as drug delivery vehicles (9).

8. Advantages of EVs relative to other synthetic vesicles

EVs have many characteristics of an ideal delivery vehicle and can be as favorable alternatives to liposomes and other synthetic vesicles as drug delivery vehicles. Unlike synthetic vesicles, EVs have the distinct advantage, firstly, their natural origins that have protein and genetic materials (such as mRNA and miRNA) in their constructions which suggest EVs could be loaded by such biological materials and make them promising drug carriers, secondly, EVs are well tolerated and less toxic in the body as evidenced by their ubiquitous distribution in biological fluids such as blood, saliva, urine, cerebrospinal fluid, amniotic fluid, bile, and breast milk and thirdly, EVs have long half-life in circulation and a notable intrinsic capability to home to target tissues. MSC EVs-mediated drug delivery is likely to be biocompatible and would not elicit immune rejection response or adverse effect (9).

9.1. Exosomes loading (in vitro- in vivo)

Extensive research has been carried out using exosomes as vehicles for drug delivery. Efficient delivery of substantial number of therapeutic cargo from EVs highly depends on a successful method of their loading (152). Several strategies can be utilized for loading therapeutically active cargo molecules into extracellular vesicles (EVs): (A) loading EV-producing cells with a therapeutic agent or in vivo drug loading, which is then released in EVs, recently researchers have shown that MSCs are able to uptake and then release entrapped drugs and suggested that MSCs are a promising factory to develop drugs with a higher cell-target specificity (Stem cell-extracellular vesicles as drug delivery systems: New frontiers for silk/curcumin nanoparticles (153), (154), (B) transfecting/infecting EV-producing cells with DNA encoding therapeutically active compounds which are then released in EVs; it was reported that miR-122- transfected adipose-derived MSC is able to effectively package miR-122 into secreted exosomes and increase chemosensitivity of hepatocellular carcinoma through alteration of miR-122-target gene expression (155), and(C) In vitro drug loading refers to drugs loading into naïve EVs. Different procedures for drug incorporation in EVs were proposed including incubation, freeze/thaw cycles, electroporation, sonication, extrusion, and permeabilization with saponin (Figure 1). Doxorubicin and paclitaxel as two potent chemotherapeutic agents were loaded into EVs released by macrophages in 3 ways including incubation at room temperature (RT),

electroporation, and sonication (156). In 2015, Haney et al. applied sonication to EVs to load catalase and demonstrated a significant loading enhancement compared to permeabilization with saponin, freeze-thaw cycles, sonication, and extrusion (157). EVs act as messengers and interact with target cells to induce changes in their physiology. Some studies have shown the interaction of EVs with recipient cells by suitable techniques, such as flow cytometry, fluorescence microscopy. EVs can be interacted by target cells through mechanisms including ligand/receptor pairs and fusion with the plasma membrane, phagocytosis, endocytosis or micropinocytosis. For instance, LFA-1, a specific ligand on DCs can capture ICAM-1-bearing DC-EVs. In some cases, induction of physiological changes occurs after binding of EVs to recipient cells, for instance during the presentation of MHC–peptide complexes to primed T cells or NKG2D ligands to NK cells. In other cases, transferring of content of EVs inside the recipient cell induces physiological changes. It depends on the recipient cells, phagocytosis in macrophages, receptor-mediated endocytosis in DCs, macropinocytosis in microglia, lipid raft–dependent endocytosis in endothelial and tumor cells(157) (Figure 1).

10. EVs rout of administration models

Since understanding of communication between cells by EV and details of their biogenesis and release lead to improved diagnosis, early detection and novel therapeutic possibilities in some diseases. EVs can be used for delivery of drugs and genes as described previously. EVs can be administrated in intravenous injection, Subcutaneous/intravenous, intranasal and in situ injection and followed in vivo (158). For instance, administration of exosome contained supper paramagnetic iron oxide nanoparticles into the footpad resulted in EVs localization into lymph nodes with magnetic resonance tracking (159). The intravenously injected exosomes loaded with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) siRNA for specific knockdown of the GAPDH gene and delivering the APP cleaving enzyme (BACE1) siRNA, down regulation of the BACE1 protein in the brain was achieved in mouse model of Alzimers disease (143). In another research intranasal administration route of curcumin-loaded exosomes from different cell types were capable for transversing of the blood brain barrier (BBB) and selectively taken up by microglial cells and induce apoptosis. In result exosomes inhibit brain inflammation and autoimmune responses in a model of experimental autoimmune encephalomyelitis and rapidly showed effectiveness. In this experiment, exosomes represented a safe and efficient delivery tool by crossing the BBB noninvasively and without being immunogenic (160). Moreover, intravenous administration of MSCs-derived EVs significantly improved functional recovery, rescued pattern separation and spatial learning impairments, promoted neurovascular remodeling (angiogenesis and neurogenesis) and reduced neuro inflammation in TBI animal models (161). Systemic administration of cell-free MSC-generated exosomes from tail route improves functional recovery and enhances neuron remodeling, neurogenesis, and angiogenesis. Based on results, axonal density and synaptophysin-positive areas were significantly increased along the ischemic boundary zone of the cortex and striatum in stroke rats treated with exosomes as EVs compared with PBS control as data shown with histopathology and immunohistochemistry (162). However, recent evidences, obtained following miR-155

loaded EVs expression, suggest that, in the blood, EVs are detectable as early as 5 min after intravenous administration with subsequent distribution in the liver, adipose tissue, lung, muscle and kidney, then decrease to less than 50% during 30 minutes, and become undetectable after 4 hours (163), so it is crucial to use proper and accurate route for administration , biodistribution, trafficking analysis of exogenously administered MSCs-derived EVs to receive more effective therapeutic response.

11. EVs as a tool for diagnosis and therapy

EVs carry nucleic acids and proteins from their host cells that are indicative of pathophysiological conditions of host cell. EVs have been recently involved in intercellular communication and in a variety of biological processes such as modulation of immune response, signal transduction, transport of genetic materials. They can be isolated from body fluids such as plasma, urea, blood, saliva, breast milk (164). EVs are widely considered to be important tool for biomarker discovery in early detection and also therapy of various diseases such as cancer and neurodegenerative diseases. Characterization of biological parameters such as protein, nucleic acid or even CD markers and lipid composition seriously are crucial for EVs potential applications. Early diagnosis of many cancers will not be possible. Therefore, it is of outstanding interest to look in more detail to the EV cargo (i.e. proteins, miRNA, mRNA) as they provide tools to monitor the status of the relevant producer cell. In this respect, the exploitation of comparative omic studies is fundamental for the detection of new biomarkers. For this reason, in proteomic and lipidomic analysis could significantly appliance the use EVs for theranostic (the combination of both therapeutic and diagnostic) approach (165). Expression level can be evaluated for diagnostic traits in compare of healthy conditions in various human diseases such as cancer or targeted for therapeutic application (166, 167). These nanoparticles can indicate the medical state of patients because of correlation between parent cell and biomarkers. For instance, it was revealed that panels of eight EV-associated proteins were up regulated in the urine of patients with bladder cancer compared to healthy subjects (168). Likewise, miRNA profiling of plasma-derived EVs identified a panel of four tumor-specific miRNAs of potential use in a screening test for lung carcinoma (169). MSC-EV can mimic the effects of their parental cell. Compared to cells, MSC-EV is very smaller and has lower possibility of rejection. For example based on Lau et al. in 2013 indicated that exosomes isolated from saliva may provide is criminatory biomarkers for pancreatic cancer (170). Also, Elements released by malignant cells such as oncogenic proteins and miRNAs, which can traverse via EVs to the tumor microenvironment and transfer of oncogenic activity to non-malignant cells. For example, mRNA and miRNA arrays have been employed to identify mRNAs and miRNAs associated with melanoma progression and metastasis in extracellular vesicles derived from melanoma. Identified RNA biomarker candidates are validated using quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR) (171). MSC-EVs could recaptulate the effects of MSCs in tumor therapies.Therefore; MSC-EVs are as alternative strategy that could overcome the limitations of cell-therapy approaches. In addition, due to their small size and transfection efficiency, EVs can act as proper nano vehicle for delivery of for example cancer or neurodegenerative disease drugs. These lipid vesicles could be engineered to deliver

therapeutic agents to target sites. For instance, it has been reported that the EVs secreted by SR4987 cells as mesenchymal cell model primed with paclitaxel (SR4987PTX) delivered active drugs and inhibited human pancreatic adenocarcinoma cells proliferation in a dose-dependent manner. This experiment demonstrated that MSCs are able to package and deliver active drugs through their micro vesicles, suggested the possibility of using MSCs as a factory to develop drugs with a higher cell-target specificity (154). Currently some database are available for recording diagnostic result of EVs content for various disease, i.e., Vesiclepedia (www.microvesicles.org), EVpedia (www.evpedia.info) and ExoCarta (www.exocarta.org) (172).

Moreover, EVs have also the potential to serve as a noninvasive intervention for successful delivery of therapeutic agents such as drugs and siRNAs to the brain. Immunotherapy represents one of the most investigated aspects in EV-mediated therapy. It is also well determined that most of the mis-folded proteins associated with neurodegeneration such as superoxide dismutase, α-synuclein, amyloid and tau - involved in amyotrophic lateral sclerosis (ALS), Parkinson's disease, Alzheimer's disease and tauopathies, respectively, are carried via EVs, which can be considered as novel biomarkers for neurodegenerative diseases and interpret such neuronal EV (nsEV) analyses to neural clinical diagnostic applications and drug development. (158, 173). Although exosomes do not replicate as an independent entity, but they can facilitate movement and spread of infection (bacterial or viral) through blood and tissues and could cause the effect in gene expression in the recipient cells through the classical toll-like receptor and NFκB pathway. In this regard EVs that can target certain tissue types and can protect them from the immune system are more preferred than liposomes, as they also have a longer half-life. So, EVs can be utilized for diagnosis or therapeutic aspects of infectious disease particularly in the pathogenesis of HIV to prevent from transmitting of infection (174). It has been shown that the level of EVs circulating in blood is significantly elevated in cancer patients. EVs vesicles purified from urine could be used for monitoring of the status of prostate cancer patients, because they contain elevated levels of 5T4 oncofetal trophoblast glycoprotein, prostate-specific antigen, and prostate-specific membrane antigen, relative to normal urinary EVs (175). Based on mRNA expression level analysis for diagnosis of cancer, extracellular vesicles purified from urine of prostate cancer patients, show increased expression of prostate cancer antigen-3 and transmembrane protease serine 2-transcriptional regulator ERG gene fusion (176). Furthermore, gastric cancer, glioblastoma, and lung cancer may also be detected using EVs, which show elevated expression of several cancer biomarkers including extracellular matrix metalloproteinase inducer, hepatocyte growth factor receptor, human epidermal growth factor receptor-2, and melanoma-associated antigen-1 for gastric cancer (177), prominin-1 for glioblastoma (178), and leucine-rich a-2-glycoprotein for lung cancer (179). In addition, circulating extra cellular vesicles purified from the blood of glioblastoma patients contain epidermal growth factor receptor variant III mRNA (180). Circulating EVs purified from the blood of gastric cancer patients exhibit significantly higher levels of mRNAs encoding melanoma-associated antigen-1 and human epidermal growth factor receptor-2 (177). The level of miR-21 is increased in circulating extracellular vesicles of patients with ovarian or esophageal cancer. In addition, levels of miR-141, miR-200a, miR-200b, miR-200c, miR-203, miR-205, as well as miR-214 are increased in the circulating EVs of ovarian cancer patients (181).

However, further studies are warranted for clinical and commercial application of the use of circulating EVs for cancer diagnosis and monitoring. Experimental biomarker sources and several proteins or RNAs in EVs have been found to be useful in monitoring of wide range of disease. But it is necessary to standardize the technique of EVs manipulation for their application in therapy and long-term EVs effectiveness in the treatment of human disease.

12. Application of EVs derived from MSCs in regenerative medicine

12.1. Immune disease treatment

MSCs can manage immune systems response through cell-cell association and discharge of paracrine molecules. It's trusted that MSCs select inhibitory chemokines and stifling ligand-receptor associations to assume a noteworthy part in immunosuppression and T cells movement direction(182). In agreement to understood MSCs immunoregulatory impacts, MSCs-determined EVs including microvesicles, exosomes and apoptotic vesicles have likewise been shown immunological concealment movement by enlistment of mitigating cytokines levels and diminishing of provocative chemokines which make them a contender for immune disorders treatment(10). What's more, MSCs-derived exosomes can likewise hinder Toll-like receptor signaling and activation that identified with innate immunomodulatory activity of MSCs(183). At the point when MSCs-inferred exosomes infused into a hypoxic pneumonic hypertension demonstrate, the inflammation suppress through pro-proliferative signaling, for example, phosphorylation of STAT3(184). The various consequences of EVs immunomodulation activities make EVs awesome immunotherapeutics alternatives. The extracellular vesicles (EVs) emitted by both prokaryotic and eukaryotic cells can convey molecular patterns of pathogen-related and injuries related, cytokines, autoantigens and the catalysts identified with tissue corrupting. What's more, EVs may be connected to diagnostics and focused on treatment in the incendiary and immune diseases (185) .In view of this speculation, MSC-derived exosomes possess an induction of peripheral tolerance. The segregated Microvesicles from co-culture amongst MSC and splenic mononuclear cells could hinder autoreactive lymphocyte multiplication and arrival of interleukin (IL)- 10 and transforming growth factor (TGF)- β in autoimmune system encephalomyelitis mouse display. Likewise, MSC-derived microvesicles have been broke down by flow cytometric techniques which its outcomes have demonstrated these cell-items can be a transporter for tolerogenic molecules including PD-L1, Gal-1, and TGF-β. This trial data recommend that MSC-derived EVS are strong organizer for peripheral tolerance induction and direction of invulnerable responses, which thought a novel obstinate hopeful as opposed to utilizing MSCs in immune system sicknesses treatment(186). Powerful techniques have been examined EVs anti-inflammatory properties for Rheumatoid Arthritis (RA). The transplantation of autologous adapted serum (ACS) exosomes intra-articular seems useful impacts on RA treatment with diminished agony in joints and decreased markers of inflammation markers in the blood test. ACS-derived exosomes will be a promising and safe treatment for different sicknesses identified with the chronic inflammation or autoimmunity because of RNA and protein transfer.. MSC-MVs smothered multiplication in the receptive lymphocyte and furthermore IL-10 and TGF-b emission Correspondingly, exosmes got from MSCs were utilized as a part of lupus murine model and

were critical to ensure MSCs function.(187). As of late MSC-derived exosomes have been infused in mouse models of allogeneic skin grafting which prompt expanding Treg levels and put off GVHD for two days. In vitro explores revealed that the enactment of MYD88-subordinate motioning in monocytes gave by exosomes expanded Tregs cells (182). Clinical utilization of MSC exosomes in a patient therapy-refractory graft-versus-host disease proved their efficacy demonstrated their adequacy and 4 months following up of appearances identified with mucosa and cutaneous enhanced while immunosuppression organization diminished. Every one of the perceptions present the MSCs exosome potential for autoimmune treatment and immunotherapy however more examinations is required to assess their and advantages and disadvantages, viability and security for immunomodulatory purposes (188, 189).

12.2. Cancer therapy

Several studies have reported that EVs are potential candidates for the treatment of different tumors. Zitvogel and colleagues were the first researchers who have used tumor peptide-pulsed dendritic cells (DCs) EVs as an alternative to DC adoptive therapy against tumors. Some studies have demonstrated that MSC derived EVs have a strong tropism to tumor environment such as their cellular origin which can provide a promising therapy for cancer treatment. It has been shown that MSC derived EVs can induce cell death by apoptosis in HepG2 (hepatoma) and Kaposi's sarcoma cancer cell lines, while necrosis has been seen in ovarian cancer cell line (Skov-3) after MSC EVs treatment (190). EVs can pass the blood–brain barrier and have been demonstrated to deliver genetic materials contained within to brain. These nano-vesicles are non-viable and the risk profile of EVs is thought to be less than that of cellular therapies. The natural ability of EVs to transport RNA suggests that these particles can be useful in gene- based cancer therapy to deliver therapeutic short interfering RNA (siRNA) and miRNAs to the target cells. Shtam et al. reported that EVs can deliver RAD51- and RAD52-siRNA into fibrosarcoma cell line and induce both gene knockdown and the massive reproductive cell death (191). EVs derived from the high expression of tumor suppressor miR-146b MSCs, inhibited glioma growth in a xenograft model (192). Two other studies also showed EV-delivered tumor suppressor miRNAs, let-7a and miR-143, inhibited tumor growth of breast and prostate cancer in vivo, respectively (147), (193). Migration of osteosarcoma cell has been reduced by EV-formed miR-143 (194). Munoz et al. have reported glioblastoma was more sensitive to chemotherapy after delivering anti-miR-9 through MSC EVs (195). In 2014, Pascucci et al. showed that MSCs as a factory are able to package and deliver paclitaxel through their MVs and possessed strong anti-proliferative activity on the human pancreatic cell line (CFPAC-1) (185). EVs from dendritic cells loaded with a chemotherapy agent doxorubicin inhibited the growth of breast cancer xenograft tumors in vivo (196). MSC derived EVs can acquire strong anti-tumor activity after priming with anti-cancer agents. EV as an endogenous and promising vehicle for delivering active molecules, such as chemotherapeutic agent, growth factors and genetic molecules, specifically to the tumor microenvironment in cancer.

12.3. Neurological disease

There are strong evidences that EVs, and in particular endosome-derived exosomes contribute to homeostasis in the CNS. Neurons communicate with each other through the secretion of EVs contributing to local synaptic plasticity, but EVs may also allow longer range communication within the CNS and influence neuronal networks located at a distance. In CNS-associated diseases, EV biogenesis, transfer or composition can alter, and cause pathology (165). Altogether, the EVs contribute to the local proliferation of neurodegenerative diseases, targeting EVs or manipulating the nature of EVs as natural carriers of miRNAs and drug delivery devices. It has been investigated that EVs as biomarkers of CNS disorders as well as therapeutic agents (197). Also, targeting EVs directly to sites for inhibiting deleterious effects seems an attractive approach. Adeno-associated viruses (AAVs) encapsulated in EVs with viral capsid proteins can deliver genetic cargo into recipient cells (198). EV-mediated delivery offers multiple advantages as these vesicles are biocompatible, can be autologous (i.e. patient-derived) and appear to use as the novel weapon for the treatment of TBI according to its advantages such as nanosize, easy administration and notably ability to cross the biological barriers especially the brain barrier. In a study of EVs as a vehicle, EVs loading with siRNA by electroporation and engineered to expose a brain-specific peptide (rabies virus glycoproteinderived peptide), specific mRNA knockdown was observed throughout the brain but was minor in the liver and spleen. The therapeutic potential of EV-mediated siRNA delivery was demonstrated by protein (62%) knockdown of BACE1, a therapeutic target in Alzheimer's disease (143). In another research for therapy approach in Alzheimer's disease with an aspect of increase in antibodies against ceramide, Dinkins *et al.* administrated subcutaneous ceramide injections to increase serum anti-ceramide IgG in mice animal model and circulating exosomes and finally aid exosome-mediated clearance of Aβ (199). In neurodegenerative disorders, neurons, and in some cases astrocytes, produce and release aggregated proteins such as a-synuclein, amyloid precursor protein (APP) and phosphorylated tau and, pathogenic PrPSc protein, inprion disorders. In demyelinating disease, myelin-stressed oligodendrocytes produce altered myelin proteins and heat shock proteins (HSPs) that may hypothetically be released in EVs. The 'disease-associated' proteins activate microglia that may intense disease or alternatively affects neurons and axons leading to dysfunction. Despite the current realization that siRNA loading into EVs has although many technical difficulties need to be overcome, targeting of EVs to the brain, a major previous biological barrier, seems at least possible. In another recent study, intrastriatal injection of exosome-mediated transfer of miRNA (in particular, miR-124a) was shown to have a role in neuron to astrocyte signaling. In the research of ALS therapy, exosomes isolated from a neuron in conditioned medium which contained small RNAs and internalized into astrocytes, increased astrocyte miR-124a and GLT1 protein levels in ALS. MiR-124a is selectively reduced in the spinal cord tissue of end-stage mouse model of ALS. In addition, exogenous delivery of miR-124a in vivo through stereotaxic injection seemed to prevent further pathological loss of GLT1 proteins in animal model (200). Traumatic brain injury (TBI) is a leading cause of death and long term disability such as behavioral, cognitive and motor deficits worldwide. At present, there are mainly two therapeutic strategies to treat TBI; one is a neuroprotective and neurorestorative treatment. In first, focus on reducing or

preventing secondary injury and neural cell death and, the second is to improve neurological recovery in CNS to promote neurovascular remodeling including angiogenesis, neurogenesis, oligodendrogenesis and dendrite or axon outgrowth (162).

In the past decade, exosomes as EVs wildly have been used in research of TBI treatment. In this respect, EVs contain various miRNAs which play a key role in modifying the phenotype and/or the physiology and modulating the cellular processes of the recipient cell, and miRNAs such as miR-21 could be potential therapeutic targets for interventions after TBI (201). In another study, Katsuda et al. (2013) have provided an important role for ASCs-derived exosomes in the context of AD, as these express high levels of Neprilysin (NEP), the most important Aβ-degrading enzyme in the brain. NEP-loaded exosomes were shown to be efficiently transferred to neuroblastoma cells and led to the decrease of both extracellular and intracellular Aβ levels. In vitro neurodegeneration model, exosomes released by dental pulp-derived MSCs on a 3D culture, rescued dopaminergic neurons from 6-OHDA induced apoptosis, thereby providing a possible treatment of Parkinson's disease (202). Based on data achieved from continuous intracerebrally injection of exosomes which loaded with Aβ on surface glycosphingolipids, nanovesicles transport into microglia in AD mouse brains, result in reductions in Aβ pathology in hippocampus and provide a novel therapeutic intervention for Alzheimer disease(203). Immunomodulation of neurological disorders by use of EV-mediated is in primary steps but important in application of EVs in therapeutic approach. and effective. For instance, use of EV derived from modified dendritic cells secreting TGF-β1 to inhibit the progression of murine experimental autoimmune encephalomyelitis (204). Lopez-Verrilli et al. (2016) have recently unraveled the potential of menstrual MSCs (MenSCs) exosomal-enriched fraction as therapeutic approach in neurodegenerative pathologies. Because MenSC exosomes induced neurite growth in cortical neurons and had a similar effect to BM-MSC exosomes on neurite outgrowth of dorsal root ganglia neurons (205). EV protein therapy as a novel strategy for delivery of catalase in Parkinson's disease to reduce oxidative stress and thus help neurons to survive is another application of EV, as prodrug actives enzymes which can convert a prodrug which crosses the blood–brain barrier into a toxic chemotherapeutic drug for schwannomas and gliomas, and the apoptosis-inducing enzyme, caspase-1 under a Schwann cell specific promoter for schwannoma (206). In the pilot immunotherapy trial for recurrent malignant gliomas, autologous exosomes isolated from tumor cells which removed by surgical craniotomy. The isolated cells, treated with an investigational new drug (an antisense molecule), that target surface receptor protein, are re-implanted (encapsulated in a small diffusion chamber) in the abdomen of the patient. Tumor cells treated with the antisense molecules, to apoptosis. Released exosomes are full of tumor antigens that together with the antisense molecules and could stimulate the immune system against tumor recurrence. The patient of this trial is currently processing. (NCT02507583) (www.clinicaltrial.gov). EVs study and analysis is an interesting target for the potential detection of new therapeutics in regenerative medicine. This potential needs to further research in EVs field, as well the study of novel techniques to produce and develop engineered EV.

12.4. Cardiovascular disease

Numerous preclinical and clinical studies within the past few years have demonstrated that cardiac stem cell-based therapy could be considered as a new and safe therapeutic procedure in heart disease. Among different stem cells that have shown beneficial effects MSCs are the most promising cells. The accessible and safety of allograft transplantation of these cells make them the most popular stem cells in cardiovascular disease therapy.(225) There have been several assumptions regarding mechanisms of MSC related cardiac repair, including transdifferentiation and cell fusion. However, the magnitude of overall physiological impact seems higher than just structural contribution of these transplanted cells. Therefore it is claimed that other components secreted by these cells may contribute to the significant therapeutic effect induced by these cells (226,227). These assumptions were further confirmed by infarct size reduction and cardiac function improvement in animal models after injection of CM of MSC and also by injection of vesicular components of the CM(228,229). These findings concurred with new discoveries in 2005 demonstrating that the extracellular vesicles derived from platelet are able to induce revascularization in chronic cardiac ischemia via VEGF secretion stimulation (230). 10 years later published studies showed that extra cellular components derived from plasma trigger cardio protective reactions in ischemia-reperfusion through induction of TLR4 (Toll-like receptor 4) (231). These findings encouraged scientists to discover and empower the components of these paracrine factors in order to recapitulate the ability of these components to develop cell-free transplant systems and hopefully to overcome the problems associated with cellular therapy. Further studies indicates that exosomes derived from cardiac progenitor cells mollify cardiomyocyte death in a murine model of ischemic-reperfusion (229,232) Besides, cardiospher-derived cells release exosomes that exert the same regenerative effect as their cells of origin in left ventricular dysfunction(233,234). Likewise, EVs from human embryonic stem cell driven cardiovascular progenitor cells improve cardiac function in post infarct heart failure model of murine like their cell counterparts (235). Not only are intact EVs considered as an alternative to cellular therapy but also the desirable designed or favorably loaded extracellular vesicles are going to be more deeply studied and examined. The assumption is that they may weather be designed to target the specific cells or used as a cargo to deliver the drugs of interest to the particular cells. For instance it has been shown that miR-126 carrying vesicles decrease atherosclerotic plaque formation in mice (236). Furthermore, miR-126 vesicles induce angiogenesis after vascular injury (237). Besides, sonic hedgehog overexpressing exosome derived from $CD34^{+}$ improve cardiac function (238). Likewise, sonic hedgehog overexpressing exosome derived from T-lymphocyte induce neoangiogenesis by NO synthesis pathway stimulation (239). Although such studies are scarce particularly in cardiovascular disease the results are promising and it resting seems that EVs whether as therapeutic vectors or as therapeutic agents are a step forwards in cardiac cell therapy.

12.5. Kidney injury treatment

EVs secret by numerous cell types into the extracellular space and extensively present circulate with our body in fluids and carry some regulatory signals such as mRNA, miRNA, proteins, and molecules of signaling. Increasing amounts of observations suggest that the

extracellular vesicles have a key role in the cell-to cell signaling, normal development and various pathological developments (207). This makes them as an intriguing diagnostic tools and an effective treatment strategy for drug delivery system, cell-free vaccines. The recently data propose that urinary EXs incorporated in mediationg cell-to-cell communication of nephrons and transfer of functional molecules such as aquaporin-2 between cells (208). The accessible information suggest, EXs also play a key role in kidney development due to Wnt proteins and beta-catenin delivery which are intense mediator of kidney developmentm. While the kidney-Exosomes impacts on the ordinary procedures of kidney are not completely illustrated, huge parts of EXs have been appeared in the different kidney disease improvement. For example, MSCs or Endothelial derived vesicles improved kidney regeneration. Biancone et al distributed that the beneficial influences of MSCs on both acute and chronic kidney injury was because of their paracrine effects. . Different examinations have been endeavored to research their paracrine impacts through MSC-derived EXs. All things considered, EXs of MSCs promoted the injured-kidney epithelium of rat by the suppression of apoptosis, increase of tubular epithelial cell proliferation (209-211). Different investigations have detailed the anti-apoptotic genes up-regulation and apoptotic genes down-regulation via a mechanism related to RNA transfer. It has likewise been exhibited the effective impacts of bone marrow and umbilical-derived EXs on cisplatin-induced nephrotoxicity. These EXs induced renal epithelium proliferation and also, reduced Bax level with increase Bcl-2 level in order to decrease apoptosis (89, 207). Another intriguing research area is kidney tumor EXs that play a key role in tumorigenesis. One experiment shown that in a SCID (immunocompromised severe combined immunodeficient) mouse model Renal carcinoma stem cells (rCSCs) derived- vesicles promote lung angiogenesis and metastasis(212). Also, EXs potential as diagnostic tools for the kidney malfunction monitoring has been shown. It's established, the urinary EX components linked to specific nephron parts, thus many of these components can be related a certain disease (213). The elevated GPRC5B or Fetuin-A, ATF3 amount in the kidney-derived EXs associated with AKI disease which be known as AKI diagnostic marker. Some markers such as Promonin-1 (CD133) found in healthy people which is lost in the end stage of kidney disease. Also, protein and RNA examination of urinary EXs demonstrate a non-obtrusive technique to diagnose several kidney diseases.In addition, protein and RNA analysis of urinary EXs prove a non-invasive method to diagnose several renal diseases. All things considered, miR-145 and miR-130a levels have been expanded in patients-bearing diabetic nephropathy, while miR-155 and miR-424 sums were diminished. It is realized that EXs include in kidney maladies, cancer and even even typical improvement of kidney. In any case, many works ought to be done to illustrate the physiological mechanisms of EXs by characterized disease mode (214-216) .

12.6. Respiratory diseases

MSC derived secretome generally utilizing as a part of preclinical analyses and various outcomes has been distributed that show they can be a novel cell-free strategies for lung disease treatment. The amazingly expansive measure of studies showed that MSCs therapeutics impacts emerge from dissolvable elements discharge. keratinocyten growth

factor (KGF) and angiopoietin-1 derived MSC support the epithelium of alveolar and endothelium against injury in acute lung injury(ALI) models and clinical trials [ISRCTN95690673](217).](217). Other considered investigated the the anti-inflammatory effects of other emitted factors such as interleukin-10 (IL-10), prostaglandin-E2 (PGE2), or transforming growth factor-β (TGF-β). In other hand, few productions demonstrated that helpful effects of MSC condition medium (CM)-cell free strategy is same as MSC cell therapy. In the lipopolysaccharide (LPS)induced-ALI model, the administration of MSC-CM avoid edema formation through insulin-like growth factor I secretion.(218). Thus, another examination demonstrated comparable outcome LPS-induction of ALI in an ex vivo lung of human which demonstrated the diminishing of irritation, edema hindrance and maintaining a strategic distance from the inundation of neutrophils and alveolar liquid ingestion bringing up in the harmed alveolus(219). In any case, MSC-CM helpful application has limited in light of the fact that there in not a standard procedure of precondition, finding ideal organization course and clinical dosage. As of late, clinical MSC-CM using confronted to the lack of condition medium institutionalization. To date, some investigators been contemplated the MSCs microvesicles therapeutic impacts in lung diseases related to acute inflammation such as ALI, pulmonary artery hypertension (PAH) and asthma (220). These gatherings found that Micorvesicles show solid impacts as their stem cell source as therapy .one study by MSC MVs have been conducted in a mouse model of ALI that be induced by endotoxin . MVs could reestablish protein permeability of lung, diminish the neutrophils influx , expanding levels of macrophage inflammatory protein-2. At long last, they reduced inflammation and avoided the pulmonary edema formation in the damaged sites (221). Gennai et al.[105] did a research by MSC MVs which prompt upgrade of alveolar of alveolar fluid clearance in a dose-dependent way, reduced lung weight after perfusion and ventilation and also a significant improvement of airway and hemodynamic parameterswhen compared to perfusion alone[105]. in addition, anti- CD44 antibody administered with MVS that attenuate these influences through internalization of the MVS into damage cells (222). the consequence of many investigations propose several mechanism actions of MSC MVs to induce beneficial effects in lung disease including:1) pre-condition of MSCs with an agonist of toll-like receptor 3 stimulate MSCs before MVS secretion then will improve bacteria phagocytosis; 2) The cyclooxygenase 2 (COX2) mRNA transfer to activated monocytes by MSC MVs cause secretion of PGE2 which resulting in anti-inflammatory M2 phenotype of monocytes; 3)it's observed the uptake of MSC MVS using CD44 receptor is critical for their effective influences in primary cultures of human monocytes or human alveolar type 2 cells. the experimental finding suggested that MSC MVS effectiveness can be similar to their parent cells so they can consider as a successful substitute for stem cell therapy in ALI and other lung disease (223).

13-exosomes and clinical trials

As of late, the International Society for Extracellular Vesicles (ISEV) demonstrated the EV-based therapies thought of administrative, clinical and fabricating.. Clinical manufacturing of mammalian vesicles employ cell culture methods of primary stem cells or cell lines for constantly secretion of EVs. Notwithstanding, their manufacture is same as other biologics

because they naturally secreted from various cell types. The most created field of EVs is cancer therapy (224, 225). The vaccine of dendritic cell-derived and tumor-derived exosomes is contemplated in Phase 1/2 trials. . Other effective ranges for unmodified EVs application MSCs, endothelial progenitors, Tregs, DCs, and NSCs, as well as of different cell types, are regenerative medicine and immunotherapy of non-malignant disorders. Exosomes derived from mesenchymal stromal cells are obtaining to the first clinical trials. At least 3 companies (ReNeuron, Capricor and Aegle Therapeutics) are developing commercial use of EVs. Capricor Inc., investigating their beneficial applications in cardiac and muscle disease (http://capricor.com) and ReNeuron Group PLC, , had practical experience in neurological and ischemic conditions (http://www.reneuron.com). Moreover, Anosys Inc., began to make up autologous DC-derived EVs vaccine for cancer therapy (http://chromos.com). Nonetheless, it is essential to mention advantages and disadvantages, while cell lines characterization are still not completely understood but primary cells studies have been shown many aspects of their capacities to avoid the immunological rejection (226), which in some cases have been reduced by autologous EVs application (227, 228). All in all, the primary cell bank generation is challenging due to low vesicle yield of these cell types and restricted passage number. The Crucell have designed a human cell-line technology to evaluate oncogenic potential of these cells. Some recent studies are studying the applied potential of EVs derived from non-mammalian cells such as bacteria, yeast and plant cells (229). One of the serious problems can be their isolation techniques for more translational trials and research studies. There are current strategies including ultrafiltration, ultracentrifugation or a polyethylene glycol 6000 precipitation method (229). However, the contamination of EVs together proteins or lipoproteins occurs which reduce our isolation purity level. Overcoming these problems, we need some reproducible methods that isolate functional EVs rapidly without any contaminations. Finally, translation of EVs into clinic needs isotonic buffers to keep PH shifts during storage process such as freezing and thawing. The optimization and validation of storage condition will conduct stability of their function and physical characteristics. Also, the temperature storage and certain materials for long-time reservation must be established since they are most effective on the reserved sample quality (230).

14. Conclusion

Although the therapeutic benefits of MSCs in immune modulation and tissue remodeling have drawn much interest, there are several limitations that impair their wide widespread use. It has been demonstrated that implanted MSCs do not survive for long time and the MSCs therapeutic benefits might be attributable to their secreted factors including cytokines, growth factors, microRNAs, proteasomes, and exosomes, which may play an important role in the regulation of numerous physiological processes (231) . Secretome-based approaches using exosomes may present considerable potential advantages over living cells; multiple experimental studies demonstrate that secretome-derived products are sufficient to significantly improve multiple biomarkers of pathophysiology in many animal models of different diseases. Various studies indicate that MSC-derived exosomes exert their effect via horizontal transfer of proteins, mRNAs and regulatory microRNAs. Anatomical origin of MSC changes the composition and effects of secretomes. For instance, hUCESCs are obtained by Pap cervical smear, so the quick obtain of large amounts of hUCESCs or

secretome-derived products for research and clinical use is ossible because of their easy isolation and high proliferative rate (232) . The complex composition of secretome-derived products from MSCs should not be an impediment for regulatory approval of a regenerative product. For example, platelet-rich plasma or amniotic fluid, which are routinely used as a regenerative therapy for multiple applications in wound healing and orthopedics include numerous growth factors and exosomes that have not completely characterized. Exosomes sourced from dendritic cells are used in the clinical-trial stage for immunotherapy of certain cancers even though the regulatory requirements for manufacturing and quality control are required. Safety tests will be based on systemic bio-distribution, biocompatibility and half-life trials (233). Despite the rapid progress in exosome research, a number of important questions remain about their role in MSC biology; including the role that endogenous niche resident MSCs, which secrete exosomes or microvesicles, play in hematopoiesis and skeletal homeostasis, as well as their role in niche maintenance under normal physiological conditions, the possibility of captured MSC by its secreted products for therapeutically use. But these problems are some of the most interesting problems in biology that do not confined to MSCs or stem cells but the broader biology community will contribute to answer. MSC-derived exosomes use has several potential advantages in human patients. First, it avoids transfer of cells which may have mutated or damaged DNA. Second, against MSCs which are too large, exosomes are small to circulate easily through capillaries. Third, while the dose of infused MSCs quickly diminishes, it is possible that MSC-derived vesicles can deliver a higher "dose" and circulates to a greater extent. Furthermore, their cost-effective strategies and possible repeated administration have been though as their advantages. However, MSC-derived are static and more cannot be produced in vivo when the cell is transplanting (155). Several significant parameters affect the utility and efficacy of MSC-derived exosomes clinical testing including optimizing reproducible methods to manufacture exosomes/ microvesicles with defined content, developing methods of storage and recovery of these products that maintain vesicle potency, and evaluating their therapeutic efficacy in well controlled, appropriately powered clinical trials that are rationally designed based on supporting scientific and translational data. Still, there are a number of challenges that need to be resolved prior to their clinical application. The amount of MSC exosomes needed to generate an equivalent effect as MSCs in tissue injury is roughly higher. Although MSCs are relatively easy to expand in vitro, their growth in culture is finite, so new batches of MSCs and more effective techniques for large-scale exosome production need to be developed (229). Furthermore, since there is still no gold standard in characterizing MSC exosomes, the obtained exosomes isolated are also heterogeneous which may cause different effects on their target cells. Moreover, as the methods used to precondition MSC in stimulating exosome release change the surface and their intracellular content, the effective methods for maintaining the homogeneity of MSC-derived exosomes need to be developed.

Conflict of interest

None.

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Figure legend:

Figure 1. An overview of different extracellular vesicle (EXs) , exosomes and microvesicles (MVs) loading techniques. Left panel: loading of EVs after their isolation via common loading methods (electroporation, incubation, sonication, extrusion, etc.). Right panels: loading of EVs via treatment/transfection of the parental cells.

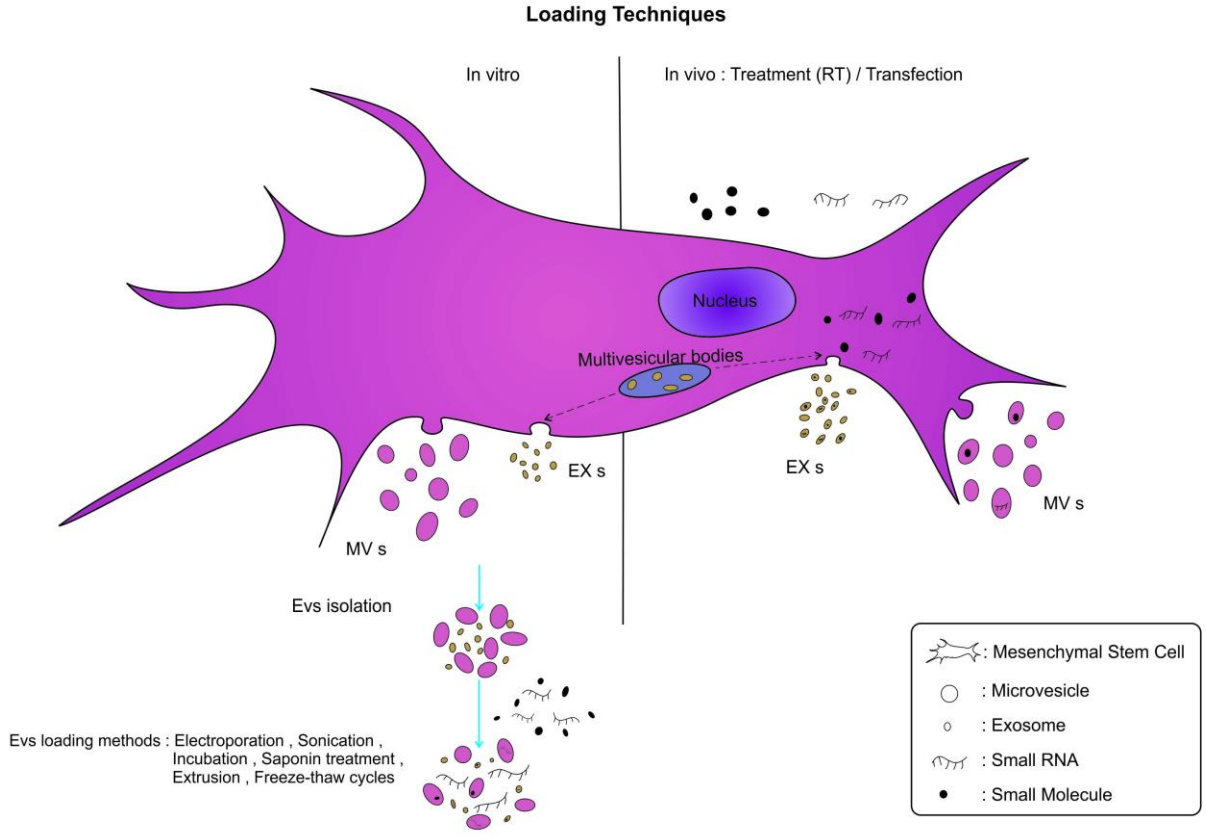

Figure1.

Table 1 Overview of therapeutic applications of EV-based gene delivery

Type/source of EV	Loading method	Target gene/cells	Drug (nucleic acid biotype)	Target effects	Ref.
Rat heart-derived H9c2	Transfection	QKI	miR-208a/b	Suppress apoptosis of cardiomyocyte	(234)
Human endothelial cells	Transfection	5-hydroxytryptamine transporter (5-HTT)	miR-195	regulation of the proliferation of Human smooth muscle cells through 5-HTT	(235)
Adipose-derived Stem Cells	Transfection	WISP2	miR-450a-5p	miR-450a-5p promoted adipogenesis through repressing expression of WISP2	(236)
human Wharton Jelly mesenchymal stromal cells	Transfection	DRP1/ Rat renal tubular epithelial cells	mir-30	ameliorate acute renal IRI by inhibiting mitochondrial fission	(237)
human liver stellate cell line (LX2)	Transfection	VEGF, CDC42, CDK1,CDK4, CDK6, and CDC25/BDEneu and BDEsp cell line	mir-195	decrease the size of cholangio carcinoma tumor, and improve survival of treated rats	(238)
HUVEC cells	Transfection	VEGF/Human gastric cell line SGC7901	mir-29a/c	miRNA-containing MVs to control gastric cancer growth by blocking angiogenesis	(144)
adipose-derived stem cells microvesicles	Transfection	factor-inhibiting HIF-1/ HUVECs	mir-31	angiogenic therapy for ischemic diseases	(239)
adipose-derived stem cells exosomes	Transfection	ADAM10, IGF1R, and CCNG1/ Hepatocellular carcinoma	miR-122	enhance HCC chemosensitivity	(240)
WT-MSCs exosomes	Transfection	Sema3A and Stat3/ cardiomyocytes	miR-223	exosomal miR-223 induced cardio-protection in sepsis	(241)

Pancreatic cancer-derived exosomes	Transfection	RFXAP	miR-212-3p	miR-212-3p transferred to dendritic cells & decrease MHC II expression and induce immune tolerance of dendritic cells	(242)
Mesenchymal stem cells exosomes	Transfection	Efna3/ HUVECs	miR-210	MSC-EVs significantly improved angiogenesis and cardiac function in post-MI heart	(243)
HEK293T, HUVEC,HRVT, hMSC	Electroporation	HEK293Ts	1000>bp dsDNA from the VA1 gene	EVs as broadly applicable DNA delivery vehicles	(139)
HBV-infected PXB-cells	Inoculation with HBV	HBV -DNA		transmitting viral DNA into hepatocytes	(134)
human glioma cancer stem cells	Transfection	Bloodstream	DNA sequence of $IDH1^{G395}$ mutation	EVs can cross the disrupted BBB into the bloodstream	(137)
HL-1, acardiomyocyte cell line	Transfection	Fibroblasts	DNA	gene expression changes	(138)
Cardiosphere-derived cells	Exo-Fect exosome transfection	Macrophages	Y RNA fragment	cardioprotection via modulation of IL-10 expression and secretion	(141)
brain endothelial bEND.3	Transfection	U-87 MG cells	VEGF siRNA	brain tumor	(244)
Human keratinocytes	Transfection	Melanocytes and/or fibroblasts	miR-675	miR-675 inhibit melanogenesis	(245)
ES-2 and SKOV3	Transfection	RMG-1, ES-2 and A2780 cell lines	MMP1	ovarian cancers	(246)
U87 glioblastoma cells	Exosome were incubate with Cy3- fluorescent hsiRNAs	Primary cortical neurons were isolated from E15.5 mouse	hsiRNAs targeting Huntingtin mRNA	Huntington's disease	(142)

HEK-293T cells	Transfection	Human schwannoma cell line HEI-193	Cytosine deaminase-uracil phosphoribosyltransferase	schwannoma tumors	(140)
The HEK 293T and CHO-K1 cells	Transfection	Rat primary pinealocyte cells	siRNA for the endogenous GTPBP1	exosome-mediated mRNA turnover pathway	(247)
Bone marrow mesenchymal stem cells	Transfection	Intestinal epithelial cells: IEC-6	miR-200b	anti-fibrotic treatment	(248)
MDA-MB-231 (breast) cancer cells	Transfection	Fibroblasts	miR-9	enhancing the switch to CAF phenotype	(249)
U87 and X12 cells	Transfection	Brain microvascular endothelial cells (HBMVEC)	miR-1	Reduce GBM invasion	(250)
human MCF-7 and MDA-MB-231 cells	transfection	HMLE cells	miR-10b	induce the invasion ability of non-malignant HMLE cells	(251)
Murine dendritic Cells	Electroporation	Systemic delivery to mice	α-Syn siRNA	Parkinson's disease	(252)
HEK-293T	Transfection	C2C12 cell lines	MOR siRNA	morphine addiction	(151)