REVIEW ARTICLE

Mesenchymal Stem Cell Secretome: Cell-free Therapeutic Strategy in Regenerative Medicine

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Abstract

BACKGROUND: Mesenchymal stem (stromal) cells (MSCs) have a multipotent character, able to differentiate into several cell types, thus MSC serve as a cell reservoir for regenerative medicine. MSC therapeutic potency more associated to their immunosuppressive and anti-inflammatory properties rather than the multipotency, by its mechanism to secrete soluble factors with paracrine actions.

CONTENT: MSC paracrine function was known to mediated partly by extracellular vesicles (EVs), which were released predominantly from the endosomal compartment contained in MSC secretome. EV contain a cargo bring micro RNA (miRNA), messenger RNA (mRNA), and proteins from their cells of origin, propose EV as a novel alternative to whole cell therapies, regarding the benefit of EV in safety and easier storage compared to the parent cells.

SUMMARY: The discovery of EVs including exosomes in MSC secretome as key of stem cells beneficial function lead to the future hope of using cell-free regenerative therapies.

KEYWORDS: MSC, secretome, conditioned media, extracellular vesicle, exosome

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Introduction

Within tissues, cells coordinating need a proper communication. Different cell types can communicate each other by soluble factors (1), cell-to-cell interactions mediated by adhesion molecules including cytonemes that connect neighboring cells for exchange surface-associated molecules, or by tunneling nanotubules that establish conduits between cells which more than only transferring surface molecules but also cytoplasmic components.(2,3)

Extracellular vesicles (EVs) are a heterogeneous collection of membrane-bound carriers release from a cell with complex cargos, including proteins, lipids and nucleic acids (*e.g.*, cell specific antigens, surface markers, adhesion molecules, ligands, receptors, enzymes, micro RNAs (miRNAs), long non-coding RNAs (lncRNAs), messenger RNAs (mRNAs), growth factors, *etc.*).(4-6) EVs consist of exosomes and microvesicles, which originate from the endosomal system or which are shed from the plasma membrane, respectively.(7) For a long time, microvesicles regarded as inert cellular debris or as the consequence of cell damage or the result of dynamic plasma membrane turnover, until recently some studies suggested them to be involved in cell communication.(8,9) In the last decade, many studies in physiology and diseases considered EVs as significant factors in inflammation and immune responses, antigen presentation, cancer progression and metastasis, immunomodulation, coagulation, tissue regeneration, organ repair, cell-cell communication, senescence, proliferation and differentiation in the body (10-12).

Exosomes perform many stimulatory or inhibitory functional outcomes including cell proliferation, apoptosis, cytokine production, immune modulation, and metastasis,

as their bioactive cargo is capable to modify the activity or properties of specific target cells.(13-17) Despite of the conventional one, exosomes add an alternative mode of paracrine and endocrine communication strategies of direct cell-cell contact and soluble, receptor-targeted hormones and cytokines.(18) Therefore, exosomes are proposed to play an important albeit role in human physiology and homeostasis, pathogenesis of major human diseases, and also become a promising source of disease-associated biomarkers and outright may be used as cell-free delivery vectors for targeted biological therapies.(19,20)

Regenerative medicine aims to restore any damaged, malfunctioning, or missing tissue. There are currently three approaches in regenerative medicine: first, cell-based approach, where cells are directly administered to restore a tissue through the cells themselves and/or the cells' paracrine functions; secondly, classical tissue engineering, using a combination of cells and a biodegradable scaffold to form a tissue; and lastly material-based approaches, which rely on bio-degradable materials, often functionalized with cellular functions.(9) Increasing evidences support that many observed effects of stem cell therapies were employed by the cells' secretion products like growth factors and cytokines, and implanted cells alone cannot survive for long without bioactive factors they produce.(6-9) This secreted paracrine factors drag a major interest to discover new therapeutics that stimulate local tissue regeneration and in tissue engineering as well.(21-23)

Biogenesis, Secretion and Functions of Exosomes and Other Extracellular Vesicles

EVs consist of exosomes, microvesicles (also known as shedding vesicles, ectosomes, and nanoparticles) and apoptotic bodies, with different size and biogenesis. By some stimulus, cell plasma membrane inwardly budding to form early endosomes. The late endosomes then subsequently form multivesicular bodies (MVBs) through characterized pathway that relies on endosomal-sorting complexes required for transport (ESCRT). ESCRT helps in MVBs invagination and cleaving the buds to form intraluminal vesicles (ILVs). During this process, certain proteins are incorporated into the invaginated membrane, later generating the parent cells' reflection in exosomes.24-29 Most ILVs are released into the extracellular space upon MVBs fusion with the plasma membrane, which are referred to as exosomes. Exosomes are the smallest EVs, with a diameter of 30-150 nm, and have an endosomal origin, compare to larger microvesicles (100 nm-1 mm) and apoptotic bodies (1-5 mm) result from the direct outward budding and fission of the plasma membrane.(30-32) Figure 1 shows the assembly, release and action process of EVs.

Typical exosomal marker proteins are tetraspanins such as cluster of differentiation (CD)9, CD63, and CD81; cytoplasmic proteins such as actin, annexins, and Ras-associated binding (RAB) proteins; MVB biogenesis molecules such as Alix and tumor susceptibility gene (TSG)101, also heat-shock proteins such as heat shock protein (HSP)70 and HSP90.(33-36) Other components defined in exosomes can also be cell-type specific such as ~1300 mRNAs (*i.e.*, about 10% of mRNAs from the parent cells) and 120 miRNAs, various other types of RNAs and of double-stranded DNA fragments, phospholipids which form the lipid bilayer, identified as about 3500 proteins and 2000 lipid species.(37-43) The presence of so many molecules is not in one single vesicle but instead those bulk materials were represented in several populations of microvesicles or exosomes as an exosome pellet or a microvesicle pellet.(41-44). So, there may be some distinct biogenesis pathways for different populations.(45)

More recently, another pathway for exosome biogenesis was characterized which involving the syndecan/syntenin complex and requires the activity of the phospholipase D (PLD)2.(16) PLD2 inactivation prevents the MVBs intraluminal vesicles formation (Figure 2). PLD2 is activated by the small G protein Arf6, and PLD activities known to coordinate the process between exosomes and microvesicle formation. Arf6 activity involved in microvesicle formation by leading to the localization of the myosin-light chain kinase at the neck of the newly forming vesicles, to promote the fission to release them from the plasma membrane.(22)

The protocol for reticulocyte exosomes purifying first developed to separate exosomes from tissue culture conditioned medium (46,47) and then improved to purify these vesicles from antigen-presenting cells based on differential centrifugation, where the smallest vesicles (including exosomes) are sedimented by ultracentrifugation at 100,000×g.(7,37,43) However, the ultracentrifugation is not a proper purification, because it open the possibility for other vesicles with similar size as protein aggregates. Many current commercially available methods without ultracentrifugation claims to be fast and simple, either (presumably) by polymer-based precipitation or immunocapture by antibody-coated beads. Nonetheless, these new tools still need to be validated for any kinds

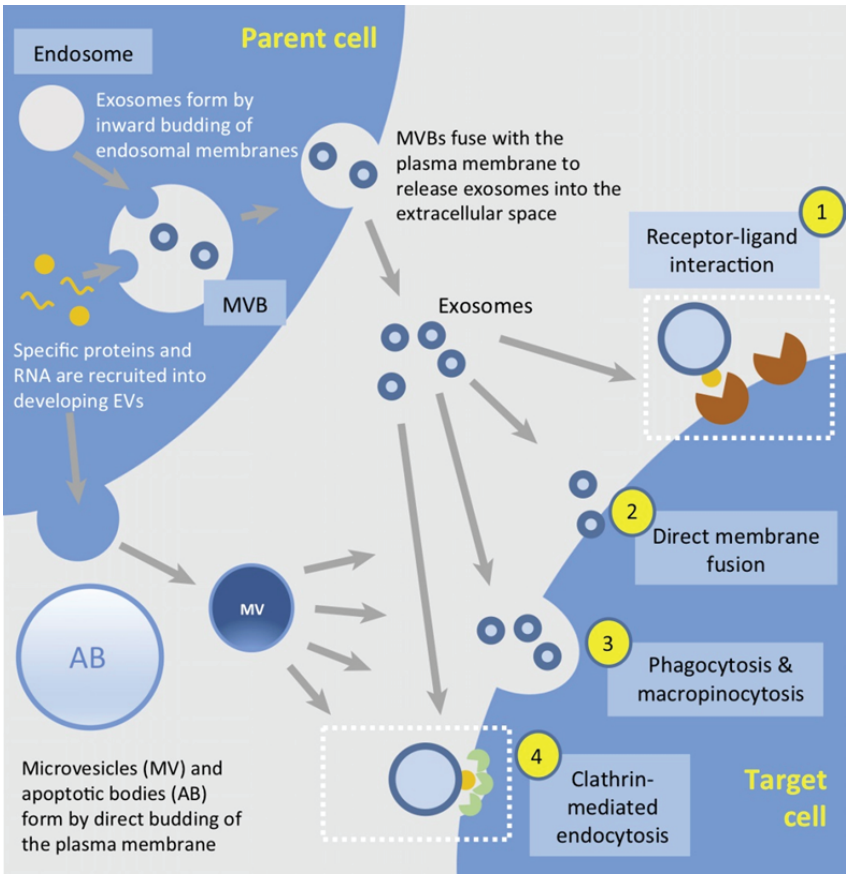

Figure 1. Assembly, release and action of EVs.(18) (Adapted with permission from Elsevier).

of vesicles were precipitated.(7) Overall composition of extracellular vesicles is shown in Figure 3.

Exosomes are secreted by various cell types including immune cells, neural and stem cells to interact with other cells, involved in many physiological processes such as antigen presentation (48), transfer of RNA (13) or tissue repair (49), resulting in physiological changes (48). This explain exosomes and its associaton with the progression of disease conditions including neurodegenerative disease, cardiovascular diseases and cancer (50-53), raising the interest to isolate exosomes as the active components of conditioned medium from human embryonic stem cell-derived MSC (49). Clinical studies performed the injection of dendritic cell-derived exosomes in melanoma patients showed tumor regression and long-term stabilization.(28) Exosomes first time proved to be used as delivery vehicle for nucleic acid cargos was exploited recently during Alzheimer's disease.(54,55)

Stem Cells in Regeneration Medicine

Regenerative medicine is defined as various approaches and actions to replace lost tissues with new tissues/cells or enhance regeneration of damaged tissues in a broad spectrum of indications (*e.g.*, myocardial infraction, osteoarthritis, lung diseases, acute kidney injuries, chronic wounds, muscular dystrophies, bone and cartilage defects, *etc.*).(9,10,56-58) There are different strategies towards tissue/organ regeneration, from cell transplantation to utilizing biomaterials alongside stem cell therapy, which are called tissue engineering.(59)

Mesenchymal stem (stromal) cells (MSCs) are multipotent, non-hematopoietic adult stem cells, with the potential to differentiate and/or transdifferentiation into osteoblasts, chondrocytes, and adipocytes as well as endothelial, cardiovascular, and neurogenic cell types, thus appeared to be the plausible solution for tissue repair and wound healing.(60-63) MSCs can be isolated from bone marrow (BM), umbilical cord, placental or adipose tissue, with the capacity for *ex vivo* expansion and ethical acceptable.(61-64) Despite of their direct role in tissue regeneration, the potency of MSC found to associated with its anti-inflammatory and/or immunosuppressive properties, as demonstrated by some studies that the differentiation capability of MSCs did not predominant the mechanisms for promoting or repairing the tissue damage in most disease but instead they found a short-lived paracrine mechanisms

among MSCs therapeutic actions.(65-69) Extensive studies explore more MSCs use as regenerative agents in spinal cord injury, multiple sclerosis, Alzheimer's disease, liver cirrhosis and hepatitis, osteoarthritis, myocardial infarction, kidney disease, inflammatory bowel disease, diabetes mellitus, knee cartilage injuries, organ transplantation, and graft-versus-host disease (http://www.clinicaltrials.gov; accessed November 2014).(66)

In acute kidney injury (AKI), MSC administration give their benefit not for differentiating into a tubular or endothelial cell phenotype, but by increasing anti-inflammatory regulation and organ-protective mediators including interleukin (IL)-10, basic fibroblast growth factor (bFGF), transforming growth factor (TGF)-α, and B-cell lymphoma 2 (Bcl-2).(70) The paracrine nature of cytoprotection in the immediate vicinity of administered MSCs in AKI. In this study, the renotropic factors, (hepatocyte growth factor, and insulin-like growth factor 1) induced by MSCs, showed to decrease apoptosis and stimulate proliferation of renal epithelial cells.(71) However, the precise mechanism of MSC's paracrine fashion is not fully understood. Some data suggested an array of soluble factors and large numbers of extracellular vesicles (EVs) released by MSCs, bring up possibilities that EVs to some degree, mediating the communication between exogenously administered MSCs and other stem cells and generating the complex paracrine regenerative actions.(72-74)

Therapeutic Potential Of The MSC Secretomes

Many growing evidences supported the MSCs paracrine actions in improving their positive clinical outcome, along with the findings of a wide range of chemokines, cytokines, growth factors and EVs secreted by MSCs, collectively termed as secretome which involved in cell viability, proliferation, angiogenesis, and immune responses.(75) The secretome is defined as the set of factors/molecules secreted to the extracellular space.(76-83) Secretome of individual cells and tissues is specific, and changes in response to fluctuations in physiological states or pathological conditions.(23,84)

MSC secretion also including vascular endothelial growth factor (VEGF), insulin-like growth factor (IGF)-1, bFGF, (TGF-β1), nerve growth factor (NGF), placental growth factor (PGF), stromal-derived growth factor (SDF-1/CXCL12), monocyte chemo-attractant protein-1 (MCP-1/CCL2), IL-6, IL-8, IL-10 and IL-13.(85-88) Different population of MSCs can secret different secretome, for instance adipose-derived MSCs were reported to have higher mRNA expression of VEGF-D, IGF-1 and IL-8, while dermal sheath and dermal papilla-derived cells secreted higher concentrations of CCL2 and leptin.(89) placenta-derived MSCs increased expression levels of

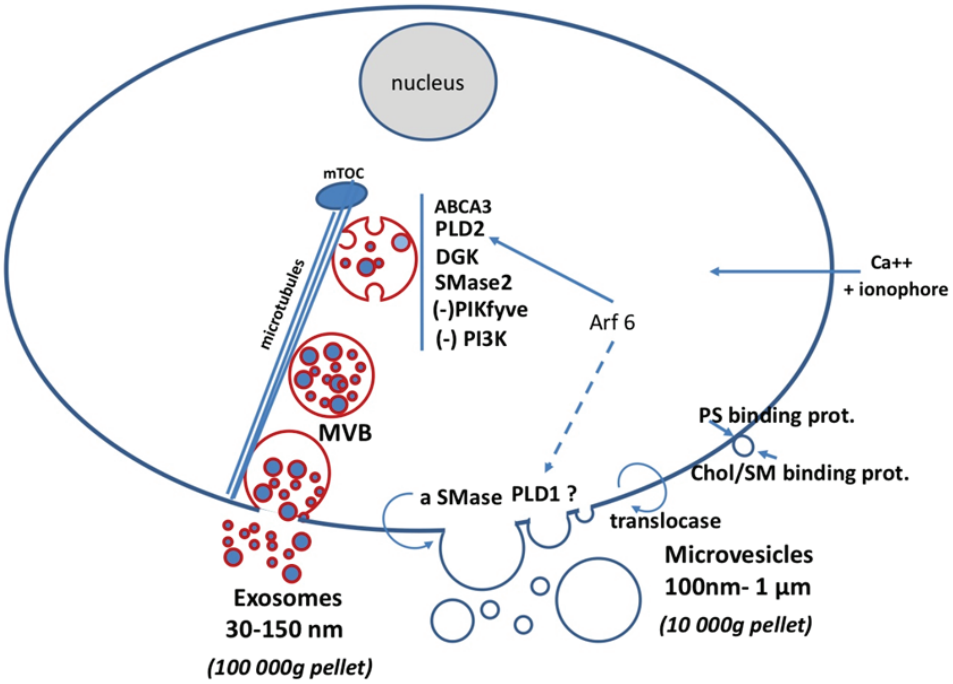

Figure 2. Lipid-related partners of exosome and microvesicle biogenesis.(45) (Adapted with permission from American Society for Biochemistry and Molecular Biology).

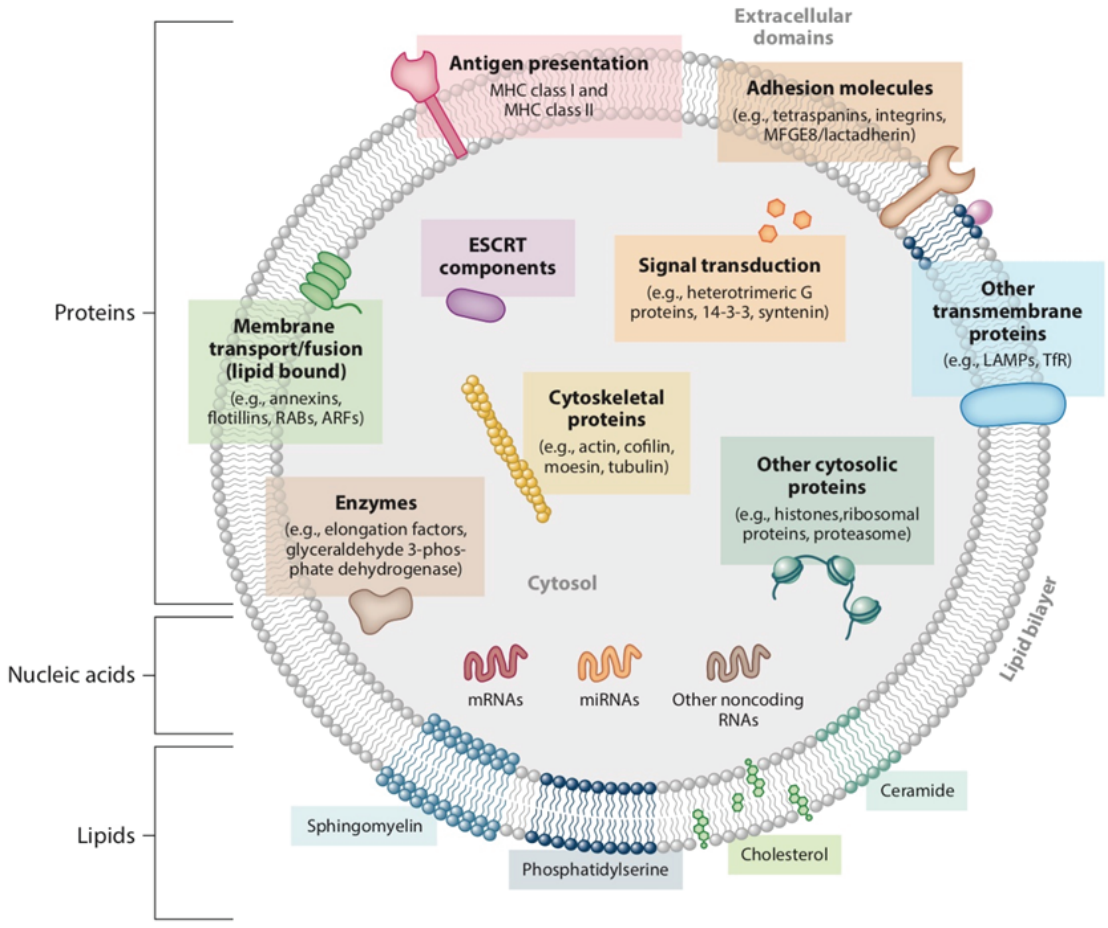

Figure 3. Overall composition of EVs.(7) (Adapted with permission from Annual Reviews). MHC: major histocompatibility complex; MFGE8: milk fat globule-EGF factor 8; ESCRT: endosomal-sorting complexes required for transport; LAPM: ysosome-associated membrane glycoproteins; TfR: transferrin receptor; RAB: Ras-associated binding; ARF: ADP-ribosylation factor.

hepatocyte growth factor (HGF), bFGF, IL-6, IL-8, IL-1α and IL-1β, while in bone marrow-derived MSC VEGF-A, NGF and angiogenin were higher.(90) The MSC secretome therefore has great potential as a regenerative therapy for stroke due to their potency in promoting angiogenesis and neurogenesis, prevent apoptosis and modulate inflammatory responses so many attempts were taken to enhance the MSC secretome (Figure 4).(75)

There are some advantages in using cell-free therapies such as MSC-sourced secretome in regenerative medicine compared to the stem-cell itself, *i.e.,* secretome application regards to be safer related to transplantation of living and proliferative cell populations in the matter of immune compatibility, tumorigenicity, emboli formation and the transmission of infections; the evaluation protocol for safety, dosage and potency of MSC-sourced secretome can be done following to conventional pharmaceutical agents; secretome can be stored for a long period without potentially toxic cryopreservative agents or loss of product potency (91-93); it is more economical and practical and avoids invasive cell collection procedures (94); possible for tailor-made mass production, providing a convenient source of bioactive factors; has lower cost and time for production, thus off-the-shelf secretome therapies could be immediately available for treatment of urgent conditions such as cerebral ischemia, myocardial infarction, or military trauma; and the biological product is available for modification adjust to desired therapeutic cell-specific effects.(23) By preventing cell apoptosis, modulating the inflammatory response and promoting endogenous repair mechanisms such as angiogenesis and neurogenesis, MSC secretome can promote tissue repair.(75)

Prospect of MSC Conditioned Medium in Regenerative Medicine

Cell-based therapy using stem cells is a promising option for treating ischemic diseases, including ischemic heart diseases and chronic limb ischemia.(95,96) However,

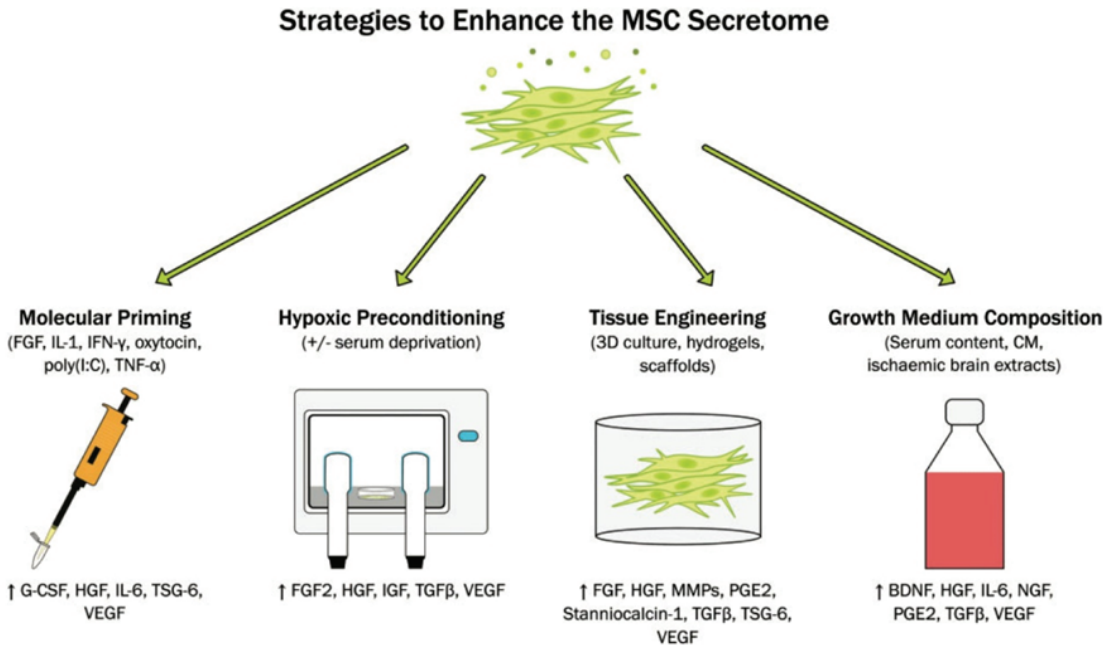

Figure 4. Summary of *in vitro* approaches that have been utilized to enhance the therapeutic potential of mesenchymal stem cell secretome.(75) (Adapted from Sage Publication)

the cell-based therapy efficacy was limited by the poor engraftment (97,98) and the potential cancer risk after stem cell transplantation (99,100). Here, stem cell conditioned medium (CM) offers a solution for a safer option.(101)

Recent evidences showed that even the secreted factor (*i.e.*, secretome, microvesicles, or exosomes) alone without the cell may repair damaged tissue in various conditions. The use of secretome found in the medium where the stem cells are cultured, or so called CM have advantages more than what we have mentioned before, as it is devoid of cells; there is no need to match the donor and the recipient to avoid rejection problems.(102) The use of CM for therapy is very appealing and may be booming in the near future, as studies on the use of CM for various diseases are accumulating.(101,103-105) The fact that stem cells secrete various growth factors was also shown by various proteomic studies, which revealed the presence of various growth factors and other cytokines in the CM.(106-109) To use CM for various human diseases, production method of the CM needs to be standardized in terms of the type and number of cells that were needed to produce the CM, culture medium and condition, and CM processing. In addition, the volume and mode of delivery are also important. As various studies used various numbers and type of cells and various doses of CM, it is important to know the number of cells that yielded the CM for one application, which may be interpolated for human studies.

In addition, for translation into patients, it is very important to analyze and to note the various cytokine contents of the various conditioned media. Further, for every CM with known cytokine content, validation of its use on various diseases needs to be conducted. Finally, the possibility of promotion of existing cancer should be tested for every CM, and caution should be taken before CM therapy to ensure that the recipient is free from cancer. Advantages of production of various CM for patients lie in the possibility of mass production by pharmaceutical companies, when production methods have been standardized. Conditioned media are not like stem cells that need a good manufacturing practice (c-GMP) facility to be applied to patients.(110) When CM has been packaged properly, it can be transported easily as drugs and does not need cryopreservation, such as that the stem cells need. However, compared to stem cells that may survive for a rather long period, CM needs to be given more frequently, as cytokines' and growth factors' half-lives are mostly shorter.(111,112), which is a disadvantage for the patients but will give more profit to pharmaceutical companies.(113)

There are various therapeutic applications for CM, including anti-photoaging properties and accelerating the wound healing with fewer scars. Moreover, it plays an important role in inducing migration and angiogenesis, preventing muscle atrophy, possessing anti-fibrotic properties and regenerating capacities. It does help in

suppressing proteolytic system and the ROS generation in muscle atrophied cells.(114-116) The diverse studies on the secreted factors derived from stem cells exhibited that the secreted soluble factors without the stem cells might provoke tissue repair in different conditions that involved in organ or tissue damage.(113,117)

CM obtained from ubmilical cord-MSCs (UC-CM) found to exert significant angiogenic and chemoattractant effects on progenitor cells, fibroblasts and stem cells, suggest a role of SDF-1/CXCR4 and MCP-1/CCR2 axes in UC-CM-induced migration. The local delivery of UC-CM may induce the recruitment of cells from the surrounding tissues and enhance the proliferation of these cells in injured tissue.(118) Recent findings suggest that MSC-CM have similar properties like MSCs and favorable antitumor characteristics as well. Therefore it is compelled to be applied for the generation of novel and targeted regenerative medicine (Figure 5).(117)

MSC-derived Exosome for Cell-free Therapy

Identification of exosomes revealed its unique protein and lipid contents, which can be used as positive markers. It mostly contain fusion proteins and transport proteins (annexins and flotilin), HSP70, CD's proteins (CD9, CD81), as well as phospholipases and other lipid related proteins.(119,120). In purpose of using MSC exosomes as cell-free regenerative medicine, the parameters of quality, reproducibility, and potency of their production should be considered well.(57)

Many techniques for exosome isolation has been developed with appreciable quantity and purity. Different technique exploits a particular trait of exosomes, such as their density, shape, size, and surface proteins to aid their isolation with the advantages and disadvantages of each. (121) Exosomes can be found in all body fluids, carrying specific information to their progenitor cells, thus exosomes is cheap, minimally invasive, and specific to be ideal biomarkers.(122,123) Many studies so far rationalize exosome as a novel form of a therapeutic intervention which is safer, cheaper, more accessible and potent, cell-free, and off- the-shelf therapy, although translation to clinical practice would require validation.(124) Studies was reported that hucMSC-Ex-mediated Wnt4 induces β-catenin activation in endothelial cells and exerts proangiogenic effects, make it prospect for use in cutaneous wound healing.(125) Another study demonstrated a cartilage regeneration in a full-thickness cartilage defect model in immunocompetent adult rats from human MSC exosomes (58), proof that exosomes also have a chance to alleviate OA via repairing and regenerating the damaged articular cartilage (126) In neurorestorative events after stroke and neural injury, exosomes showed as important intercellular players where either naturally occurring or engineered exosomes derived from stem/progenitor cells provide therapeutic benefits. (72,127,128) Suggest that exosomes not only cross the

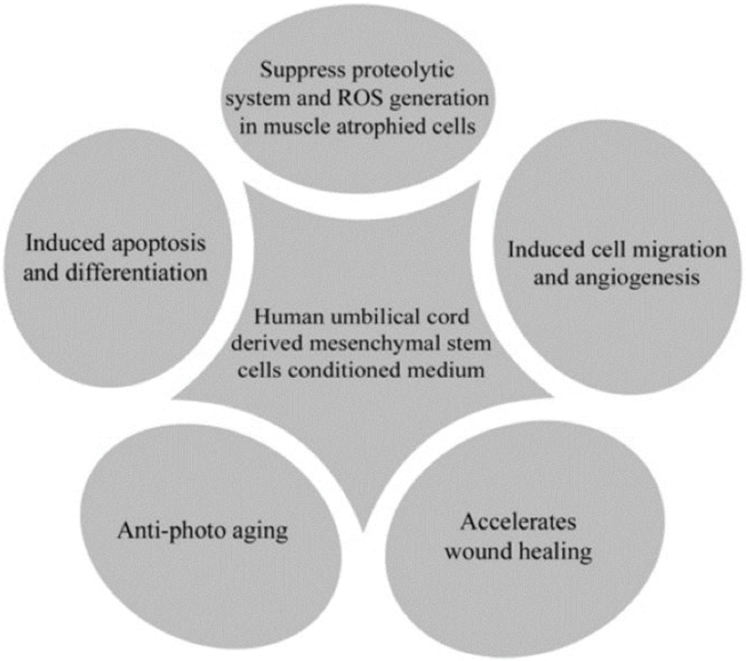

Figure 5. Clinical applications of conditioned medium (CM) derived from human umbilical cord-MSCs.(117) (Adapted with permission from International Journal of Hematology-Oncology and Stem Cell Research).

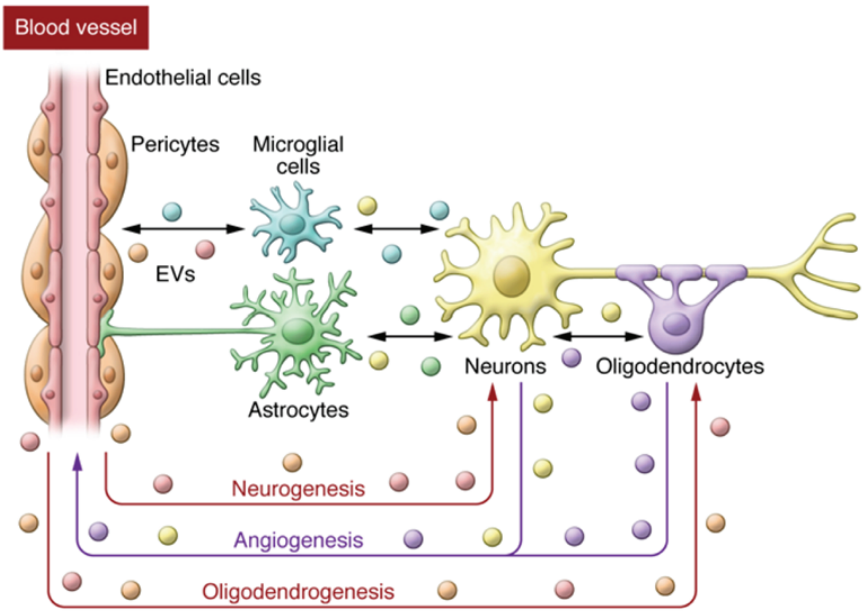

Figure 6. Potential of exosome-mediated intercellular communication in brain remodeling after stroke.(129) (Adapted with permission from American Society for Clinical Investigation).

blood brain barrier (BBB), but also deliver functional cargo to trigger gene expression in specific recipient cell types in the brain.(129)

In the case of ischemic injury, exosomes was known to improve heart function by stimulating neovascularization and restrain the inflammation response.(130) Many future studies will reveal the clinical potential of exosomes including their cargo property, targeting function and different sources of exosomes, to enable exosomes application in targeted tissue (Figure 6).(120)

Conclusion

Secretome-based approaches showed many potential advantages over the living cells themselves, including manufacturing, storage, handling, packaging, product shelf life and their potential as a ready-to-go biological therapeutic agent. Besides, exosomes also fulfill the requirements to be potential biomarkers of pathophysiology in many different diseases. However, we still need a clear consensus about the optimum culture conditions, separations, characterizations and stability and preconditioning strategies to maximize the regenerative potential of the MSC secretome.

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