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THERAPEUTIC POTENTIAL OF MSC DERIVED EXTRACELLULAR VESICLES

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Tämän tutkielman tarkoitus on lyhyesti esitellä ajankohtainen katsaus multipotenttien mesenkymaalisten stromaalisten solujen ja niiden erittämien solunulkoisten vesikkelien, mahdollisiin käyttötapoihin terapeuttisena menetelmänä. Multipotentit mesenkymaaliset stromaaliset solut tunnetaan paremmin mesenkymaalisina kantasoluina. Mesenkymaalisia kantasoluja on tutkittu vuosikymmenien ajan niiden osoittaman immunosupressiivisen sekä regeneratiivisen potentiaalin innoittamana. Mesenkymaalisilla kantasoluilla tehdyt prekliiniset tutkimukset ovat olleet lupaavia, mutta tulosten siirtäminen kliiniseen käytäntöön on ollut haastavaa. Tutkimusalueen yksi suurimpia haasteita on perustutkimuksen vajavaisuus, koska tutkimusta on suurelti tehty lupaavien näyttöjen innoittamana, ilman perusteellista taustatyötä. Mesenkymaalisten kantasolujen tutkimus vaatiikin parempaa yhtenäisyyttä käytettyjen menetelmien osalta, jotta tuloksien vertailtavuus ja ymmärrys mesenkymaalisten kantasolujen perusbiologiasta paranisi. Mesenkymaalisilla kantasoluilla on havaittu olevan parantava vaikutus useisiin erillaisiin patogeenisiin tiloihin kuten palovammat, haavautumat, tulehdustilat, sydäninfarktit,arpeutuminen ja reuma. Niiden toiminnan ajatellaan pohjautuvan niiden erittämiin molekyyleihin ja solunulkoisiin vesikkeleihin sekä suoraan kontaktiin immuunijärjestelmän solujen kanssa. Mesenkymaaliset kantasolut hillitsevät immuunijärjestelmän toimintaa ja ohjaavat kudosvaurion ympäröimää mikroympäristöä paranemiselle optimaalista tilaa kohti. Niiden kyky alentaa kudossiirteiden hylkimiseen johtavaa HVGD tilaa on pidetty yhtenä merkittävimpinä niiden havaituista ominaisuuksista.
Solunulkoiset vesikkelit ovat kalvorakenteisia vesikkeleitä, jotka kantavat sisällään molekyylejä kuten proteiineja, lipidejä ja nukleiinihappoja. Niiden on ajateltu suurelta osin olevan vastuussa mesenkymaalisten kantasolujen parantavien vaikutusten takana. Tutkimukset ovat osoittaneet mahdolliseksi mesenkymaalisten kantasolujen ohjaamisen biologisilla ja fysikaalisilla merkeillä kohti halutunlaisen erityisprofiilin luomiseen. Muunmuassa solujen altistaminen hypoksisille olosuhteille on todettu parantavan solunulkoisten vesikkelien parantavaa vaikutusta eläinmalleissa. Solunulkoisten vesikkelien käyttö terapeuttisena menetelmänä poistaisi soluterapian kohtaamia haasteita kuten solujen puutteelisen migraatiokyvyn kudosvaurioalueelle sekä mahdollisen syöpäriskin. Vaikka mesenkymaalisia kantasoluja on pidetty hyvänä vaihtoehtona allogeenisille hoidoille,niiden oletetun vajavaisen MHC ekspression vuoksi, on solunulkoisien vesikkelien käyttö luultavammin parempi vaihtoehto hylkimisreaktiota ajatellen.Solunulkoisilla vesikkeleillä on paljon terapeuttista potentiaalia, mutta myös niihin kohdistuva tutkimus kärsii perustutkimuksen puutteesta ja tulosten epäyhtenäisyydestä.

Avainsanat: Mesenkymaalinen kantasolu, solunulkoinen vesikkeli, immunosuppressio, regeneraatio, terapeuttinen relevanssi.

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1. INTRODUCTION

The aim of this thesis is to give a concise review to the therapeutic potential of MSCs and MSC derived extracellular vesicles as well as the challenges faced in turning them into therapeutic product. To understand the nature of MSC derived extracellular vesicles one must first understand what drives the perceived therapeutic functions of the cells themselves.

Multipotent mesenchymal stromal cells, or more commonly known as, Mesenchymal stem cells (MSCs), have been a hot topic in the field of cell therapy for generation's time. For the past decade MSC's have been highly researched cell therapy tool thanks to their easy harvesting and expansion as well as their ability to suppress inflammation and promote regeneration. MSCs have shown promise in animal models but so far, their use in the clinical setting has proven difficult. The ambiguity of MSC isolation protocols, multiple tissue sources and their elusive identity has complicated the comparability of studies conducted with MSCs and this the investigation of their true identity. The popularity of MCSs has also created a 'dark side' as the so called stem cell tourism has become prominent worldwide, bringing about the application of unproven stem cell treatments especially among professional athletes.

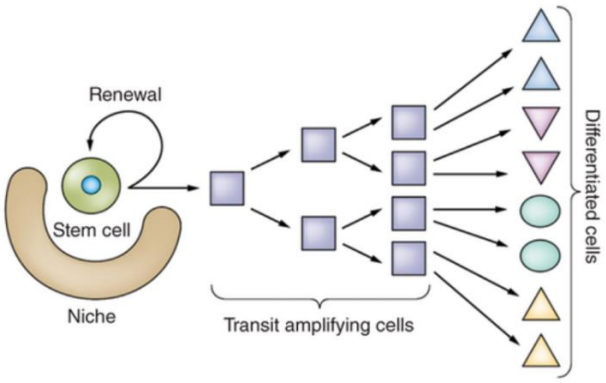
Although MSCs were first thought to function through direct cell replacement, it is now widely accepted that paracrine action plays a big part in the immunomodulatory and regenerational functions of MSCs. This is suggested to be carried out, at least partly, by extracellular vesicles (EVs). EVs have also been found to be prominent disease biomarkers. Although the physiological function and role of EVs remains an open question, MSC derived EVs have shown promise in being a cell free alternative for carrying out the therapeutic properties of MSCs.

Both MSCs and MSC derived EVs have been widely researched for therapeutic purposes for a wide range of pathologic states. Recently the outbreak of Covid-19 and the resulting acute respiratory distress syndrome (ARDS) have created a need for immunomodulatory therapeutics as the condition is caused by uncontrollable inflammatory response leading to severe alveolar damage and thus respiratory failure and currently has limited treatment options. MSCs and MSC derived EVs have received a lot of interest as a potential therapeutic tool to combat this devastating disease.

2. MULTIPOTENT MESENCHYMAL STROMAL CELLS'

Commonly referred to as mesenchymal stem/stromal cells, MSCs are spindle shaped pluripotent cells that reside in many different adult tissues. The term 'multipotent mesenchymal stromal cells' is now recommended by the International Society for Cellular Therapy (ISCT) as the official designation, although the terms 'mesenchymal stem cell' and mesenchymal stromal cell remain in popular usage. The term 'mesenchymal stem cell' produces 480 192 hits in the Tampere Univerisities Library Andor search and the term 'mesenchymal stromal cells' produces 206 321 hits.

A stem cell is defined as a cell that has unlimited self-renewing capability, being clonogenic and thus being able to form a colony of identical daughter cells and being able to differentiate into several types of differentiated cells (Weiner, 2008). Term multipotency refers to stem cells that are partially committed to a particular lineage and retain the ability to differentiate to subset of cell types, also called progenitor cells. Adult tissues contain niches of multipotent adult stem cells, which serve to maintain regenerating tissues. Figure 1 schematically describes the stem cell definition. Although MSCs were first discovered in 1968 by Friedenstein et al (Friedenstein et al., 1968) and widely researched ever since, their true identity remains elusive. The 'stemness' of MSCs has also been brought to question by their limited renewing capacity *in vitro*, as MSCs are not capable of dividing indefinitely while maintaining their multipotency (Brooke et al., 2007).



'Figure 1 schematic representation of stem cell identity. Stem cells live and are maintained in specific microenvironments or niches where their "stemness" is protected. They multiply and form colonies of cells that are "ready" to commit to different lineages and ultimately become differentiated into adult cells. (Weiner, 2008)

2.1 MSC Identity

MSCs were first discovered and isolated from mouse bone marrow, which remains the most popular method of isolation alongside with adipose tissue. MSCs can also be obtained from multiple other tissues including dental pulp, muscle, liver, placenta and cord blood (Konala et al., 2015). As

MSCs can be derived from readily available “unwanted” adult tissue sources such as placenta and umbilical cord which would be discarded as biological waste as well as adipose tissue from elective surgery, they represent an ethical source of stem cell material in contrast to embryonic stem cells.

The expanding field of MSC research and the enigmatic nature of MSCs themselves pose the problem of properly identifying MSCs. As many research laboratories use different isolation and identification protocols, the interpretation of variability in outcomes can prove difficult, together with the understanding of the true nature and functionality of the MSCs. ISCT has set the minimal requirement for identifying MSC in order to assure comparability between studies as ambiguity is likely to hinder MSC research. MSCs must be plastic adherent in culture. They should also be over 95% positive for surface markers CD105, CD73 and CD90. As MSCs are not known to express haemapoetic antigens they should also be 98% negative for CD45, CD34, CD14 or CD11b, CD79alpha or CD19 and HLA class II as listed in table 1. As the third requirement, the cells claimed as MSCs need to show differentiation capability in culture to osteoblast, adipocyte and chrondoblast.

Table 1 Summarized criteria for MSC identification for research purposes as comprised by ISCT and reported by Dominici et al.

Minimal criteria for MSC identification			
1. Adhere to plastic in standard culture conditions			
2	Positive	Negative	
	CD105	CD45	
	CD73	CD34	
	CD90	CD14	or CD11b
		CD79	or CD19
		HLA-DR	
3 *in vitro* differentiation to osteoblast, adipocyte and chondroblast			

It is worth noting that the listed characteristics are those of culture expanded MSCs and weather this pattern truly describes the freshy isolated and more importantly the native MSCs is still an open question (Dominici et al., 2006). As Girousse et al point out in their review, the phenotype of MSCs in culture may vary depending on the isolation methods. They also stress that the CD34 expression is present in native adipose tissue MSCs but is then abolished by the culture expansion (Girousse et al., 2020). Thus, the identification methods of MSCs need updating while the research keeps

advancing but the parameters provided by ISCT function as a valid starting point in harmonizing MSC research.

2.2 MSCs in cell therapy

The attraction of MSCs as cell therapy tools stems greatly from their easy isolation and expansion as well as their proposed immuno-privileged, immune-regulatory and regenerational nature. At the time of writing this thesis, there are 1434 ongoing clinical trials in the United States corresponding to search word “MSC”, linked to such conditions as Peyronie's Disease, Congenital Heart Disease (CHD), Prostate Cancer, burns, Ovarian Cancer, Autism, Multiple Sclerosis, Crohn's Disease, Asthma, Cerebral Palsy and more (clinicaltrials.gov). Observations, such as ones by Bartholomew et al. that MSC could dampen the reaction of the immune system towards allografts and extend their survival *in vivo*, together with the reports of MSCs reducing T-cell proliferation *in vitro*, (Bartholomew et al., 2002) were among those that drew interest towards MSCs as cell therapy tools. It has been shown, that MSCs in fact can interfere with multiple components of the immune system – both innate and adaptive. Spaggiari et al showed that MSCs can regulate NK cell proliferation (Spaggiari et al., 2006) and Kavanagh and Mahon established that allogenic MSCs can induce regulatory T cell activity (Kavanagh & Mahon, 2011). There are many promising reports of MSCs being able to assist in regeneration processes such as wound healing and act as a in situ enhancer of the body’s repair mechanisms. For example, Sergio et al. demonstrated MSCs being effective in inducing corneal wound healing in dogs in the cases where normal corneal wound healing was defective due to major damage (Ario et al., 2019). MSCs have also been suggested to reduce inflammation in pathological states such as arthritis, as described by Medina et al. who tested human adipose tissue derived MSCs in rabbits (Medina et al., 2018). In other words, MSCs seem to have the capacity to interpret signs of distress and respond to the changes in the milieu in and bring about favourable healing microenvironment. However, as described by Zhou et al, despite the promising results of MSC in animal disease models, the clinical trials applying MSC for use in human diseases, have been largely disappointing (Zhou et al., 2021). For example, Cheng et al report, that although MSCs are described as immunosuppressive, they are unable to bring about positive effects in Lupus patients (Cheng et al., 2019). Much of this seem to be due to the fact, that a lot about the *in vivo* functions of MSCs are largely unknown and much more needs to be discovered before their use can be optimized. The following sections cover aspects of MSCs that have been discovered so far.

The suggested immune-evasive nature of MSCs is credited to the observation that culture expanded MSCs have been shown to typically express lower levels of MHC class I surface receptors, compared to somatic cells (Y. Wang et al., 2019) and no MHC class II receptors or costimulatory

molecules (Ankrum et al., 2014). The MHCs are cell surface receptors that present peptides degraded by the cell to the cells of the immune system which allows for the immune system to perform surveillance of “non-self” entities within the human body. MHCs play a huge role in transplant rejection and Graft-vs-host-disease (GVHD) and are the major hurdle for allogenic cell transplantation (Nakamura et al., 2019). The proposed ability of MSCs to evade rejection makes them a candidate for allogenic use. However, as Ankrum, Ong and Karp conclude in their review, the immune-privileged role of MCSs may have been overestimated, as the *in vivo* phenotype of MSCs may be more MHC-expressive than the culture expanded MSCs, and allo-MSCs may not be able to persist and thus exert their desired effects (Ankrum et al., 2014). Ankrum et al. strongly critique the popular immune-privileged paradigm and the suggestion of the ‘universal donor’, the thought that MSCs could be collected from volunteer donors without HLA-matching. This said, the potential of MSCs as an allo-transplant has great therapeutic relevance because it would make them readily available for patients without the need for the expansion period for autologous cell population. Also, as MSCs are stem cells or stem cell-like cells their numbers dwindle in the aging individual (Rebolj et al., 2018) and thus gaining the appropriate number of cells for expansion could pose a challenge. On top of this, the fitness of the autologous cell graft could be compromised, if the MSC of an aging individual are malfunctioning or incapable to expand in culture while maintaining their therapeutic phenotype (Ahmed et al., 2017).

Much of the early research of MSCs acted under the assumption that MSCs were able to migrate to injured tissues and differentiate into the cells of said tissue and thus act as “replacement tool kit” of the body. For example, a study conducted by Horwitz et al. in 2002 found that patients suffering from osteogenesis imperfecta who received two 10- to 15-min infusions of MSCs experienced notable benefits in gains in body length and bone mineralization and close to no side-effects (Horwitz et al., 2002). They concluded that it was the MSCs ability to engraft and directly replace cells that carried out the effects. As the function of stem cells in general is to replace lost somatic cell material as they perish, this assumption cannot be faulted. However, the paradigm has changed since then and it is now accepted that direct replacement of the lost cell material plays a minuscule, if any, part in MSCs physiological functions and they exert most of their effects via their secretome. MSCs are suggested to function as ‘shepherds’ in injured tissues, shaping the microenvironment towards a regenerative phenotype. As Caplan and Dennis summarized in 2006 e MSCs secrete a variety of cytokines and growth factors that have both paracrine and autocrine activities and exert trophic effects (Caplan & Dennis, 2006). Prockop et al have demonstrated that MSCs respond to the damaged tissue by secreting large quantities of bioactive molecules such as cytokines, antioxidants, proangiogenic substances, trophic factors which in turn create a healing environment by bringing down inflammation and inhibiting cell death and recruiting reparative cells of the native tissue (Prockop D. J. 2009). As Phinney and Pittinger sum up, MSCs can limit inflammation via cytokines,

release growth factors in order to create a healing microenvironment, alter the host immune response by secreting immune-modulatory proteins and recruit endogenous repair machinery (Phinney & Pittenger, 2017).

Research has shown that when administered intravenously or locally, MSCs can migrate to the site of tissue injury such as cardiac infarct. The inflammation process upon injury including the released chemokines, cytokines and growth factors provide a road map for the mobilized MSCs which have been proposed to use a homing mechanism similar to circulating white blood cells (Chavakis et al., 2008; Ullah et al., 2019) as presented in figure 2 below.

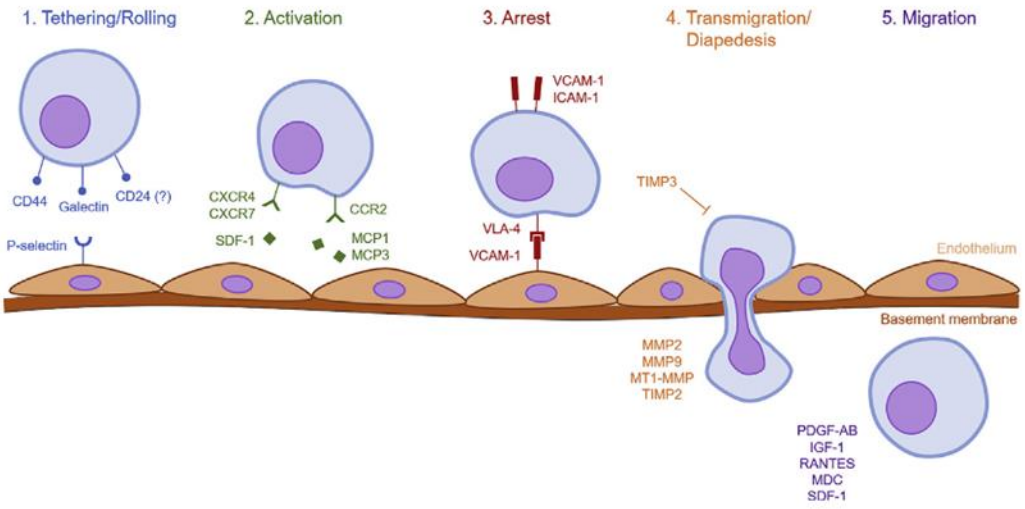


Figure 2 The proposed mechanism of MSC homing to tissues as represented schematically by Ullah et al. The MSCs express surface receptors capable of interacting with the endothelial cell receptors P-selectin and VCAM-1 which enable the MSC interaction and arrest to endothelial cell surface and the consequent cell diapedesis through the cellular junctions. MSCs also secrete matrix-metalloproteases which allows them to migrate through the dense extracellular matrix towards the site of injury (Ullah et al., 2019).

However, the engraftment process has been relatively ineffective as most MSCs get caught in lung capillaries (Scarfe et al., 2018) and are cleared by the immune system. Assuming that MSCs have to be on site to exert their effects, the inefficiency in the homing process presents a huge issue in therapeutic use of MSCs. Ullah et al. suggest several strategies in their review to improve MSC homing including overexpression of homing receptors, coadministration of vasodilators, direct in situ injections or MSCs and implantation of MSCs with scaffolds (Ullah et al., 2019). Interestingly, in some cases MSCs have been shown to exert their immune-suppressive effect from distance (English, 2013; Roddy et al., 2011), although in many cases it seems that MSCs require direct contact with immune cells in order to function accordingly (English, 2013). The detection of MSCs in the blood stream (Xu & Li, 2014) and their increase in some pathological states (Bian et al., 2009), such as bone fracture and large burns stream (Xu & Li, 2014), suggests that mobilization of

MSCs *in vivo* is a physiological phenomenon that occurs during injured state. As MSCs are found in almost all adult tissues, and MSC identification *in vivo* remains problematic, it is an open question which tissue resident MSCs are prone to mobilization, and which exert their effects locally. Due to their global presence in the human body, and the similarities in surface markers, it has been postulated that MSCs could be pericytes *in vivo* (Caplan, 2008).

3. EXTRACELLULAR VESICLES

Intercellular communication is a key factor in maintaining tissue homeostasis. Cells communicate with each other by direct contact, secreting soluble signalling molecules or by releasing extracellular vesicles. Extracellular vesicles (EVs) are nano-sized membranous structures carrying cell-derived biomolecules including proteins, carbohydrates and nucleic acids. EVs were first thought to be experimental artefacts, cell debris or waste products (Pan & Johnstone, 1983) but are now known to be secreted by most types of cells to the extracellular space in both physiological and pathological conditions. Commonly recognized extra vesicular subtypes are exosomes, microvesicles and apoptotic bodies. Oncosomes, ectosomes, membrane particles and exosome-like vesicles have also been mentioned under the definition of EVs, although under the scope of this thesis these definitions shall remain irrelevant. The different subtypes of EVs differ in their biogenesis, release pathway, size, cargo and function.

3.1 Extracellular vesicle types

Exosomes, which are often mistakenly referred as synonyms to extracellular vesicles, are the smallest of EVs. Their size is reported between 30-200nm, depending on the source. They originate by inward budding of the multivesicular endosome (MVE), also known as multivesicular body (MVB), forming nanoscale membranous vesicles containing cytosolic contents. Exosomes are released to the extracellular space when the MVB fuses with the plasma membrane releasing the vesicles. The molecular content of exosomes varies greatly and is dependent on the host cells physiological state and cell type. However, exosome cargo is not simply a sampling of its host cells cytosolic content, but the content profile can vary from the cell of origin. This implicates an existence of a controlled sorting process. With the notion that not all multivesicular bodies are secreted, as some fuse with lysosomes for degradation purposes, this is also an indication that exosome release is not simply a purge of unwanted material. However, exosome release could also help the cell to relieve itself from harmful contents present in the endosomal pathway (Ståhl et al., 2019). The endosomal sorting complex required for transport (ESCRT) is involved in the sorting of the exosome cargo and is often found on the exosome membrane, thus serving as a marker for exosome identification. 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 the sorting process (Andaloussi et al., 2013; Gurung et al., 2021).

Microvesicles (MVs) are formed by shedding of the plasma membrane. They are generally bigger and more variable in size than exosomes,100-1000nm as reported. Microvesicles carry membrane

proteins present on the plasma membrane (PM), as could be expected. MVs also reflect the physiological environment encountered by the host cell as they can also carry cytokines, chemokines and other signalling molecules in interaction with membrane receptors present on the cells PM and thus also MV originating from it. Apart from proteins associated with the plasma membrane, MVs also carry genetic material including mRNA and miRNAs. The release of microvesicles is dependent on asymmetric phospholipid distribution of PM caused by calcium ions (Konala et al., 2015).

Apoptotic bodies are the biggest EVs. Their size is reported to be up to 5000nm, although there is some variation in the reporting of their minimum size which varies between 50-100nm (Caruso & Poon, 2018; Ståhl et al., 2019). Apoptotic bodies are formed during apoptosis when the cellular contents are packaged into membrane bound vesicles in order to be cleared out by the immune cells. As pointed out by Sarah Caruso and Ivan K. H. Poon (Caruso & Poon, 2018), Apoptotic bodies are not simply 'trash bags' of the human body but they play a signalling role as well. Caruso and Poon describe apoptotic bodies having antigen presenting role and thus can educate the immune system regarding the reason behind the programmed cellular death that occurred. In other words, they have a role in immunomodulation.

In the context of this thesis the term EV refers to exosomes and microvesicles. In the case of exosomes versus microvesicles, the evidence regarding which EV type is the acting agent in physiological or pathological processes is lacking, as their sizes and cargos overlap (Ciferri et al., 2021)

*Table 2 Summary of some of the characteristics of the three types of extracellular vesicles. *the proposed size of the exosome varies somewhat in different sources. (Andaloussi et al., 2013; Gurung et al., 2021; Ståhl et al., 2019)*

Characteristics of extracellular vesicles

	Exosomes	**Microvesicles**	**Apoptotic bodies**
Size	30-200nm*	50–1000nm	50-5000nm
Origin	Endosomal pathway	Plasma membrane	Plasma membrane
Markers	Tetraspanins (such as TSPAN29 and TSPAN30), ESCRT machinery, PDCD6IP, TSG101, flotillin, MFGE8	Integrins, selectins, CD40 ligand	Extensive amounts of phosphatidylserine
Contents	mRNA, microRNA (miRNA) and other non-coding RNAs; cytoplasmic and membrane proteins including receptors and major histocompatibility complex (MHC) molecules	mRNA, miRNA, non-coding RNAs, cytoplasmic proteins and membrane proteins, including receptors	Cell debris, nuclear structres, cell organelles
Time of release	Ten minutes +	A few seconds	Unknown

Release	Constitutive or upon activation	Constitutive or upon activation	Apoptosis
Shape	Spheroid	Irregular	Variable
Density	1.13-1.19g/ml	1.04-1.07g/ml	1.16-1.28g/ml

3.2 EV interaction with cells

EVs carry bioactive molecules safely packaged into their membranous lumen. They have been implicated in processes such as stem cell maintenance, blood coagulation, tissue repair and immunosurveillance. They can thus be regarded as “signalosomes” (Andaloussi et al., 2013). The EVs secreted by MSCs have been credited to function in paracrine and endocrine signalling events (Lee et al., 2019). When EVs encounter cells, there are three different ways of interaction, also schematically represented in figure 3: receptor interaction, fusion with plasma membrane and unloading of the cargo to the cell cytoplasm or internalization of the whole vesicle. The direct interaction with the recipient cell through receptor mediated contact is often related to immunomodulatory and apoptotic functions. Internalization of the EV is reported to be the common way of transferring cargo as membrane fusion is suggested to be pH dependent and more common in tumour environment (Gurung et al., 2021).

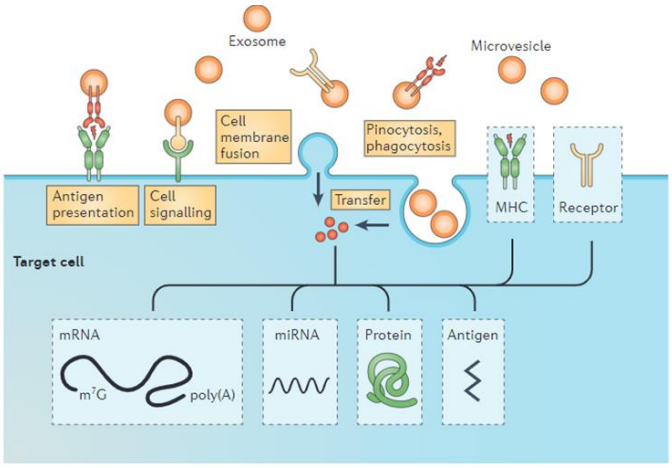


Figure 3 The recipient cell interactions with EVs as described by Andalousi et al. Evs can interact with cells via proteins on their membrane surface and start signalling cascades and thus cause changes in the recipient cells behaviour. EVs can also act as antigen presenters. EVs can also fuse with the plasma membrane and thus purge their cargo into the cell cytoplasm, or they can be taken up in their entirety by the recipient cell via pino- or phagocytosis.(Andaloussi et al., 2013)

3.3 EV therapeutic effect

As MSCs have been shown to secrete factors with cytoprotective and regenerative properties, the notion that the essence of these cells could be captured by using their secretome as a therapeutic product is quite captivating. The word secretome refers to the whole bulk of molecules and vesicles

secreted by the cells. As there are some complications in using MSC cell-based therapy, such as insufficient homing and intervention by the immune system as well as the risk of tumour formation common to any stem cell therapy, EVs as cell free therapy could offer a way to use MSCs in a safer and more efficient way. The use of EVs as a topical application to prevent scarring (Zhu et al., 2020) or co-application to alleviate graft versus host disease after transplantation (Fujii et al., n.d.), are examples of proposed therapeutical uses for MSC derived EVs. The use of EVs as biomarkers to detect pathogenesis is also quite a widely researched topic, as pathological cells have been shown to secrete EVs with distinct characteristics of healthy cells. EV secretion is also suggested to be higher in pathological states (Soung et al., 2017). As EVs are present in all bodily fluids they present and exciting alternative for disease diagnosis. If the information carried out by these nanovesicles could be harnessed into diagnostic tool, it would greatly reduce the need for invasive diagnostic procedures and could lower the requirements of symptoms for diagnosing diseases such as cancer (Ciferri et al., 2021). This topic is however outside the scope of this thesis. Table 3 exhibits a few examples of recent publications related to the research efforts in order use of MSC derived EVs in therapeutic functions.

Table 3 Recent publications in the field of MSC derived extracellular vesicles.

MSC DERIVED EVS IN RESEARCH

AUTHOR	Article	Year
VERÉB, Z., PÓLISKA, S., ALBERT, R. ET AL	"Role of Human Corneal Stroma-Derived Mesenchymal-Like Stem Cells in Corneal Immunity and Wound Healing"	2015
SAMAEEKIA ET AL	"Effect of Human Corneal Mesenchymal Stromal Cell derived Exosomes on Corneal Epithelial Wound Healing "	2018
ZUO ET AL	"BM-MSC-derived exosomes alleviate radiation-induced bone loss by restoring the function of recipient BM-MSCs and activating Wnt/β-catenin signalling"	2019
CHENG ET AL	"MSCs-derived exosomes attenuate ischemia-reperfusion brain injury and inhibit microglia apoptosis might via exosomal miR-26a-5p mediated suppression of CDK6"	2021
WANG ET AL	"Cardiac microvascular functions improved by MSC-derived exosomes attenuate cardiac fibrosis after ischemia–reperfusion via PDGFR-β modulation"	2021
XIE, FENG ET AL	“Engineering Extracellular Vesicles Enriched with Palmitoylated ACE2 as COVID-19 Therapy.”	2021

WARNECKE, ATHANASIA ET AL	"First-in-human Intracochlear Application of Human Stromal Cell-derived Extracellular Vesicles."	2021
SHENG, YONGJIA ET AL	"MSC Derived EV Loaded with miRNA-22 Inhibits the Inflammatory Response and Nerve Function Recovery after Spinal Cord Injury in Rats."	2021

As mentioned before MSCs "read" their surroundings and answer by secreting paracrine factors and EVs to make their environment favourable for healing. As demonstrated by Waterman et al. cultured MSCs can be directed towards a desired phenotype and secretome by offering them different signals, in their case either pro-inflammatory or immunosuppressive phenotype using Toll-Like-receptor signals (Waterman et al., 2010). Other preconditioning methods include likes of hypoxia, thrombin, LPS and hydrogen peroxide. Sung et al. showed that thrombin preconditioning of MSCs boosted the EVs ability to induce cutaneous wound healing (Sung et al., 2019).

The obvious downfall of using MSC derived EVs over the actual cells is the fixed nature of the EVs. It goes without saying that EVs do not posses the ability of MSCs to react to their environment. Also, it remains an open question to which extend it is the EVs that execute the healing functions of MSCs. It remains unproven that the soluble factors are not the ones in play for performing the proposed functions of Evs, or to which extend the presence and cell-to-cell contact with immune cells is needed to perform the immunosuppressive tasks. Another important question to pose is, can the true nature of the MSC be duplicated in a laboratory setting lacking the input MSCs receive *in vivo*? As MSC are present in many tissues it is yet to be demonstrated that all EV secreting MSCs are equal in their healing functions. Considering all of this, can the ultimate secretome ever be created in a cell culture vessel? As it is still upon speculation how do the MSCs "know" how to secrete the appropriate factors in order to manage the healing environment, creating therapeutic EVs needs more investigation of the identity of MSCs and the function of EVs they secrete.

Even though there are plenty of open questions regarding MSC derived EVs they have shown a lot of promise as a therapeutic tool as they have demonstrated to alleviate pathology in many disease models (Hall et al., n.d.; Sheng et al., 2021; X. Wang et al., 2021; Zuo et al., 2019). Due to their nano size and membranous structure, they can spread widely within the body and can even cross the blood brain barrier (Saint-Pol et al., 2020). They are also potentially immuno-privileged: Alvarez-Erviti et al have shown that exosomes do not trigger significant immune response in mice even when applied repeatedly (Alvarez-Erviti et al., 2011). The fact that EVs are membranous makes their cargo protected and molecules susceptible for destruction, such as nucleic acids, can be transported over long distances. Lack of understanding regarding EV targeting strategies *in vivo* poses problems regarding how to get the MSC derived EVs to the desired cells. The notion that Evs do carry proteins that can interact with cell membranes could offer a possibility for genetically

enhanced EVs which surface proteanome would be directed towards interaction with desired targets.

3.4 EV isolation

As with MSCs ambiguity of identification and variety of isolation protocols hinders the collective research efforts concerning EVs. Not only is it unclear which EV type exerts the effects of MSCs, the presence of soluble active biomolecules in the bodily fluids and MSC secretome, such as extracellular RNA and non-EV carriers such as lipoproteins, which often co-isolate with EVs, complicates the interpretation of EV participation in biological processes (Li et al., 2017). International Society for Extracellular Vesicles has issued a position statement for minimal experimental requirements for definition of EVs. ISEV describes a set of considerations starting from the isolation techniques, nomenclature of the isolated product and the reporting of the isolation and analytic processes used. The most importantly they recommend analysing the supposed EVs for known positive markers for EVs as well as negative markers to rule out the presence of coisolating contaminants. ISEV recommends the characterization of the single EVs by two different methods such as eletron microscopy and nano particle tracking analysis. There is also a strong recommendation for dose dependent analysis of the EV effect on studied subject (Théry et al., n.d.).

One of the biggest challenges in researching EVs and bringing them into therapeutic tools is the difficulty of isolation and purification of these nano sized vesicles. In order to create accurate data and study the applications of EVs they need to be specifically isolated and separated form interfering components and cellular debris. When assessing the EV contents and their effect on biological processes the isolation process needs to be highly accurate to avoid wrong conclusions. On top of this, the isolation protocols used by research groups need to be standardized in order to make research results comparable. For the purpose of bringing EVs into clinical use the isolation techniques need to be robust, sensitive and highly selective to be cost effective and clinically relevant. Isolation techniques include ultracentrifugation, size-based isolations such as filtering, immunoaffinity capture based techniques, EV precipitation and microfluid based techniques (Li et al., 2017).

Ultracentrifugation separates particles in suspension according to their density, size and shape. Denser and bigger particles are pelleted on the bottom first. The particles in suspension can be separated sequentially according to their physical properties by applying different centrifugal forces. The purpose of ultracentrifugation is to create exceptionally high centrifugal forces to separate miniscule particles. Due to the overlap in EV sizes and densities this technique is often plagued by contaminations and EV loss.Ultrafiltration is an alternative size-based separation method to ultracentrifugation. It does not require complex equipment and is fast and cost-effective.

However, the use of force applied may result in breakage of the particles and thus cause EV loss and hamper downstream analysis. Also, as in ultracentrifugation the overlap of EV characteristics can hinder the separation of the vesicle populations.The presence of proteins and receptors on EV surfaces offer the opportinuty for immunoaffinity capture based techniques where the EV samples are run through a pillar enriched in antibodies matching for EV surface receptors. They enable high specifity of isolation but are slow and low capacity and most likely not suitable for high scale clinical use. Comercial percitipation kits for EVs are also available but they often result in copercipitation of contaminants, are labor intensive as they require pre- and post clean up of the samples and require a long run time. The microfluid based techniques often implement multiple different approaches of isolation by using several properties of EVs. These devices enable fast and low cost isolation of EVs on a microscale, but have low sample capacity. They are also unsuitable for clinical use due to lack of standardization and method validation (Li et al., 2017).

Bringing EVs to therapeutic use requires an effective workflow , an example of which is sumarized in the figure 4 below. Isolation of MCSs needs to be effective, robust, safe and reliable from viable, safe and readily available tissue sources. The cells need to then be characterized as MSCs by the minimal criteria of identifying MSCs presented earlier. Also need for further analysis of MSC identity might arise as the research for identifying MSC therapeutic potential advances. The number of MSCs then need to be expanded in culture in order to obtain ideal number of cells for EV production. This step might also require MSC priming with bioactive molecules or physiological cues such as hypoxia in order to obtain the desired secretome. The expansion step needs to be optimized for EV production but also for maintaining the proper MSC identity as the culture conditions may result in losing some of the MSC *in vivo* functions which govern the cells' ability to produce trophic factors. The expanded cells need to then be cultured for the ideal time span and the conditioned media needs to then be collected for EV isolation. EV isolation protocol needs to be such that allows for isolation in an accurate and cost-effective way. The isolation technique needs to guarantee purity of the isolate but also a large yield of EVs. The isolated EVs need to be characterized in an effective way to ensure the isolate quality. The isolate then needs to be stored in such way that enables quick implementation of the stored isolate.

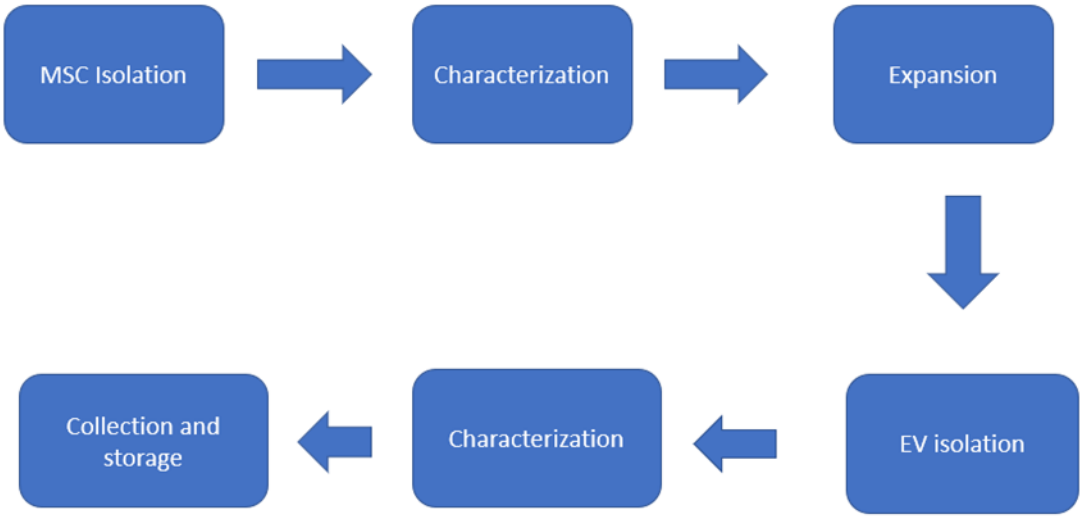


Figure 4 The workflow of making MSC derived EVs. After MSC isolation the cells need to be characterized by the minimal requirements introduced earlier in this thesis: adherence to plastic, the appropriate surface markers and differentiation into osteoblast, adipocyte and chondroblast. The cell numbers are then expanded by cell culture passaging and the media from the cell culture is collected and the EVs are then isolated. The EVs produced by the cell cultures need to be characterized to ensure absence of contaminants and the presence of EVs. The EVs can then be collected in appropriate concentrations and cryostored for later use.

4. CONCLUSIONS

Multipotent mesenchymal stromal cells and their secretome offers an exciting premise for therapeutic applications in the field of regenerational medicine. MSCs ability to shape the site of injury towards a healing microenvironment is truly a unique characteristic and harnessing this ability could mean an unseen breakthrough for cell therapy or cell-based therapy applications. The extracellular vesicles secreted by these cells could potentially carry the healing properties of these cells. If the regenerational and immunomodulatory aspect of MSCs could be captured in their secretome it could mean a ready-to-go clinical do-it-all application. This however, requires breaking the code on what makes the MSC tick. MSCs are able to respond to the molecular factors mobilized by the injured environment or pathological state and act accordingly secreting trophic factors and interacting with the immune cells. Whether it is possibly to wake up this process in a laboratory setting is one of the biggest deciding factors of therapeutic use of MSC derived EVs. There are a lot of reports praising the effect of EVs on multiple pathological states but the translation into clinical application remains undone.

The problematic part of MSC research is the large amount of promising data in animal disease models but an unquestionable lack of basic research. The ambiguous research methods and even unprecise reporting have led to a situation where the acquired data is hard to interpret and the true identity of the MSCs and their secretome remains a mystery. It has been even brought up to question whether the suggested phenotype of MSCs even exist *in vivo* and whether the MSC as we know it, is just a product of cell culture environment. Since the identification methods of MSCs have been developed referencing culture expanded MSCs the field might be accidentally ignoring the true holy grail of cell therapy tools. The lack of surface markers and reliable isolation techniques make it difficult to analyse MSC derived EVs and map their contents and effects. All in all, the field needs elaboration on the very basics of MSC and EV biology.

5. REFERENCES

Ahmed, A. S. I., Sheng, M. H., Wasnik, S., Baylink, D. J., & Lau, K.-H. W. (2017). Effect of aging on stem cells. World Journal of Experimental Medicine, 7(1), 1. https://doi.org/10.5493/WJEM.V7.I1.1

Alvarez-Erviti, L., Seow, Y., Yin, H., Betts, C., Lakhal, S., & A Wood, M. J. (2011). Delivery of siRNA to the mouse brain by systemic injection of targeted exosomes. https://doi.org/10.1038/nbt.1807

Andaloussi, S. el, Mäger, I., Breakefield, X. O., & A Wood, M. J. (2013). Extracellular vesicles: biology and emerging therapeutic opportunities. https://doi.org/10.1038/nrd3978

Ankrum, J. A., Ong, J. F., & Karp, J. M. (2014). Mesenchymal stem cells: immune evasive, not immune privileged. Nature Biotechnology 2014 32:3, 32(3), 252–260. https://doi.org/10.1038/nbt.2816

Ario, M., Ergio, S., Falc, A., Dos, H., Sena Brunel, S., Peixer, M. A. S., Dallago, B. S. L., Costa, F. F., Mauro Queiroz, L., Campbell, P., & Malard, P. F. (2019). Effect of allogeneic mesenchymal stem cells (MSCs) on corneal wound healing in dogs. https://doi.org/10.1016/j.jtcme.2019.04.006

Bartholomew, A., Sturgeon, C., Siatskas, M., Ferrer, K., McIntosh, K., Patil, S., Hardy, W., Devine, S., Ucker, D., Deans, R., Moseley, A., & Hoffman, R. (2002). Mesenchymal stem cells suppress lymphocyte proliferation *in vitro* and prolong skin graft survival *in vivo*. Experimental Hematology, 30(1), 42–48. https://doi.org/10.1016/S0301-472X(01)00769-X

Bian, Z.-Y., Li, G., Gan, Y.-K., Hao, Y.-Q., Xu, W.-T., & Tang, T.-T. (2009). Increased Number of Mesenchymal Stem Cell-like Cells in Peripheral Blood of Patients with Bone Sarcomas [Article]. Archives of Medical Research, 40(3), 163–168. https://doi.org/10.1016/j.arcmed.2009.01.002

Brooke, G., Cook, M., Blair, C., Han, R., Heazlewood, C., Jones, B., Kambouris, M., Kollar, K., McTaggart, S., Pelekanos, R., Rice, A., Rossetti, T., & Atkinson, K. (2007). Therapeutic applications of mesenchymal stromal cells [Article]. Seminars in Cell & Developmental Biology, 18(6), 846–858. https://doi.org/10.1016/j.semcdb.2007.09.012

Caplan, A. I. (2008). All MSCs Are Pericytes? In Cell Stem Cell (Vol. 3, Issue 3). https://doi.org/10.1016/j.stem.2008.08.008

Caplan, A. I., & Dennis, J. E. (2006). Mesenchymal stem cells as trophic mediators [Article]. Journal of Cellular Biochemistry, 98(5), 1076–1084. https://doi.org/10.1002/jcb.20886

Caruso, S., & Poon, I. K. H. (2018). Apoptotic cell-derived extracellular vesicles: More than just debris. Frontiers in Immunology, 9(JUN), 1486. https://doi.org/10.3389/FIMMU.2018.01486/BIBTEX

Chavakis, E., Urbich, C., & Dimmeler, S. (2008). Homing and engraftment of progenitor cells: A prerequisite for cell therapy. Journal of Molecular and Cellular Cardiology, 45(4), 514–522. https://doi.org/10.1016/J.YJMCC.2008.01.004

Cheng, R.-J., Xiong, A.-J., Li, Y.-H., Pan, S.-Y., Zhang, Q.-P., Zhao, Y., Liu, Y., & Marion, T. N. (2019). Mesenchymal Stem Cells: Allogeneic MSC May Be Immunosuppressive but Autologous MSC Are Dysfunctional in Lupus Patients [Article]. Frontiers in Cell and Developmental Biology, 7, 285–285. https://doi.org/10.3389/fcell.2019.00285

Ciferri, M. C., Quarto, R., & Tasso, R. (2021). Extracellular vesicles as biomarkers and therapeutic tools: From pre-clinical to clinical applications [Article]. Biology (Basel, Switzerland), 10(5), 359. https://doi.org/10.3390/biology10050359

Dominici, M., le Blanc, K., Mueller, I., Slaper-Cortenbach, I., Marini, F. C., Krause, D. S., Deans, R. J., Keating, A., Prockop, D. J., & Horwitz, E. M. (2006). Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. Cytotherapy, 8(4), 315–317. https://doi.org/10.1080/14653240600855905

English, K. (2013). Mechanisms of mesenchymal stromal cell immunomodulation. Immunology and Cell Biology, 91(1), 19–26. https://doi.org/10.1038/ICB.2012.56

Friedenstein, A. J., Petrakova, K. v, Kurolesova, A. I., & Frolova, G. P. (1968). Heterotopic of bone marrow. Analysis of precursor cells for osteogenic and hematopoietic tissues [Article]. Transplantation, 6(2), 230–247.

Fujii, S., Miura, Y., Fujishiro, A., Shindo, T., Shimazu, Y., Hirai, H., Tahara, H., Takaori-Kondo, A., Ichinohe, T., & Maekawa, T. (n.d.). Graft-Versus-Host Disease Amelioration by Human Bone Marrow Mesenchymal Stromal/Stem Cell-Derived Extracellular Vesicles Is Associated with Peripheral Preservation of Naive T Cell Populations. https://doi.org/10.1002/stem.2759

Girousse, A., Mathieu, M., Sastourné-Arrey, Q., Monferran, S., Casteilla, L., & Sengenès, C. (2020). Endogenous Mobilization of Mesenchymal Stromal Cells: A Pathway for Interorgan Communication? [Article]. Frontiers in Cell and Developmental Biology, 8, 598520–598520. https://doi.org/10.3389/fcell.2020.598520

Gurung, S., Perocheau, D., Touramanidou, L., & Baruteau, J. (2021). The exosome journey: from biogenesis to uptake and intracellular signalling. Cell Communication and Signaling 2021 19:1, 19(1), 1–19. https://doi.org/10.1186/S12964-021-00730-1

Hall, J., Prabhakar, S., Leonora Balaj, •, Charles, •, Lai, P., Cerione, R. A., Xandra, •, & Breakefield, O. (n.d.). Delivery of Therapeutic Proteins via Extracellular Vesicles: Review and Potential Treatments for Parkinson's Disease, Glioma, and Schwannoma. https://doi.org/10.1007/s10571-015-0309-0

Horwitz, E. M., Gordon, P. L., Koo, W. K. K., Marx, J. C., Neel, M. D., McNall, R. Y., Muul, L., & Hofmann, T. (2002). Isolated Allogeneic Bone Marrow-Derived Mesenchymal Cells Engraft and Stimulate Growth in Children with Osteogenesis Imperfecta: Implications for Cell Therapy of Bone [Article]. Proceedings of the National Academy of Sciences - PNAS, 99(13), 8932–8937. https://doi.org/10.1073/pnas.132252399

Kavanagh, H., & Mahon, B. P. (2011). Allogeneic mesenchymal stem cells prevent allergic airway inflammation by inducing murine regulatory T cells. Allergy, 66(4), 523–531. https://doi.org/10.1111/J.1398-9995.2010.02509.X

Konala, V. B. R., Mamidi, M. K., Bhonde, R., Das, A. K., Pochampally, R., & Pal, R. (2015). The current landscape of the mesenchymal stromal cell secretome: A new paradigm for cell-free regeneration [Article]. Cytotherapy (Oxford, England), 18(1), 13–24. https://doi.org/10.1016/j.jcyt.2015.10.008

Li, P., Kaslan, M., Han Lee, S., Yao, J., & Gao, Z. (2017). Progress in Exosome Isolation Techniques. Theranostics, 7(3), 789–804. https://doi.org/10.7150/thno.18133

Medina, J. P., Pérez-Baos, S., Naredo, E., López-Reyes, A., Herrero-Beaumont, G., & Largo, R. (2018). AB0075 Intraarterial injection of human adipose-derived mesenchymal stem cells (HAD-MSCS) attenuates inflammation in acute arthritis model [Article]. Annals of the Rheumatic Diseases, 77(Suppl 2), 1236. https://doi.org/10.1136/annrheumdis-2018-eular.5489

Nakamura, T., Shirouzu, T., Nakata, K., Yoshimura, N., & Ushigome, H. (2019). The Role of Major Histocompatibility Complex in Organ Transplantation- Donor Specific Anti-Major Histocompatibility Complex Antibodies Analysis Goes to the Next Stage -. International Journal of Molecular Sciences, 20(18). https://doi.org/10.3390/IJMS20184544

Pan, B.-T., & Johnstone, R. M. (1983). Fate of the transferrin receptor during maturation of sheep reticulocytes *in vitro*: Selective externalization of the receptor [Article]. Cell, 33(3), 967–978. https://doi.org/10.1016/0092-8674(83)90040-5

Phinney, D. G., & Pittenger, M. F. (2017). Concise Review: MSC-Derived Exosomes for Cell-Free Therapy [Article]. Stem Cells (Dayton, Ohio), 35(4), 851–858. https://doi.org/10.1002/stem.2575

Prockop D. J. (2009). (n.d.). Repair of tissues by adult stem/progenitor cells (MSCs): controversies, myths, and changing paradigms. Molecular therapy : the journal of the American Society of Gene Therapy, 17(6), 939–946. https://doi.org/10.1038/mt.2009.62. https://doi.org/10.1038/mt.2009.62

Rebolj, K., Veber, M., Drobnič, M., & Maličev, E. (2018). Hematopoietic stem cell and mesenchymal stem cell population size in bone marrow samples depends on patient's age and harvesting technique [Article]. Cytotechnology (Dordrecht), 70(6), 1575–1583. https://doi.org/10.1007/s10616-018-0250-4

Roddy, G. W., Oh, J. Y., Lee, R. H., Bartosh, T. J., Ylostalo, J., Coble, K., Rosa, R. H., & Prockop, D. J. (2011). Action at a Distance: Systemically Administered Adult Stem/Progenitor Cells (MSCs) Reduce Inflammatory Damage to the Cornea Without Engraftment and Primarily by Secretion of TNF-α Stimulated Gene/Protein 6. Stem Cells, 29(10), 1572–1579. https://doi.org/10.1002/STEM.708

Saint-Pol, J., Gosselet, F., Duban-Deweer, S., Pottiez, G., & Karamanos, Y. (2020). Targeting and Crossing the Blood-Brain Barrier with Extracellular Vesicles. Cells, 9(4). https://doi.org/10.3390/CELLS9040851

Scarfe, L., Taylor, A., Sharkey, J., Harwood, R., Barrow, M., Comenge, J., Beeken, L., Astley, C., Santeramo, I., Hutchinson, C., Ressel, L., Smythe, J., Austin, E., Levy, R., Rosseinsky, M. J., Adams, D. J., Poptani, H., Park, B. K., Murray, P., & Wilm, B. (2018). Non-invasive imaging reveals conditions that impact distribution and persistence of cells after *in vivo* administration. Stem Cell Research and Therapy, 9(1), 1–17. https://doi.org/10.1186/S13287-018-1076-X/FIGURES/6

Sheng, Y., Zhou, | Xiaohong, Wang, J., Shen, H., Wu, S., Guo, W., Yang, | Yi, & Yang, Y. (2021). O R I G I N A L A R T I C L E MSC derived EV loaded with miRNA-22 inhibits the inflammatory response and nerve function recovery after spinal cord injury in rats. J Cell Mol Med, 25, 10268–10278. https://doi.org/10.1111/jcmm.16965

Soung, Y. H., Ford, S., Zhang, V., & Chung, J. (2017). Exosomes in cancer diagnostics. In Cancers (Vol. 9, Issue 1). https://doi.org/10.3390/cancers9010008

Spaggiari, G. M., Capobianco, A., Becchetti, S., Mingari, M. C., & Moretta, L. (2006). Mesenchymal stem cell-natural killer cell interactions: evidence that activated NK cells are capable of killing MSCs, whereas MSCs can inhibit IL-2-induced NK-cell proliferation. Blood, 107(4), 1484–1490. https://doi.org/10.1182/BLOOD-2005-07-2775

Ståhl, A. lie, Johansson, K., Mossberg, M., Kahn, R., & Karpman, D. (2019). Exosomes and microvesicles in normal physiology, pathophysiology, and renal diseases. Pediatric Nephrology (Berlin, Germany), 34(1), 11. https://doi.org/10.1007/S00467-017-3816-Z

Sung, D. K., Chang, Y. S., Sung, S. I., Ahn, S. Y., & Park, W. S. (2019). Thrombin Preconditioning of Extracellular Vesicles Derived from Mesenchymal Stem Cells Accelerates Cutaneous Wound Healing by Boosting Their Biogenesis and Enriching Cargo Content. Journal of Clinical Medicine, 8(4). https://doi.org/10.3390/JCM8040533

Ullah, M., Liu, D. D., & Thakor, A. S. (2019). Mesenchymal Stromal Cell Homing: Mechanisms and Strategies for Improvement. IScience, 15, 421. https://doi.org/10.1016/J.ISCI.2019.05.004

Wang, X., Bai, L., Liu, X., Shen, W., Tian, H., Liu, W., & Yu, B. (2021). Cardiac microvascular functions improved by MSC-derived exosomes attenuate cardiac fibrosis after ischemia–reperfusion via PDGFR-β modulation [Article]. International Journal of Cardiology, 344, 13–24. https://doi.org/10.1016/j.ijcard.2021.09.017

Wang, Y., Huang, J., Gong, L., Yu, D., An, C., Bunpetch, V., Dai, J., Huang, H., Zou, X., Ouyang, H., & Liu, H. (2019). The Plasticity of Mesenchymal Stem Cells in Regulating Surface HLA-I. IScience, 15, 66. https://doi.org/10.1016/J.ISCI.2019.04.011

Waterman, R. S., Tomchuck, S. L., Henkle, S. L., & Betancourt, A. M. (2010). A New Mesenchymal Stem Cell (MSC) Paradigm: Polarization into a Pro-Inflammatory MSC1 or an Immunosuppressive MSC2 Phenotype. PLoS ONE, 5(4). https://doi.org/10.1371/JOURNAL.PONE.0010088

Weiner, L. P. (2008). Definitions and Criteria for Stem Cells. Methods in Molecular Biology, 438, 3–8. https://doi.org/10.1007/978-1-59745-133-8_1

Xu, L., & Li, G. (2014). Circulating mesenchymal stem cells and their clinical implications [Article]. Journal of Orthopaedic Translation, 2(1), 1–7. https://doi.org/10.1016/j.jot.2013.11.002

Zhou, T., Yuan, Z., Weng, J., Pei, D., Du, X., He, C., & Lai, P. (2021). Challenges and advances in clinical applications of mesenchymal stromal cells. In Journal of Hematology and Oncology (Vol. 14, Issue 1). https://doi.org/10.1186/s13045-021-01037-x

Zhu, Y.-Z., Hu, X., Zhang, J., Wang, Z.-H., Wu, S., & Yi, Y.-Y. (2020). Extracellular Vesicles Derived From Human Adipose-Derived Stem Cell Prevent the Formation of Hypertrophic Scar in a Rabbit Model. https://doi.org/10.1097/SAP.0000000000002357

Zuo, R., Liu, M., Wang, Y., Li, J., Wang, W., Wu, J., Sun, C., Li, B., Wang, Z., Lan, W., Zhang, C., Shi, C., & Zhou, Y. (2019). BM-MSC-derived exosomes alleviate radiation-induced bone loss by restoring the function of recipient BM-MSCs and activating Wnt/β-catenin signaling [Article]. Stem Cell Research & Therapy, 10(1), 30–30. https://doi.org/10.1186/s13287-018-1121-9