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Isolation, Characterisation and function of Extracellular Vesicles derived from Mesenchymal Stem Cells

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Abstract

Mesenchymal stem cells (MSCs) are capable of self-renewal and differentiation into osteoblasts, chondrocytes and adipocytes. MSCs have been investigated in both animal models as well as human clinical trials for tissue regeneration with various degrees of success. Recent research suggests that MSC therapeutic efficacy may be mediated by small extracellular vesicles (EVs). EVs are lipid bilayer membraned vesicles bearing proteins, lipids and nucleic acids. They mediate intercellular communication between adjacent neighbour cells or distant cells to facilitate diverse biological and pathological processes. Their potential for therapeutic, diagnostic and biotechnological application have attracted enormous interest in many areas of medical research including regenerative medicine and may have the potential to address several unmet medical needs. However, the lack of standardization of the isolation of MSC-derived EVs and analysis methods restrict the utility of MSC-derived EVs in clinical settings. In this thesis, I firstly focused on optimisation of MSC-derived EV isolation method. Four MSC derived EV samples were isolated from human adipose derived MSC culture medium by differential ultracentrifugation, with three different ultracentrifuge durations to investigate the influence of ultracentrifuge time on quality and quantity of MSC-derived EVs. Additionally, a commercial kit was used to extract EVs from MSC cultured medium and compared it with the ultracentrifugation method. Samples were then characterised for the particle concentration, protein concentration, particle size and detection of EV protein markers by western blot and FACS. When all the results were compared across the five different isolation methods, 70 minutes of differential ultracentrifugation was optimal to isolate high quality and quantity of MSC-derived EVs from MSC cultured medium.

Furthermore, EVs could be taken up by recipient cells to trigger phenotypic effects. Understanding the EV uptake mechanisms can lead to the development of the EV therapeutic strategies. However, EV uptake mechanisms and intracellular fate of EVs still remain elusive. An experiment was designed to investigate MSC derived EV uptake by MSCs. MSC-derived EVs isolated from younger individual, via our previously optimised method, were fluorescently labelled *in vitro* and incubated with the parental MSCs as well as MSCs from an older individual, to compare MSC derived EV uptake in the cells by confocal microscopy and flow cytometry. In addition, we examined the differences in EV uptakes between freshly isolated MSC-derived EVs and frozen MSC-derived EVs. The results confirmed that both freshly isolated and frozen MSC-derived EVs could be taken up by MSCs, including their parental MSCs. Freshly isolated MSC-derived EVs appeared to be more bioactive as they gave much stronger intracellular fluorescent signals than frozen EVs. These results suggest that using freshly isolated MSC-derived EVs is the best condition for further MSC-derived EV functional analysis.

The functional effects of MSC-derived EV on MSCs were examined by qPCR technique, comparing MSCs incubated with or without MSC-derived EVs. Freshly isolated MSC-derived EVs derived from a young patient with osteoarthritis (OA) due to a sport injury were added into MSC cell lines derived from their parental young patient or an older OA patient. Three groups of genes included pluripotent, differentiation and MSC cell surface marker genes were investigated by qPCR. This comparison highlighted that MSC-derived EVs from a younger individual have significant effects on old aged MSCs by increasing expression of genes for pluripotency, differentiation gene and MSC surface markers except CD90 gene. These in vitro experimental results suggest that MSC-derived EVs could potentially have therapeutic potential as they are able to alter gene expression in the recipient cells.

Overall, the isolation and characterisation methods and the potential benefits of EVs described in this thesis would encourage further research to explore and discover the therapeutic potential of MSC-derived EVs. I believe one day MSC-derived EVs can become an “off-the shelf” cell-free therapeutic.

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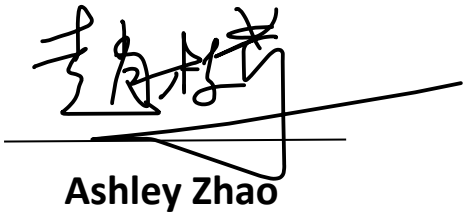
Declaration

I, Ashley Zhao declare this submitted thesis contains no material which has not been accepted for the award to the candidature of any other degree or diploma, except where due reference is made in the text of the examinable outcome. To the best of my knowledge, this thesis contains no materials previously published or written by another author except where due reference is made in the text of the examinable outcome; and where the work is based on joint research or publications, discloses the relative contributions of the respective workers or authors.



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Publications

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List of Abbreviations

MSCs	Mesenchymal stem cells
EV	Extracellular Vesicle
OA	Osteoarthritis
ISCT	International Society for Cellular Therapy
ISEV	International Society of Extracellular Vesicles
MVB	Multivesicular bodies
ILV	Intraluminal Vesicle
ESCRT	the Endosomal Sorting Complex Required for Transport
PI(3)P	phosphatidylinositol 3-phosphate
PS	phosphatidylserine
FACS	fluorescence-activated cell sorter
SEM	scanning electron microscopy

Chapter 1

Literature Review

Extracellular vesicles (EVs), which are released by all eukaryotic cells and some prokaryotic cells, contain nucleic acids, proteins and lipids and are present in many biological fluids. These EVs play important biological roles in cellular homeostasis and the spreading of biomolecules to their neighbouring cells, and therefore possess significant potentials in biotechnology (Colombo, Raposo, & Thery, 2014; Gould & Raposo, 2013). Mesenchymal stem cells (MSCs) are multipotent stem cells and have been used as ideal candidates for tissue regeneration and tissue engineering in the last three decades (Klimczak & Kozlowska, 2016). More recently MSC-derived EVs have been gained much attention for their therapeutic potential, studied in both animal models and various clinical applications in many disease areas (Gatti et al., 2011; Lai, Chen, & Lim, 2011; Rani, Ryan, Griffin, & Ritter, 2015; Zhang et al., 2018; Zhao, Shah, Cromer, & Sumer, 2020). MSC-derived EVs may be used as an alternative, cell-free therapy instead of MSCs as they have several advantages, such as they have lower immunogenicity, capacity to cross biological barriers and less safety concerns. In this thesis, we focus on the biology and the potential therapeutic properties of MSC-derived EVs.

This chapter starts by providing introductions to MSCs, discusses EVs which includes the details of EV biogenesis, composition, EV uptake and function, and current methods of isolation and characterisation. MSC-derived EVs are then described and finally, an outline of the thesis is provided.

1.1 Mesenchymal stem cells

MSCs are originally derived from mesenchyme which is developed from the mesoderm (Barry & Murphy, 2004). MSCs are multipotent stem cells which have the ability to self-renew and differentiate into connective and skeletal tissues such as bone, fat, cartilage and muscle (Bianco, 2014). MSCs were firstly described as stromal precursors by Friedenstein and colleagues in 1974 (Friedenstein, Chailakhyan, Latsinik, Panasyvk, & Keiliss-Borok, 1974). The term Mesenchymal stem cell is coined by Caplan in 1991 (Caplan, 1991). The main roles of resident MSCs are self-repair and maintenance of tissue homeostasis in *vivo*, and thus MSCs are relatively abundant throughout the body. Due to their plastic adherence property, MSCs can be easily isolated from various organs and tissues such as bone marrow, adipose tissue, muscle tissue, skin, teeth, periosteum, trabecular bone, synovium, skeletal tissues, brain, spleen, liver, kidney, thymus, pancreas and blood vessels (Bianco, 2014; da Silva Meirelles, Chagastelles, & Nardi, 2006). MSCs are considered to be ideal candidates for tissue regeneration and tissue engineering, and have dramatically increased biological and clinical interest over the last three decades (Klimczak & Kozlowska, 2016). Because MSCs are heterogeneous, and have different tissue sources within the body, there is non-consensus on the isolation and expansion methods. Thus, there were difficulties and confusion in comparing MSCs across studies and experimental outcomes. The Mesenchymal Stem Cell Committee of the International Society for Cellular Therapy (ISCT) has proposed three minimal criteria to define human MSC (Dominici et al., 2006).

1. MSC must be plastic adherent in tissue culture flasks maintained in standard culture conditions.
2. Over 95% of MSC population must express CD105, CD73 and CD90 and lack expression (≤2%) of CD45, CD34, CD14 or CD11b, CD79α or CD19 and HLA class II.
3. MSCs must be able to differentiate into osteoblasts, adipocytes and chondrocytes in *vitro* under standard differentiating conditions.

MSCs can be expanded *in vitro* due to their self-renewal capacity and also can be differentiated into various cell types due to their multipotent properties (Pittenger et al., 1999). Meanwhile, transplanted MSCs can secrete a wide range of bioactive factors to build up a regenerative local microenvironment in a paracrine manner (Rehman et al., 2004). Due to their capacity for differentiation, trophic, and paracrine functions, MSCs have become a powerful tool in regenerative medicine and in clinical therapeutic applications such as in cardiovascular, neural and orthopaedic therapeutic applications (Barry & Murphy, 2004). There are over two thousand MSC clinical trials registered on clinicaltrials.gov in many diseases such as bronchopulmonary dysplasia, multiple sclerosis, autoimmune diseases, alzheimer's disease, liver diseases, osteoarthritis, kidney disease, myocardial infarction, and graft versus host disease. Furthermore, this Masters degree project is a collaboration with Magellan Stem Cell Centre which involved in treating osteoarthritis patients by using MSCs. Here, we focus on the details of osteoarthritis, a degenerative medical condition for which currently there is no cure or long-term treatment available, and the use of autologous MSCs to treat this chronic condition.

Osteoarthritis (OA), is one of the most common chronic disabling diseases, is usually associated with the synovial joints which formed between two bones' ends covered with articular cartilage, a capsule filled with the synovial fluid, ligaments, tendons, muscles, blood vessels and nerves (Goldring & Goldring, 2007). Any changes in those structural components lead to anabolic or catabolic responses in other components (Säämänen, Arokoski, Jurvelin, & Kiviranta, 2010). An abnormality in synovial joint tissues causes the stress in the joint, resulting in degeneration of articular cartilage, associated with hypertrophy of bone and thickening of the capsule, and eventually lead to the clinical symptoms of OA such as stiffness, pain, limitation of movement, and variable degrees of local inflammation (Eckstein, Burstein, & Link, 2006; Woolf & Pfleger, 2003). The non-neural, non-lymphatic, nonvascular articular cartilage is restricted in self repair. Therefore the progressive degeneration of articular cartilage is considered as irreversible stage degeneration (Loeser, Goldring, Scanzello, & Goldring, 2012). In the 21st century, using MSCs to treat OA provides an exciting promise for regeneration of the damaged articular cartilage. In the clinical studies, autologous MSCs isolated from a patient's own bone marrow or adipose tissues, purified, cultured and injected back into the affected joint under ultra sound guidance have shown great promise (Shah, Zhao, & Sumer, 2018). Such MSC therapy for OA has resulted in good outcomes for over 70% of the treated patients. However, various studies have shown that there are non-responders to this therapy shown by the various studies (Boyd et al., 2019). To understand the underlying reasons for non-responders to MSCs therapy for OA, compared to the responders, the biology of MSCs therapeutic efficacy needs to be unravelled and the exact mechanism of action needs to be established.

In a variety of MSC therapeutic applications, MSCs were initially used to replace injured cells based on their differentiation potential. However, less than 1% of the transplanted MSCs could reach to the target tissue, such as the infarcted myocardium in treatment of myocardial infarction (Barbash et al., 2003). Nonetheless, MSCs could restore the heart function more rapidly when compared with the

slow and inefficient differentiation process of cardiomyocytes (Lai et al., 2011). MSCs have also demonstrated, both *in vitro* and *in vivo*, the modulation of immune responses, tissue repair and regeneration in many MSC therapeutic applications. It has been proposed that MSCs exert their beneficial effects by paracrine secretion rather than from their differentiation (Baglio et al., 2015). Thus MSCs effect on tissue repair may be by altering the microenvironment via paracrine signalling (Meirelles, Fontes, Covas, & Caplan, 2009), rather than by cell differentiation, which is how the most MSC clinical trials were rationalized. However, to date, none of the identified soluble secreted mediators discovered have been shown to sufficiently mediate the MSC therapeutic effects (Ghannam, Bouffi, Djouad, Jorgensen, & Noel, 2010). Subsequently many studies have revealed that the paracrine effects of MSCs are mediated, at least in part, by secretion of EVs, especially exosomes (Bruno et al., 2012; Lai et al., 2010). Therefore, current research focus on the mechanism of therapeutic actions of MSCs, attributed to paracrine efficacy, now focused on investigation of EVs, which are described in the next section.

1.2 Extracellular vesicles (EVs)

All eukaryotic cells, as well as some prokaryotic cells release EVs packed with cellular cargo containing nucleic acids, proteins and lipids into extracellular space (Colombo et al., 2014). Released EVs are present in many biological fluids such as serum, cerebrospinal fluid, saliva, urine, nasal secretions and breast milk. EVs play important biological roles, contribute to many diseases and possess significant potential in biotechnology (Gould & Raposo, 2013). Many different names have been used for EVs in the different independent discovery and led to confusing nomenclature. As the extracellular vesicle field keeps growing, the International Society for Extracellular Vesicles (ISEV) was launched in 2011 and aimed to advance extracellular vesicle research globally. The term EVs was introduced by ISEV to describe preparations of vesicles isolated from biofluids and cell cultures (Gould & Raposo, 2013). Based on their biogenesis pathway, EVs can be classified into three main classes (Table 1). They are exosomes (40-120 nm), microvesicles (50-1000 nm) and apoptotic bodies (500-2000 nm) (El Andaloussi, Maeger, Breakefield, & Wood, 2013). More recently, the experimentally prepared EVs by many isolation methods have been suggested to broadly divide into two main categories: exosomes and microvesicles (van Niel, D'Angelo, & Raposo, 2018).

Table1: Different types of EVs

Vesicles	Size (nm)	Density (g/mL)	Origins
Exosomes	40-120	1.13-1.19	Intraluminal budding of MVBs, release by fusion of MVBs with cell membrane
Microvesicles	50-1000	Not well defined	Outward budding of cell membrane
Apoptotic bodies	500-2000	1.16-1.28	Outward blebbing of apoptotic cell membrane

Exosomes are formed in the endocytic pathway. During early endosomal development they mature towards the late endosome processes, Intraluminal vesicles (ILVs) form by inward budding of the limiting membrane and accumulate in the lumen of endosomal compartments (Colombo et al., 2014). These vesicle-containing endosomes are referred as multivesicular bodies/endosomes (MVBs). MVBs have two fates to fuse with lysosomes to degrade, or fuse with the plasma membrane to release ILVs known as exosomes (Harding, Heuser, & Stahl, 2013). Therefore, Exosomes are derived from

endocytic pathway, generated from the intraluminal budding of multivesiclar bodies, and released by fusion of multivesiclar bodies with the plasma membrane (El Andaloussi et al., 2013). Microvesicles directly bud from the plasma membrane (Figure 1). Even though they are generated at different sites within the cell (Microvesicles at the plasma membrane and exosomes at the limiting membrane of the multivesiclar body), the common intracellular mechanisms and sorting machineries are involved in both microvesicle and exosome biogenesis. This also further results in difficulty to distinguish between EV subtypes (Colombo et al., 2014).

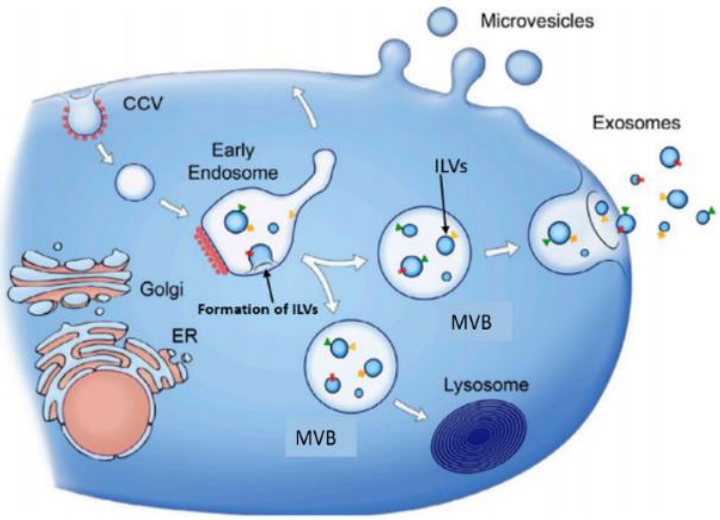


Figure 1: Release of MVs and exosomes. The image is adapted from Raposo & Stoorvogel, 2013. Firstly the early endosome forms through endocytosis. Then ILVs form by inward budding of the limiting membrane and accumulate in the lumen to generate MVBs. MVBs have two fates, to fuse with lysosomes to degrade of to fuse with the plasma membrane to release ILVs known as exosomes. Microvesicles bud directly from the plasma membrane.

Exosomes play important biological roles in many aspects of biology such as intercellular vesicle traffic, immunity, neurobiology, and microbiology, contribute to many diseases such as cancer, neurodegenerative disorders and HIV. Most importantly, exosomes possess tremendous therapeutic and medical biotechnological potential e.g., clinical and therapeutic applications using exosome instead of cells for cell therapy, and may serve important clinical tools such as biomarkers, drug delivery device and vaccines (Harding et al., 2013; Raposo & Stoorvogel, 2013). In this section of literature review, we will focus, specifically on exosomes.

1.2.1 Exosomes

1.2.1.1 History

The term exosome was firstly used for the plasma membrane derived vesicles by Trams and colleagues in 1981 (Trams, Lauter, Salem, & Heine, 1981). Later, the releasing of nanovesicles by mammalian reticulocytes through endosomal pathway was discovered by Pan, Harding and colleagues in the early 1980s (Harding, Heuser, & Stahl, 1984; Pan & Johnstone, 1983; Pan, Teng, Wu, Adam, & Johnstone, 1985). The mammalian reticulocytes secreted nanovesicles formed within multivesicular endosomes/bodies (MVEs or MVBs) and fused with the cell membrane during maturation process. The secreted vesicles were considered as waste products, containing membrane proteins such as transferrin receptors, which become useless in mature red blood cells. In 1987, Rose Johnstone

proposed the term exosome to refer to the released membrane vesicles derived by MVBs (Johnstone, Adam, Hammond, Orr, & Turbide, 1987). After a decade of its discovery, exosomes have been isolated from cell culture media of B lymphocytes by differential centrifugation followed by sucrose density gradients (Raposo et al., 1996). The released exosomes contained compact MHC II can result in T cell responses in recipient cells. Thus, exosomes were speculated to function as transport vehicles. Soon after, exosomes derived from dendritic cells have been represented their therapeutic function for suppressing tumor growth (Raposo et al., 1996; Zitvogel et al., 1998). An important breakthrough came when it was discovered that exosomes carried nucleic acids - mRNA and miRNA (Valadi et al., 2007). Exosomal mRNA can be transferred and translated to proteins in the recipient cells. Exosomal miRNA are believed to be involved in specific gene expression and protein translation in recipient cells. Therefore, Exosomes are considered to play an important role in intercellular communication through transfer of proteins, lipids and nucleic acids into recipient cells (El Andaloussi et al., 2013; Mathivanan, Ji, & Simpson, 2010; Simons & Raposo, 2009). Exosomes are secreted by various cell types including hematopoietic, non-hematopoietic and epithelial cells, nervous tissue and cancer cells and are present in the culture supernatant of these cells. As mentioned earlier, exosomes are also present in biological fluid such as plasma, saliva, breast milk, urine, amniotic fluid, ascites fluid, cerebrospinal fluid (Rezaie et al., 2018) and help facilitate various cellular activities.

1.2.1.2 Exosome biogenesis

Exosomes are ILVs with a variety of different sizes. They are internalised into the lumen of the endosomal compartments and released by fusion with the plasma membrane (Raposo & Stoorvogel, 2013). Exosome biogenesis firstly starts in the endosomal system where cells internalize macromolecules and particles to form the early endosomes in the plasma membrane through endocytosis (Huotari & Helenius, 2011). The early endosomes communicate with the *trans-Golgi network* (TGN) through bidirectional vesicle exchange during maturation of the late endosomes. The early endosomes are located towards the peripheral cell membrane with tubular shape and late endosomes are spherical and closed to the nucleus (Mellman, 1996). They further mature into late endosomes, ILVs invaginate from the outer endosomal membrane and bud into the lumen of endosomes (Colombo et al., 2014). The early endosomes are weakly acidic and contain a relatively low Ca^{2+} concentration (Huotari & Helenius, 2011) and are regulated of maturation to the late endosomes when Rab5 switches to Rab7. The late endosomes with spherical shape, formation of ILVs, low luminal pH range (from pH6 to 6.0-4.9) with decreased buoyant density and increased negative surface charge (Huotari & Helenius, 2011) generally are known as MVBs which was firstly discovered by Palay and Palade in 1955 (Palay & Palade, 1955). The different subpopulations of MVBs coexist in cells and have two fates—degradation or secretory (Colombo et al., 2014; Keller, Sanderson, Stoeck, & Altevogt, 2006). MVBs can fuse with lysosomes for degradation of their membrane and contents. Alternatively, MVBs might fuse with plasma membrane to release ILVs as exosomes into the extracellular space. The cholesterol-riched MVBs, MVBs bearing the tetraspanin CD63, lysosomal-associated membrane proteins LAMP1 and LAMP2, MHC II are prone to undergo secretive pathway (Mobius et al., 2002; Raposo et al., 1996). Destination of MVBs is likely regulated by the dynamic changes of the composition in the limiting membrane of MVBs where lipids and proteins continuously are internalised into ILVs (van Niel et al., 2018).

To generate exosomes, many cellular processes are involved, such as MVBs/ILVs formation during the early endosomes mature into MVBs, trafficking and fusion of MVBs to the plasma membrane (Hessvik & Llorente, 2018).

1.2.1.2.1 Formation of ILVs

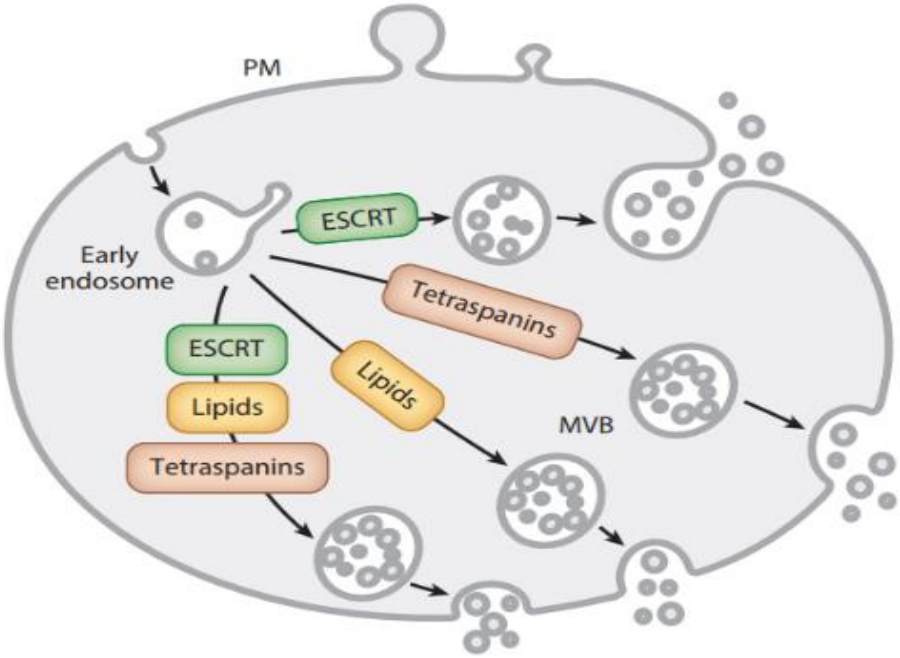


Figure 2: Formation of ILVs involved various machineries. The image is adapted from Colombo et al., 2014. Multiple machineries are involved in formation of IVLs to generate MVBs. Each machinery (ESCRT, Lipids and tetraspanins machinery) can generate MVBs. However, it is unknown whether each of them acts in the different MVBs, or they can act together on the same MVBs.

Several cellular mechanisms are involved in the formation of ILVs and maturation of MVBs. There is the endosomal sorting complex required for transport (ESCRT) dependent and ESCRT-independent mechanisms which involved lipids, tetraspanins (Figure 2), or heat shock proteins. The different mechanisms might be involved in the same endosomal compartment to form different subpopulation of MVBs, or different machineries might be used to target the same cargo during MVB maturation (Buschow et al., 2010). Meanwhile, several mechanisms simultaneously act on ILV formation to sort diverse cargos at different stage of MVB maturation resulting different subpopulation of IVLs/MVBs co-exist (Edgar, Eden, & Futter, 2014). However, it still unknown how the different mechanism acts on the different population of MVBs, and how the multiple mechanisms take place in same MVB (Colombo et al., 2014).

1.2.1.2.2 ESCRT-dependent sorting mechanism

The best-described mechanism for formation of ILVs is the endosomal sorting complex required for transport (ESCRT)-dependent machinery. The ESCRTs were firstly discovered for their central role of sorting membrane proteins from endosomes to lysosomes (Katzmann, Babst, & Emr, 2001). ILVs are formed from early endosomes by the inward budding of the limiting membrane, and then process the

scission of the narrow neck of bud to release bud into the endosomal lumen. ESCRT proteins sort the ubiquitinated proteins into these buds (Hurley, 2015). The four ESCRT complexes ESCRT-0, ESCRT-I, ESCRT-II, ESCRT-III were well described for formation of ILVs into the interior of MVBs in the early 2000s (Babst, Katzmann, Snyder, Wendland, & Emr, 2002; Katzmann et al., 2001; Katzmann, Stefan, Babst, & Emr, 2003).

Table 2: The ESCRT complexes. Adapted from Hanson & Cashikar, 2012

ESCRT complexes	Protein name	Function
ESCRT-0	Hrs	Cluster cargos and recruit ESCRT-I
	STAM1	
ESCRT-I	TSG101/Vps23	Attach to ubiquitin, recruit ESCRT-II
	Vps28	
	Vps37	
	Mvb12	
ESCRT-II	Vps36	Attach to ubiquitin and ESCRT-I, induce inward budding formation and recruit ESCRT-III
	Vps22	
	Vps25 X 2	
ESCRT-III	Vps20/CHMP6	Attach to ESCRT-I and II, narrow the neck of the inward budding vesicle
	Snf7/Vps32/CHMP4	
	Vps24/CHMP3	
	Vps2/CHMP2	
Accessory proteins	Alix	Attach to ESCRT-III to control ILV formation
	Did2/CHMP1	Recruit Vps4
	Doa4	Remove ubiquitin for recycle, disassemble ESCRT complexes to complete cycle
	Vps4	
	Vta1/LIP5	

ESCRT machinery contains approximately 30 proteins to congregate into four distinct heteropolymeric complexes ESCRT-0, ESCRT-I, ESCRT-II, ESCRT-III and different accessory molecules such as Vps4, Vta1, Alix which only transiently located on the cytosolic side of the endosomal membrane. Each complex contains different subunits plays different roles for formation and dissection of ILVs through inward budding of the MVB membrane (Table 2)(Hanson & Cashikar, 2012).

Ubiquitination is one of the important sorting signals for sequestration of cargo in ILVs (Fevrier & Raposo, 2004). The ubiquitinated cargo (on the lysine residues) accumulate at the limiting membrane (Katzmann et al., 2001) which contains a mosaic of subdomains located mostly in the tubular extensions of early endosomes (Bonifacino & Rojas, 2006). Clathrin likely plays a role for organization the protein sorting machinery or retaining Ub-sorting receptors at the limiting membrane (Piper & Katzmann, 2007). The accumulated ubiquitinated proteins can be identified and captured by ESCRT machinery which is shown in Figure 3.

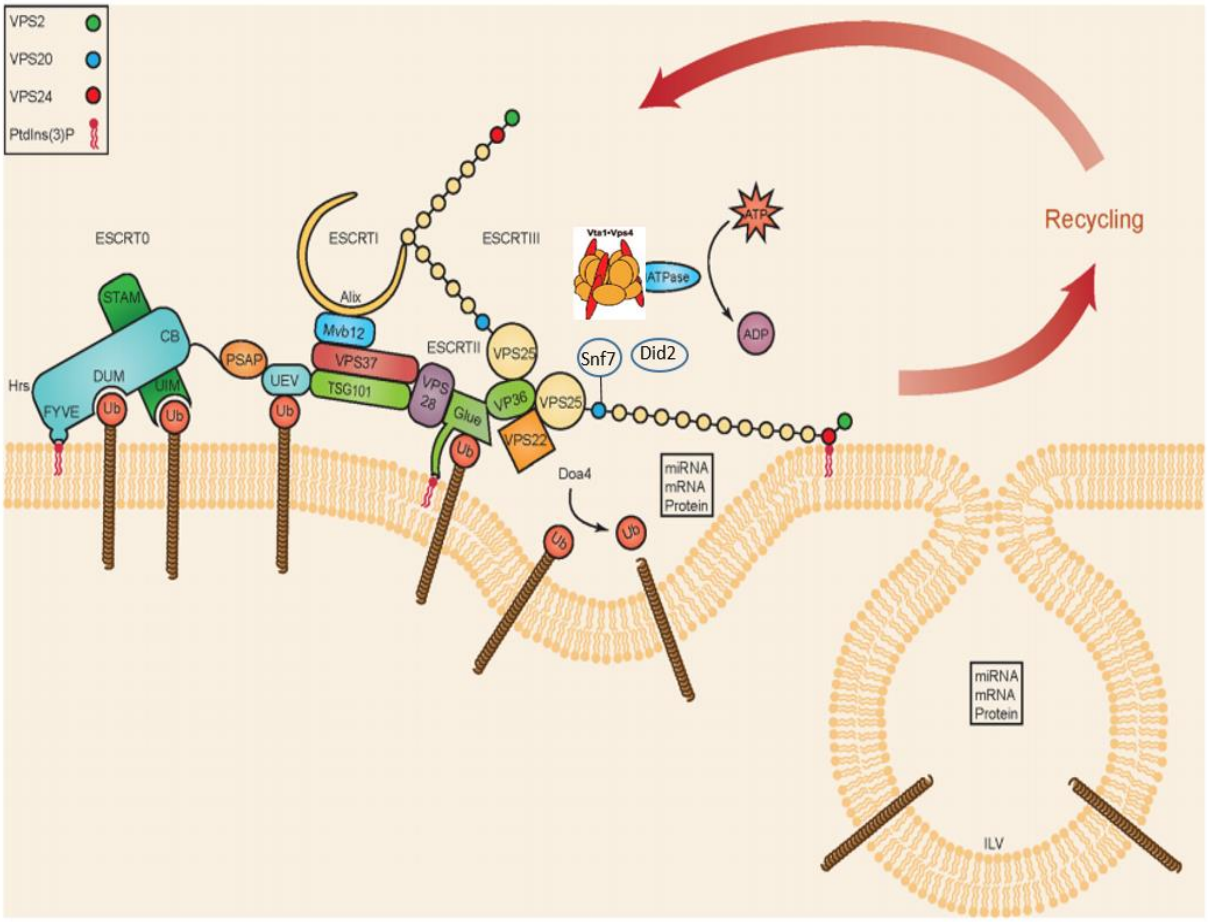


Figure 3: The ESCRT-dependent sorting mechanism. The image is adapted from Rezaie et al., 2018.
ESCRT mechanism is constitute of four complexes: ESCRT-0, -I, -II, -III which are recruited on the cytosolic side of endosomal membrane. Firstly ESCRT-0 is recruited, bind with PI(3)P and ubiquitinated proteins, interact with TSG101 subunit of ESCRT-I to recruit ESCRT-I complex and followed to recruit ESCRT-II complex and initiate the inward curvature on the endosomal membrane. ESCRT-II recalls ESCRT-III to extend away the budding vesicle from cytoplasm and narrow the neck of the inward budding vesicles. The ESCRT associated proteins Alix, Did2, Vps4, Vta1 and Doa4 are recruited by ESCRT-III to control formation of ILVs, cleave the vesicles, remove ubiquitin for recycle and disassemble ESCRT system.

ESCRT-0 consists of two subunits-HRS and STAM. HRS firstly recruits the flat clathrin coat and this HRS-clathrin coat might concentrate ubiquitylated cargo into microdomains and then readily engage with ESCRT-I (Williams & Urbe, 2007). ESCRT-0 contains five ubiquitin-binding motifs including a FYVE domain for binding with phosphatidylinositol 3-phosphate PI(3)P, a PSAP motif for recruitment of TSG101 in ESCRT-I complex, a clathrin-binding motif (Hanson & Cashikar, 2012), a UIM motif for binding ubiquitinated proteins (Williams & Urbe, 2007). PI(3)P is generated by phosphatidylinositol 3-kinase (PI(3)K) and Vps34 recruited by Rab5 GTPase. PI(3)P is enriched on the cytosolic face of early endosomes but not in late endosomes (Huotari & Helenius, 2011). It recruits many proteins to specialized endosomal subdomain (Piper & Katzmann, 2007). ESCRT-0 complex firstly is recruited by binding PI(3)P and ubiquitinated cargo on the endosomal membrane (Katzmann et al., 2003). Then direct interaction between ESCRT-0 with TSG101 in ESCRT-I complex recruits ESCRT-I to the exosomal membrane and this recruitment is also enhanced by ubiquitinated cargo.

ESCRT-I is essential for sorting cargo into MVBs (Katzmann et al., 2001) and contains TSG101, VPS28,VPS37 and MVB12 (Hurley, 2008). ESCRT-I recruits ESCRT-II followed recruitment of ESCRT-III subunits to form a large polymer on the endosomal membrane (Piper & Katzmann, 2007). ESCRT-II

consists of each Vps22, Vps36 and two copies of Vps25 (Babst et al., 2002). The C-terminal winged-helix of Vps25 interacts with Vps20 in ESCRT-III complex to recruit ESCRT-III (Teo, Perisic, Gonzalez, & Williams, 2004; Williams & Urbe, 2007). ESCRT-I/ESCRT-II system is one of the core ESCRT machinery which function as one branch of ESCRT pathway (the other branch is ALIX) to feed into ESCRT-III and Vps4 scission machinery (Hurley, 2015).

ESCRT-III consist of Vps2 (charged multivesiclar body protein CHMP2 in mammals), Vps24 (CHMP3), Snf7 (CHMP4) and Vps20 (CHMP6) proteins to form two subcomplexes (Williams & Urbe, 2007). Vps20 recruits Snf7 and follows the recruitment of Vps24. Snf7 forms loose and flat circular array on the endosomal membrane by binding with a co-expressed Vps4 to develop a curved membrane-binding surface and line tubules extended away from cytoplasm (Hanson, Roth, Lin, & Heuser, 2008). Snf7 also recruits ESCRT-III associated protein Alix which acts as stabilizing Snf7 and recruitment of deubiquitinating enzyme Doa4 (Henne, Buchkovich, & Emr, 2011). Vps24 provide membrane-binding interface to stabilize ESCRT-III on the endosomal membrane (Williams & Urbe, 2007). Vps24 and Vps2 form tubules to narrow the neck of the inward budding vesicle. Not as other complexes, ESCRT-III has no ubiquitin-recognizing module. However, ESCRT-III can directly bind to the Vps28 component in ESCRT-I complex and the ESCRT-II component Vps25. Therefore, ESCRT-III may also position the protein sorting machinery (Hanson & Cashikar, 2012).

There are few ESCRT associated proteins such as Alix, Did2, Vps4, Vta1 (LIP5 in mammal) and Doa4. Alix is recruited by Snf7. It interacts with syntenin and syndecan to control the formation of ILVs (Baietti et al., 2012). ESCRT-III related protein Did2 (CHMP1) recruits Vps4. The vesicle scission step is controlled by Vps4 (Lata et al., 2008). C-terminal microtubule-interacting and transport (MIT) interacting region of ESCRT-III bind to the MIT domain of the AAA+ ATPase Vps4 which consists of two hexameric rings (Williams & Urbe, 2007). Vps4 and Vta1 (LIP5 in mammals) form a complex and play a role to disassemble ESCRT-III and the other ESCRT complexes to complete the cycle. Without Vps4 function, ILV formation is inhibited therefore the ESCRT complexes would accumulate on the endosomal membrane (Hanson & Cashikar, 2012). ESCRT-III binds to the deubiquitinated protein Doa4 to remove ubiquitin for recycle, and also facilitates the disassembly of ESCRT machinery from the endosomal membrane (Williams & Urbe, 2007).

Therefore, the ESCRT machinery starts from the interaction of the ESCRT-0 complex with the ubiquitylated proteins which are originated from Golgi apparatus. ESCRT-0 complex interacts with TSG101 subunit of the ESCRT-I complex to recruit ESCRT-II and start inward budding of the ILVs into lumen of the MVBs. ESCRT-II recalls the ESCRT-III complex to narrow the neck of the inward budding vesicles. Finally, Vps4 and Vta1 cleave the ILV into free vesicle and disassemble ESCRT complexes. Some ESCRT components and accessory proteins such as TSG101, HRS and ALIX are retained in the ILVs and became important protein markers of exosomes. However, there is an argument if they are the specific markers for exosomes since ESCRT-I/II/III and their accessory molecules are associated with various budding and membrane scission processes such as microvesicle releasing, wound repair on plasma membrane, neuron pruning, membrane abscission in cytokinesis, nucleus envelope reformation, cellular autophagy processes etc (Hurley, 2015). Alternatively, ESCRT-0 has been just described in exosome secretion, not in plasma membrane budding and scission processes. Therefore, ESCRT-0 components might be the specific markers to demonstrate the endosomal origin (Mathieu, Martin-Jaular, Lavieu, & Thery, 2019).

1.2.1.2.3 ESCRT-independent sorting system

Without ESCRTs, ILVs can still be formed in MVBs through ESCRT-independent mechanism. Many studies suggested the ESCRT-independent mechanisms are involved in ILV formation and exosome biogenesis. The ESCRT-independent mechanisms involve lipids (ceramide, cholesterol and PLD2), tetraspanins, or heat shock proteins (Kowal, Tkach, & Thery, 2014).

Exosomes are enriched in ceramide. Ceramide is cone-shaped structure to induce inward curvature of the membrane to promote membrane invagination. Ceramides are important to promote lateral phase separation and domain formation, to induce membrane permeabilization, transbilayer lipid movements and membrane fusion and fission (Goni & Alonso, 2006), and furthermore to facilitate vesicle biogenesis (Simons & Raposo 2009). The depletion of the ESCRT subunits such as Hrs, TSG101, Alix or Vps4, exosomes enriched in proteinlipid protein (PLP) and CD63 were still secreted through ceramide-dependent sorting mechanism. Ceramide is formed after the hydrolytic sphingomyelin by sphingomyelinase. Depletion of sphingomyelinase reduced exosome release (Trajkovic et al., 2008). Ceramide could be subjected to many metabolic destination included sphingosine 1-phosphate to active MVB Gi-protein-coupled sphingosine phosphate receptor which is essential to sort cargo into exosomal ILVs and to mature MVBs (Kajimoto, Okada, Miya, Zhang, & Nakamura, 2013). Cholesterol is enriched in ILVs. If block MVB formation, the large cholesterol-rich endosomes are accumulated (Bishop & Woodmane, 2000). Meanwhile, inducing of cholesterol within endosomes can stimulate the formation of ILVs. Therefore, cholesterol facilitate the budding process of ILVs (Piper & Katzmann, 2007). Phospholipase D2 (PLD2) is enriched in exosomes as well. It involves in hydrolysis of phosphatidylcholine to phosphatidic acid (PA) which function as ceramide could induce inward curvature to form ILVs in the limiting membrane of MVBs (Ghossoub et al., 2014).

Tetraspanins involve into the sorting system (Zoller, 2009). Tetraspanins are transmembrane proteins which cross the membrane four times with a small intracellular loop, one small extracellular loop and one large extracellular loop. They can associate with cholesterol and gangliosides to form higher order tetraspanin in microdomains. Tetraspanins are highly expressed in membranes of various endocytic organelles to involve into a multitude of biological processes (Perez-Hernandez et al., 2013). Tetraspanin-enriched microdomains are specific membrane platforms which tetraspanins molecularly associate with lipids, selected transmembrane proteins such as integrins and metalloproteinases. Tetraspanin-enriched microdomains are used as entryways by some pathogens such as hepatitis C, HIV and papilloma virus. Tetraspanins could form cluster and dynamic membrane platforms with other transmembrane and cytosolic proteins to regulate the formation of the microdomains and furthermore for the internalization (Charrin, Jouannet, Boucheix, & Rubinstein, 2014). Tetraspanin-enriched microdomains ligands form a network of interactions and play a pivotal role in stabilizing the exosomal structure. Tetraspanins such as CD63 are enriched in MVBs and involve in the formation of ILVs in ESCRT-independent and ceramide-independent system (Simons & Raposo, 2009; van Niel et al., 2011). Tetraspanin CD81 with a cone-like structure is regulated their function by cholesterol bound within the intramembrane cavity (Zimmerman et al., 2016). Several cone-shaped tetraspanins clustered together might induce the microdomain inward budding (van Niel et al., 2018). Also, CD81 proposed as exosomal protein sorting platforms since the deletion of CD81 impaired the proportion

of selective proteins into exosomes (Perez-Hernandez et al., 2013). The tetraspanins CD9, CD81, CD82 have the role for sorting various cargos into exosomes (Buschow et al., 2010).

The major heparan sulfate-presenting protein-syndecans are found in exosomes (Baietti et al., 2012). Syntenins-the specific cytoplasmic adaptor proteins can sort syndecans into exosomes. The YPXL motif of syntenin directly binds to ALIX to support syndecan exosome formation. A small integral membrane protein of lysosomes/late endosomes SIMPLE can also regulate CD63- and Alix-exosome secretion but not flotillion-exosomes (Zhu et al. 2013). Finally, the heat shock cognate 70kDa protein (HSC70) and the chaperones heat shock 70kDa (HSP) interacts with transferrin receptor (TfR) of reticulocyte to recruit TfR into exosomes (Geminard, de Gassart, Blanc, & Vidal, 2004). Therefore, hsc70 acts as an adaptor to involve in the sorting selective cytosolic proteins into ILVs (Sahu et al., 2011).

Overall, exosome formation is quite complex which not only involves in many mechanisms, but is also highly depended on the cell type and could be impacted by other biological and pathological stimuli (van Niel et al., 2018). Many studies have discovered significant ILV formation pathways, however, the exosome biogenesis is still not exhaustively studied yet since the current knowledge of exosome biogenesis is not fully specific to the exosome secretion, and also not valid in all cell types (Mathieu et al., 2019). Further studies on exosome biogenesis are still needed.

1.2.1.3 MVB trafficking and fusion mechanisms

Once the late endosomes become fully matured MVB, MVBs are transported to the cell periphery and fuse with the plasma membrane to release ILVs. The mechanism of MVB mobilization, docking and fusion still remain unknown. The cytoskeleton including actin and microtubules, associated molecular motors such as kinesins and myosins, molecular switches (small GTPases), the fusion machinery such as tethering factors and SNAREs are involved in these procedure (Raposo & Stoorvogel, 2013). Proteins and protein complexes called tethers are the initial interaction between a vesicle and the target membrane and involve in nearly all membrane-trafficking events. Tethers work together with Rab proteins to direct the vesicle targeting (Cai, Reinisch, & Ferro-Novick, 2007).

Rab proteins compose of the largest family of monomeric small GTPases and function as molecular switches between GTP- and GDP-bound conformations which the conversion is caused by nucleotide exchange. The activated Rab proteins are GTP-bound form, and they recruit Rab effectors onto the cytosolic side of the intracellular membranes. Through their effectors, Rab GTPase regulate vesicle formation, trafficking and fusion, mobility through interaction of vesicles with cytoskeletal components, tethering/docking of vesicles to the target compartment (Zerial & McBride, 2001). The Rab family play a significant role in MVB trafficking to the plasma membrane for exosome secretion (Hsu et al., 2010; Ostrowski et al., 2010; Stenmark, 2009).The first Rab protein discovered in exosome secretion was Rab11 in K562 cells. K562 cells reduced TfR exosome secretion under the inhibition of Rab11 (Savina, Vidal, & Colombo, 2002). Rab7 plays a role of regulating late endosomal traffic downstream of MVBs and sequestration of cargo (Vanlandingham & Ceresa, 2009). Knockdown of Rab7 results in large endosomes and severely reduce exosome releasing (Baietti et al., 2012). Five Rab proteins Rab2b, Rab9a, Rab5a, Rab27a, Rab27b involve in the exosome secretion of Hela cells. Also silencing Rab27a and Rab27b effectors-Slp4 and Slac2b respectively could alter Rab27a and Rab27b and inhibit exosome secretion. Furthermore, knockdown of Rabs27a and Rab27b inhibit exosome

secretion by reducing MVBs docking to the plasma membrane, but without modifying exosome protein composition. Thus Rab27a and Rab27b play a key role for promoting MVBs to the cell periphery and docking to the plasma membrane (Ostrowski et al., 2010). Rab27 might also be involved in the trafficking and tethering of MVBs to the plasma membrane (Raposo & Stoorvogel, 2013). Moreover, inhibition of Rab35 function causes endosomal vesicle accumulate in the intracellular space and result in reduced exosome secretion (Hsu et al., 2010). These results implicate Rab35 in MVB trafficking.

Thery and colleagues hypothesised that multiple EV secretion machineries are regulated by Rab proteins (Figure 4) (Colombo et al., 2014). The factors involved in exosome secretion are Rab27-dependent machinery for late endosomes, Rab35- and Rab11-dependent machinery for early or recycling endosomes and Rab7-dependent machinery for late endosomes to release Alix/syntenin-exosomes.

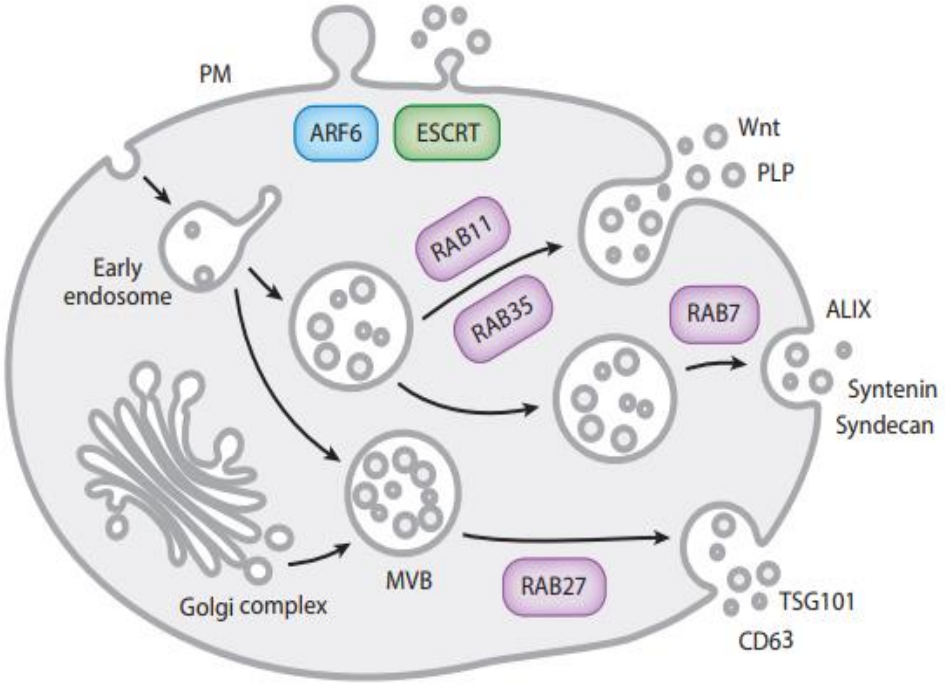


Figure 4: The multiple secretion machineries of EVs. The image is adapted from Colombo et al., 2014. RAB proteins involve in the different MVB secretion. RAB11 and Rab35 act on early MVBs and RAB7 and RAB27 act on the late MVBs to promote exosome releasing.

Recently, the Ras-related GTPase homolog (Ral-1) is reported to involve in the MVB formation and fusion with the plasma membrane (Hyenne et al., 2015). The depletion of Ral-1 reduces the exosome secretion. Additionally, the increasing intracellular Ca^{2+} level condition induced Rab11a-Munc13-4 dependent trafficking pathway to elevate exosome release in human cancer cell line (Messenger, Woo, Sun, & Martin, 2018). Overall, mechanism of exosome secretion can be influenced by many aspects such as cell line, environment, some inductions and analysis via different techniques. MVB trafficking requires the actin and microtubule cytoskeletons, motor proteins to transport and tether MVBs to the plasma membrane (Granger, McNee, Allan, & Woodman, 2014). However, their role in EV secretion have not been explored their role of EV secretion due to their multiple cellular function. The diverse non-specific effects would be induced by targeting them to study their roles in MVB

trafficking and docking (Mathieu et al., 2019). After MVBs dock to the plasma membrane, the Soluble N-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs) regulate the fusion of the lipid bilayers with the plasma membrane to release ILVs (Essandoh & Fan, 2015). Many proteins are involved in the intracellular fusion reactions. SNAREs are the core fusion engine in membrane fusion and are recycled after each fusion event (Jahn & Scheller, 2006).

SNARE proteins have a simple domain structure—SNARE motif. The typical SNARE proteins consist of a different type of folded N-terminal domain, a SNARE motif and a C-terminal transmembrane domain. According to the SNARE motif, SNARE proteins are classified into four subfamilies Qa-, Qb-, Qc-(t-) and R- (v-) SNAREs which are highly conserved and diverged early in eukaryotic evolution (Fasshauer, Sutton, Brunger, & Jahn, 1998). They are assembled in a *trans* configuration and formed as a helical core complex which is mediated by the SNARE motifs. The assembly starts at the N termini of the SNARE motif followed by a zipper-like fashion towards the C-terminal membrane anchors. The function of SNARE complexes is to provide the mechanical force exerted on the membrane to proceed the fusion of two lipid bilayers and then distort membranes to form a fusion pore to release contents of vesicle (Jahn & Scheller, 2006). The tethering proteins also play a crucial role to fusion pore formation (D'Agostino, Risselada, Lurick, Ungermann, & Mayer, 2017). SNARE complexes are transformed from *trans*-configuration to *cis*-configuration when the fusion is completed. Furthermore, SNARE complexes are disassembled by a hexameric AAA+ protein NSF (provides energy of disassembly) combined with cofactors SNAPs (soluble NSF attachment proteins) which can bind to the middle of the SNARE complexes (Jahn & Scheller, 2006). The diagram in Figure 5 shows the MVB fusion with the plasma membrane and the SNARE conformational cycle.

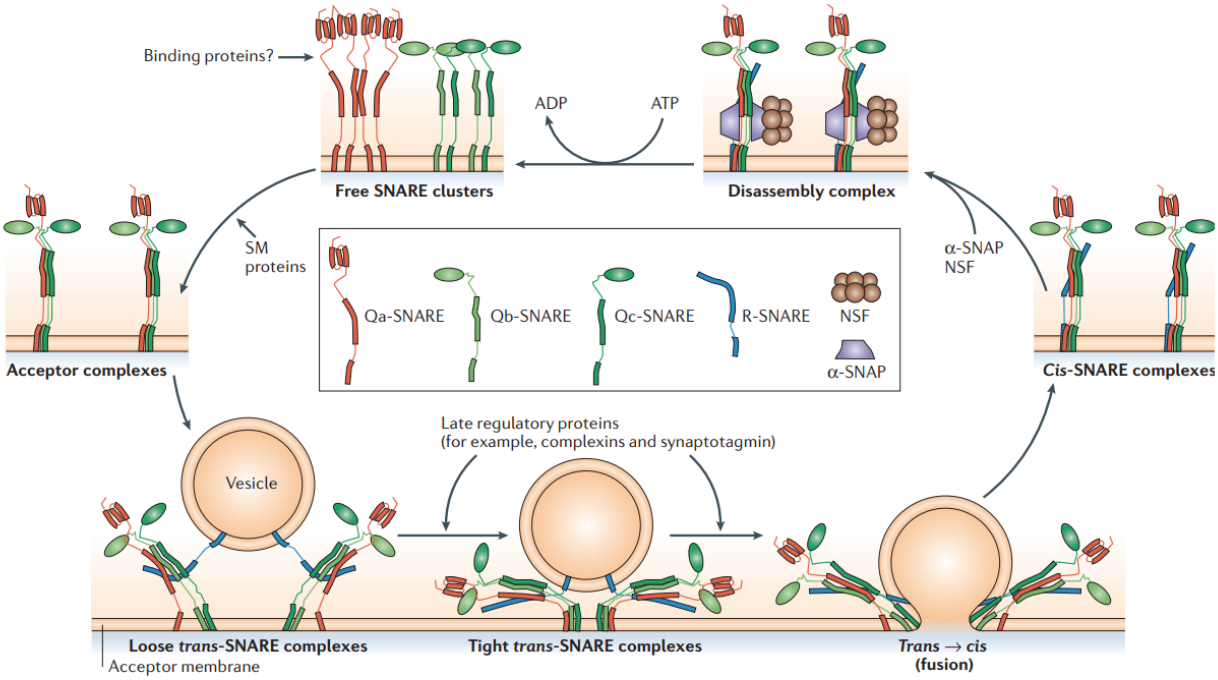


Figure 5: Fusion MVB with plasma membrane and the SNARE conformational cycle. The image is taken from Jahn & Scheller., 2006. Three Q-SNAREs on the membrane assembled into acceptor complex interact with R-SNARE on a vesicle resulting in formation of a four-helical *trans*-complex to form a fusion pore. Then *trans*-complex relaxes into a *cis*-configuration and is disassembled by sorting.

However, the molecular machinery for MVB fusion with plasma membrane to release exosomes is not well understood (Hessvik & Llorente, 2018). Vesicle-associated membrane protein 7 (VAMP7) is one of the R-SNARE proteins (Fader, Sanchez, Mestre, & Colombo, 2009). The study showed VAMP7 is essential for exosome release in human K562 cells. Inhibition of VAMP7 impaired SNARE complexes formation, results in MVBs enlarged and accumulated in the cell periphery and reducing exosome secretion. Ykt6 is one of the R-SNAREs with longin domain. It can use the lipid anchor to transiently associate with membrane (Daste, Galli, & Tareste, 2015). The inhibition of Ykt6 leads to reduced TSG101- and Wnt-exosomes secretion in HEK293 cells , and also reduced TSG101-exosome release in A549 human cells (Ruiz-Martinez et al., 2016). One study also showed the absence of Q-SNARE protein-syntaxin 5 (SYX5) caused MVB accumulation under the plasma membrane (Hyenne et al., 2015).

1.2.1.4 Sorting of cargo into exosomes

Exosomes contain heterogeneous proteins, lipids and nucleic acids. However, it is still unclear how the cargos are sorted into ILVs (Hessvik & Llorente, 2018). A study showed the ubiquitinated proteins can be transferred into exosome (Cheng & Schorey, 2016). The ubiquitination might be a mechanism to sort target proteins into exosomes through ESCRT system (Smith, Jackson, & Schorey, 2015). Some proteins such as MHC II do not rely on ubiquitination to sort into exosomes. The proteins such as HSC71, HSP90, 14-3-3 epsilon, CD20 and pyruvate kinase type M2 (PKM2) might help to sort MHC II into exosomes (Buschow et al., 2010). Lipids are also important for sorting specific proteins into exosomes (Hessvik & Llorente, 2018). The lipid raft domains in the exosomal membrane can associate to the specific proteins such as lyn, flotillin-1 and stomatin and release those proteins into extracellular space (de Gassart, Geminard, Fevrier, Raposo, & Vidal, 2003). Sphingosine 1-phophate can sort proteins such as CD63, CD81 and flotillin into exosomes (Kajimoto et al., 2013). Lipids such as ceramide also play a role for RNA sorting into exosomes. Inhibition of ceramide impairs exosomal miRNA secretion (Kosaka et al., 2010). RNA molecules are packaged into exosomes with highly selectivity. mRNA localization rely on the interaction between *cis*-acting elements and *trans*-acting factors (Jambhekar & Derisi, 2007). The short specific motifs of RNAs are enriched in exosomes but not in cytosolic RNAs. Those motifs might function as cis-acting elements targeting RNAs into exosomes (Batagov, Kuznetsov, & Kurochkin, 2011). Later on, another study showed the sumoylated hnRNPA2B1 (heterogeneous nuclear ribonucleoprotein A2B1) protein could recognise and bind to those motifs of miRNAs resulting in loading miRNAs into exosomes (Villarroya-Beltri et al., 2013). The RNA binding complex ESCRT-II may function as sorting RNAs into exosome (Raposo & Stoorvogel, 2013). Also, MVBs are sites of miRNA-loading RISC accumulation that function as miRNA loading (Gibbings, Ciaudo, Erhardt, & Voinnet, 2009). More recently, mutant KRAS protein serves as sorting miRNAs into exosomes (Cha et al., 2015). Arrestin-domain containing protein 1 (Arrdc1), an adaptor for the Nedd4 family of ubiquitin ligases not only regulates the release of exosomes and microvesicles, but also regulates the sorting of protein cargo into EVs (Anand et al., 2018). However, most findings of cargo sorting in exosome are still in the early stage. More investigations are needed to interpret the integral exosomal sorting system.

1.2.1.5 Composition of exosomes

The composition of each subtype of exosomes is unknown as it is difficult to purify them by the differential ultracentrifugation or elimination of subpopulations by immunoisolation (Colombo et al.,

2014). Also, the composition of exosomes depends on the donor cell type and is influenced by different cellular conditions or treatments (Hessvik & Llorente, 2018). The composition of exosomes might play a role in regulation of exosome formation (Ostrowski et al., 2010). Here the current state of exosome contents is summarised (Figure 6).

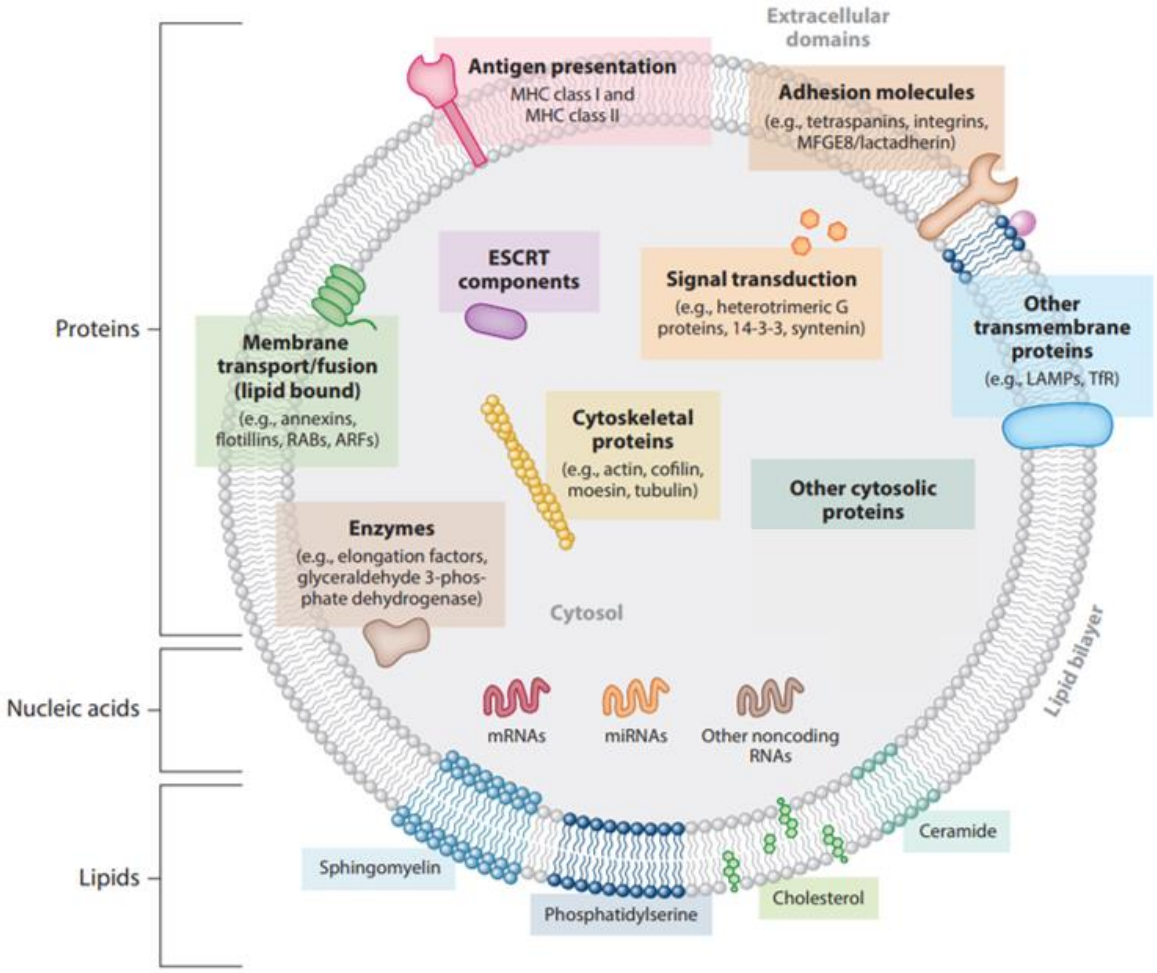


Figure 6: Composition of exosomes. The image is taken from Colombo et al., 2014. Schematic diagram represents the contents of exosome including proteins, lipids, and nucleic acids.

1.2.1.5.1 Proteins

Investigating the presence of the proteins in the exosomes, compare with varied sources of exosomes and understanding their underlying function remain a necessity and provide a huge potential to unravel the function of exosomes in the cellular mechanisms. Because of exosome origin, exosomes contain the endosome associated proteins such as Rab GTPase, SNAREs, Annexins and flotillin which are involved in the MVB trafficking and fusion, the ESCRT proteins such as TSG101 and Alix which are involved in ILV formation, the membrane proteins such as tetraspanins (CD63, CD81, CD82 etc…), glycosylphosphatidylinositol (GPI)-anchored proteins and flotillin which are clustered into microdomains at plasma/endosomal membrane (Raposo & Stoorvogel, 2013). Tetraspanins belong to a superfamily of proteins, highly enriched in EVs, involved in biogenesis, cargo selection, targeting of EVs, and widely used as EV markers (Andreu & Yanez-Mo, 2014). Exosomal proteins are absent of proteins from the nucleus, mitochondria, endoplasmic reticulum and the Golgi complex. They are

normally the proteins from endosomes, the plasma membrane, the cytosol and sometimes depending on the secreted cell types (Colombo et al., 2014).

1.2.1.5.2 Lipids

Cholesterol, sphingomyelin and hexosylceramides are enriched in exosomes which reflects the formation of ILVs from the endosomal membrane. Exosome fatty acids are mostly saturated or monosaturated (Raposo & Stoorvogel, 2013). It was found that lipids were selectively included in exosomes. Glycosphingolipids, sphingolipids, cholesterol and phosphatidylserine (PS) are enriched in exosomes compared with the donor cells (Llorente et al., 2013; Skotland, Hessvik, Sandvig, & Llorente, 2019). More investigation indicated the lipids in exosome are derived from endocytosed lipid rafts in the plasma membrane (Skotland et al., 2019; Tan et al., 2013) and elevated cholesterol, sphingomyelin and anionic lipids comparing with the plasma membrane of cells which generated EVs. Interestingly, phosphatidylserines and phosphatidylethanolamines are in the outer leaflet of EV membrane instead of the inner leaflet as found on the plasma membrane. This might be important for EV function since phosphatidylserine exposure might facilitate EV uptake (Russell et al., 2019). The unique EV lipid composition has been suggested to play a role in increasing EV circulation times *in vivo* (Russell et al., 2019). However, another study found that EV lipids did not promote extended circulation times (Smyth et al., 2015). Until now, the cellular function of EV lipids remains largely unknown.

1.2.1.5.3 Nucleic acids

One important discovery in exosome research is that they carry the functional mRNA and miRNA to mediate cell to cell communication and have critical roles in transcription and gene expression. Micro RNAs (miRNAs) are endogenous non-coding RNA (ncRNA) with approximately 22 nucleotides and can interact with mRNAs through recognition of imperfect complementary sites to influence the target mRNAs (Bartel, 2004). Therefore, they play an important role in posttranscriptional regulation of gene expression. Even though EV miRNAs were estimated to be less than one copy per EV (Chevillet et al., 2014), some EVs might be enriched with certain miRNAs. Furthermore, each miRNA can multi-regulate the expression of several proteins (Tanase, Ogrezeanu, & Badiu, 2012). Therefore, recently more researchers are interested in and working on EV miRNA discovery. The first exosomal RNA study reported approximately 121 miRNAs and 1300 mRNAs specifically packaged into exosomes of mast cells. The mouse exosomal mRNA can be transferred into human mast cells and can be translated into proteins in human mast cells (Valadi et al., 2007). miRNAs in exosomes can be transferred into the target cells to activate or repress their cellular pathways at the post-transcription level, making these important influencers in vital cellular processes. When comparing these with the donor cells, exosomes contain small RNAs but no or little ribosomal RNAs. Exosomes might serve as a diagnostic biomarker (Kosaka et al., 2010; Pegtel et al., 2010). Distinct miRNA signature were found in circulating exosomes released by prion-infected neuronal cells (GT1-7) (Bellingham, Coleman, & Hill, 2012). When comparing with non-infected exosomes, the infected exosomes represented miRNAs let-7b, let-7i, miR-128a, miR-21, miR-222, miR-29b, miR-342-3p and miR-424 with increasing levels and miR-146 with decreasing levels. Therefore, miRNAs in circulating exosomes can be used as diagnostic biomarkers for prion disease. Also, long non-protein coding RNAs are found to be enriched in exosomes (Batagov et al., 2011). Long noncoding RNAs have important role in numbers of cellular processes such as chromatin remodelling, transcriptional and post-transcriptional regulation. Moreover, exosomes have been found to carry the enriched 3'-untranslated regions (UTRs) of mRNAs

which can serve as binding sites for RNA-binding proteins to modulate stability and translational efficiency of mRNAs, and miRNA target site for repression of mRNAs (Batagov & Kurochkin, 2013).

1.2.2 Microvesicles

Similar to exosomes, many machineries are involved in microvesicle biogenesis. Unlike exosome biogenesis which was intensively studied, microvesicle biogenesis has only recently started to emerge (van Niel et al., 2018). Microvesicles classified are also as ectosomes as they are directly generated from the plasma membrane (Heijnen, Schiel, Fijnheer, Geuze, & Sixma, 1999). Microvesicles are generated by the formation of outward buds in specific sites of the membrane and then released into extracellular space by fission (J. Ratajczak, Wysoczynski, Hayek, Janowska-Wieczorek, & Ratajczak, 2006). Several molecular rearrangements included changes in lipid and protein composition and even Ca^{2+} level at the specific sites of membrane elicit in membrane budding (Pap, Pallinger, Pasztoi, & Falus, 2009; Piccin, Murphy, & Smith, 2007). Ca^{2+} level changes could alter the lipid composition of the plasma membrane. Externalization of phosphatidylserine also play a role for the microvesicle formation(Al-Nedawi, Meehan, & Rak, 2009). Microvesicles are enriched with cholesterol, and it was shown that microvesicles are raised from cholesterol-rich lipid rafts (del Conde, Shrimpton, Thiagarajan, & Lopez, 2005). Depletion of cholesterol could significantly reduce microvesicle shedding. Cell shape maintenance proteins such as cytoskeletal elements and their regulators are also involved in microvesicle biogenesis (Minciacchi, Freeman, & Di Vizio, 2015). The regulators of actin dynamics, RhoA (a member of the small GTPases family) and its downstream associated protein ROCK and LIM kinases are essential for microvesicle biogenesis (Li, Antonyak, Zhang, & Cerione, 2012). A calcium dependent enzyme, calpain which regulates cytoskeletal proteins involved in microvesicle shedding (Crespin, Vidal, Picard, Lacombe, & Fontenay, 2009). Inhibitory of calpain could supress PAK1/1 activation to decrease polymerization of actin, formation of filopodia, and furthermore interfere the generation of microvesicles. ARF6 also play a key role for microvesicle formation and shedding (Muralidharan-Chari et al., 2009). ARF6-GTP-dependent activation of phospholipase D recruits the extracellular signal-regulated kinase (ERK) to the plasma membrane, and then ERK phosphorylates and activates myosin light-chain kinase (MLCK) which is an important regulator of actin polymerization and myosin activity. This process is essential for microvesicle releasing, while inhibition of ARF6 could block microvesicle shedding (Muralidharan-Chari et al., 2009). Both exosomes and microvesicles together play important roles in cellular physiological and pathological processes.

1.2.3 The external factors impact EV releasing

The yield and contents of EVs can be drastically altered by different parameters of cell culture such as cell type, culture medium, passage number, seeding density, oxygen tension, treatments of cells (Ludwig, Whiteside, & Reichert, 2019). Different cell type needs different medium composition to meet cellular requirements. Cell culture medium is a crucial factor while EVs recovered from cell culture supernatants. Culture medium should be carefully chosen and reported in publications, as all components in the culture medium including amino acids, vitamins, glucose, antibiotics and serum, impact the production of EVs and their cargo, therefore. For example, glucose level of culture media can impact EV yield and cargo molecules (Burger et al., 2017; Garcia, Ontoria-Oviedo, Gonzalez-King, Diez-Juan, & Sepulveda, 2015; Rice et al., 2015). High glucose increases EV formation, EV mean size, capacity of EV bioactivity, and significantly alters EV composition (Burger et al., 2017; Rice et al., 2015).

In contrast, in glucose starvation (no-glucose) can also increase EV secretion. EV proteins in high-glucose culture condition are more related to cellular development processes, whereas EV proteins in non-glucose condition are more related to metabolic processes and signalling pathways for promotion of energy acquisition (Garcia et al., 2015). Whilst glucose condition also modulates miRNA content of EVs which EVs in glucose starvation condition contained a broader range of miRNAs compared with EVs in high-glucose condition. Fetal bovine serum (FBS) is popular used component in most cell culture, contains bio-active EVs which may influence cultured cells and their EV formation, also their co-isolation may interference downstream EV analysis. However, without FBS in culture media could result in cell starvation which impact cellular behaviour and EV composition (Abramowicz et al., 2018). Using serum-free media can also alter the quantity of EVs. The added antibiotics in the culture media alter external surface of EVs and furthermore affect the adhesion property of EVs. Hypoxic culture condition increases EV secretion, alters EV composition such as tetraspanins, upregulated miRNAs, pro-angiogenic proteins which may change EV function.

The cell passage number does not affect production rate and size distribution of MSC-derived EVs, but can significantly reduce the vascularization bioactivity of EVs which may furthermore reduce therapeutic potential of EVs (Patel et al., 2017). The decreasing cell seeding density increases EV production but has not much effect on the vascularization bioactivity of EVs. As an important culture environmental factor, hypoxia can increase EV releasing in pancreatic cancer cells, and also cause a various change in size distribution especially in extreme hypoxia (Patton, Zubair, Khan, Singh, & Singh, 2020). Different cell lines have various increasing responses of EV releasing under hypoxic culture condition. Hypoxia can alter EV contents and furthermore change their bioactivity and function (Kucharzewska et al., 2013; Ludwig, Razzo, Yerneni, & Whiteside, 2019; Namazi et al., 2018). MSC-derived EVs from hypoxic condition are more potent than normoxic EVs (Almeria et al., 2019). The culture container also appears to be a factor for cellular EV releasing as cells cultured in a bioreactor can produce 100 times higher EVs than in dishes (Palviainen et al., 2019).

1.2.4 Function of EVs

Endosomal exosomes are considered as mediators that effect recipient cells. However, it is difficult to efficiently separate exosomes from other subtype EVs by current isolation method since not only the formation and secretion of ILVs involve multiple mechanism resulting heterogeneous exosomes, overlapped EVs in several biophysical properties (Mateescu et al., 2017). Furthermore, there is currently no consensus on markers to distinguish exosomes from other EVs. In additionally, microvesicles have been reported to play a roles in intracellular communication, such as in cancer cells (Al-Nedawi et al., 2008). Current isolated small EVs are heterogeneous in size, origin and molecular composition with unknown portion of exosomes (Witwer et al., 2013), therefore they may contain a mixture of endosomal and non-endosomal small EVs (Kowal et al., 2016) and even some non-vesicular molecules such as various dense lipoproteins (Karimi et al., 2018). Nevertheless, many studies have discovered the significant EV function to target cells and demonstrated their potential in many pathophysiological fields such as cancer, immune responses, various diseases and regenerative therapeutics (El Andaloussi et al., 2013; Yanez-Mo et al., 2015). Many studies mention the function of exosomes only, however, the prepared EVs are mixed with different subtypes which indicates that the function of EVs are elicited by the multiple EV types (Mathieu et al., 2019). Nowadays, Exosomes and microvesicles are considered to play an important role of intercellular communication between in cells

or distant cells (Raposo & Stoorvogel, 2013). Once EVs are released into extracellular space, EVs target the recipient cells; deliver their contents to evoke functional responses in the recipient cells. EVs can bind to target cells through receptor ligand interaction (Raposo et al., 1996), attach or fuse with target cell membrane to deliver their contents (Clayton et al., 2004) or be internalized through endocytosis by the target cells (Morelli et al., 2004) to modulate the recipient cells. EVs are considered as 'signalosomes' to display important role in the regulation of normal physiological processes such as stem cell maintenance, tissue repair, immune surveillance, blood coagulation and in pathological propagation processes such as cancer and neurodegenerative diseases (El Andaloussi et al., 2013). Further studies of EV is essential to understand more clearly cell biology, and especially to discover EV clinical applications such as biomarkers, regenerative medicine, drug delivery and even vaccine (van Niel et al., 2018).

Many studies have shown the diverse biological functions of EVs. EVs released by B lymphocytes presented MHC-peptide complexes to specific T cells which suggested exosomes play a role in adaptive immune responses (Bobrie, Colombo, Raposo, & Thery, 2011; Thery, Ostrowski, & Segura, 2009). Proteins and mRNAs of EVs can be transferred into target cells and mRNAs can be translated into corresponding proteins (Ratajczak, Miekus, et al., 2006). Selective mRNAs and miRNAs were found in mast cell exosomes (Valadi et al., 2007). Genetic communication between cells might have occurred by the trafficking of exosomes through the systemic circulation similarly as hormones to impact the recipient cells. EVs derived from stem cells play a pivotal role in tissue regeneration (Lai et al., 2010; M. Z. Ratajczak et al., 2012). Exosomes not only play important biological roles in many aspects of biology such as intercellular vesicle traffic, immunity, neurobiology and microbiology, but also have important role in disease pathogenesis such as tumour progression, neurodegenerative propagation and HIV and prion spread (El Andaloussi et al., 2013). Tumor cells can release EVs into microenvironments to elicit tumor progression via numerous mechanisms such as promoting angiogenesis, suppressing immune responses and tumor cell migration in metastases (Haga et al., 2015; Kim et al., 2013; Rak & Guha, 2012; Skog et al., 2008). EVs of cancer cells have the potential to be unique diagnostic biomarkers of cancer (Rak & Guha, 2012; Zhang & Grizzle, 2011). Based on the pathogenesis function of EV, inhibition of EVs can become therapeutic target through inhibiting EV formation, releasing, uptake, and specific EV component blocking (El Andaloussi et al., 2013).

EVs have innate therapeutic potential. Exosomes can be used as a vector for gene therapy (Alvarez-Erviti et al., 2011). The main obstacle of successful gene therapy in clinical trials is vehicles used to transfer therapeutic genes. Finding or designing vectors with improved safety, specificity, and efficiency have become one of the new areas of research for gene therapy. The membrane- based exosomes have been proposed as a natural shuttle for gene transferring because they can easily cross biological barriers including the blood-brain barrier. Also, exosomes have the potential to be used as novel vaccine since their ability to carry antigens. One study showed the ubiquitinated antigens could be delivered into exosomes and those transforming exosome elicited a T cell response (Cheng & Schorey, 2016). Depending on the development of exosome technology, more pre-clinical and clinical applications of exosome have been registered in public clinical trial database https://clinicaltrials.gov. Using the key search words of "exosomes" and "extracellular vesicles" in the clinical trials website (https://clinicaltrials.gov/) reveals 213 and 80 registered clinical trials, respectively.

EVs are considered as central biological agents for intercellular communication, have therapeutic potential and have been subjected to intense investigation. However, their biological properties and mechanisms of EVs still remain elusive. Some studies have reported even contradictory results probably due to differences of cell culture conditions, purification protocols or lack of EVs characterisation (El Andaloussi et al., 2013).

1.2.5 Extracellular vesicle uptakes

EVs carry proteins, lipids and nucleic acids can be released by most cells and taken up by recipient cells to trigger phenotypic effects (Raposo & Stahl, 2019). They play an important role in cellular communication between cells. The lipid bilayer membraned EVs can protect their contents, transit through the extracellular ambience, and internalise into recipient cells. Understanding of EV uptake mechanisms can lead to the development of EV therapeutic strategies such as inhibition of interaction between cancer EVs with the health surrounding cells, clinical applications, or design of more efficient and sophisticated EV drug delivery (El Andaloussi et al., 2013).

Heterogeneous EVs enter recipient cells by different specific pathways depending on the EV size and/or their surface components, and recipient cell type (van Niel et al., 2018). EV uptake pathways are as heterogeneous as EVs themselves. The different cell types take up heterogeneous EVs through different pathway which is a highly specific process (Mulcahy, Pink, & Carter, 2014). To enter the recipient cell, EVs must firstly bind with the membrane of the target cells. These step still remains unclear, whether EVs bind to target cells through a non-specific process or a receptor-dependent pathway (Mulcahy et al., 2014). In previous studies, many molecules presented in EVs are involved in the binding process. For example the adhesion molecules such as integrins, and exosomal tetraspanin complexes, ECM proteins, lectins, glycolipids, proteoglycans lipids, Negatively charged PS- and phosphatidylglycerol-containing liposomes (Andreu & Yanez-Mo, 2014; Clayton et al., 2004; French, Antonyak, & Cerione, 2017; Morelli et al., 2004; Mulcahy et al., 2014; Murphy et al., 2019). Also EV uptake might be more dependent on the target cell types (Mathieu et al., 2019) and membrane receptors on target cells (Record, Carayon, Poirot, & Silvente-Poirot, 2014). To study the mechanisms of EV uptake, many techniques have been used to elucidate the internalisation of EVs. Examples include, using inhibitor to block various pathways, employing antibodies to shutdown interaction between receptor and ligand, and using RNAi technique to knockdown target genes. There is evidence that protein-protein interaction between surface proteins of EVs and the target cells mediates EV attachment and internalization resulting EVs enter the target cells and deliver their contents with various mechanisms (Mulcahy et al., 2014).

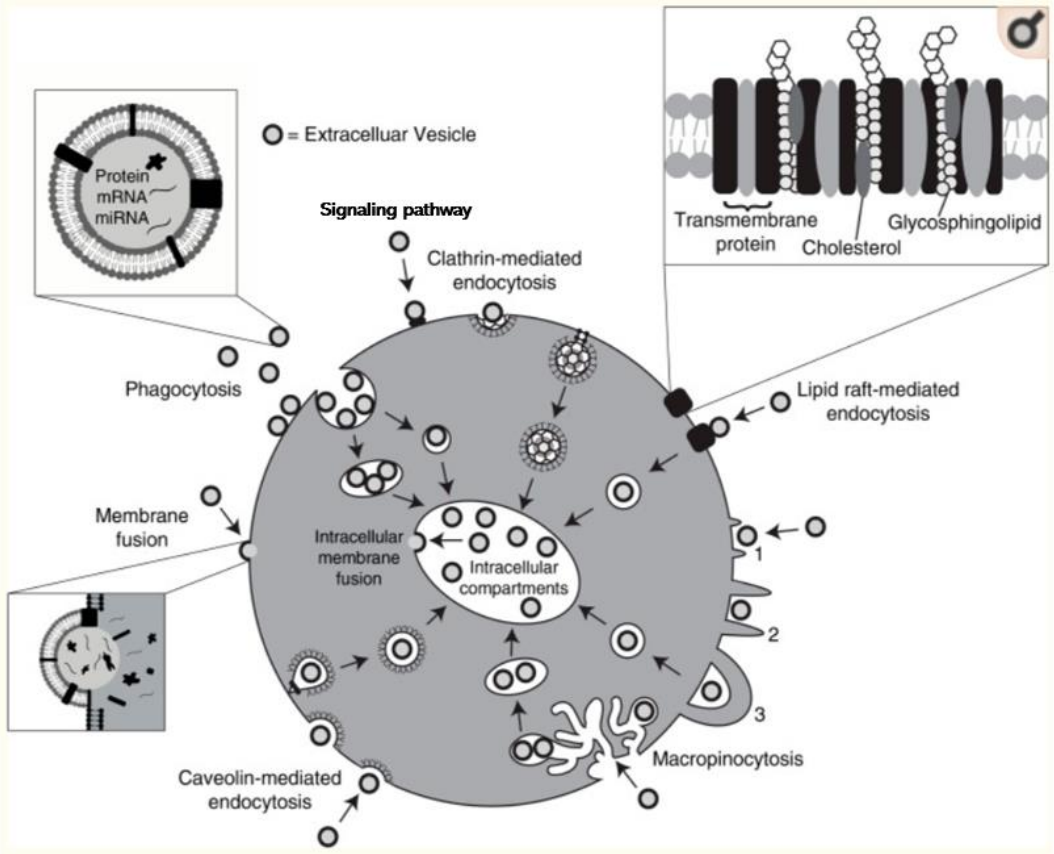


Figure 7: Multiple pathways of EV influence on the target cell. The diagram was adapted from (Mulcahy et al., 2014). EVs impact the target cell by signalling pathway, fusion with the plasma membrane, and endocytic pathways which include clathrin-mediated endocytosis and clathrin-independent endocytic pathways known as caveolin-mediated endocytosis, lipid raft-mediated endocytosis, phagocytosis, and micropinocytosis.

After EVs binds to appropriate receptors on the targeted cells through receptor ligand interaction, there are three major EV uptake pathways to impact/enter the recipient cells (Figure 7).

1. EVs result in activation of the recipient cell receptor via signalling pathway.
2. EVs fuse with target cell membrane to deliver their contents.
3. EVs are internalized through endocytosis to modulate the recipient cells.

1.2.5.1 Signalling pathway

There are few studies which show EVs impact on recipient cells through cellular signalling pathway by acting at the cell surface without delivering EV contents. For example, EVs derived from B lymphocytes can activate T cell responses by EV-bound MHC class II molecules (Raposo et al., 1996). Different subtypes of EVs derived from dendritic cells could induce CD4+ T-cell activation (Tkach et al., 2017). EV-Associated IFN- γ/Ifngr1 Complex Activates the Stat1 pathway in recipient cells (Cossetti et al., 2014). Microglial EVs presented N-arachidonoylethanolamine (AEA) could active type-1 cannabinoid receptors (CB1) to inhibit presynaptic transmission in the recipient cells (Gabrielli et al., 2015). EVs derived from embryonic stem cells could have triggered the activation of two signalling kinases through interaction with integrins of recipient cell (Desrochers, Bordeleau, Reinhart-King, Cerione, & Antonyak, 2016).

1.2.5.2 Fusion pathway

EVs could directly fuse with the plasma membrane of recipient cells to release their contents into cytoplasm of the recipient cell. This is a key step which regulate gene expression of the target cells (van Niel et al., 2018). Direct of fusion EVs with the plasma membrane is also important in order to exchange the transmembrane proteins and lipids. A study demonstrated that EVs derived from bone marrow dendritic cells fused/semi-fused with recipient dendritic cells to release EV miRNAs into cytosol of target dendritic cells (Montecalvo et al., 2012). EVs derived from monocyte/macrophage fused with activated platelets to transfer EV contents (del Conde et al., 2005). In metastatic melanoma cell EVs were taken up by melanoma cells by fusion and enhanced in a low pH condition (Parolini et al., 2009).

1.2.5.3 Endocytic pathway

Endocytic mechanism of EV uptake is the main entry route rather than the fusion pathway is the most commonly studied mechanism (Mulcahy et al., 2014). Endocytosis plays a key role in regulating the composition of the plasma membrane and thus the control of interaction between cells with their microenvironment (Doherty & McMahon, 2009). There is some evidence that verified that EVs can be internalised into target cells as early as 15 minutes (Fabbri et al., 2012; Feng et al., 2010). EV uptake is an energy-requiring process since the internalisation of EVs can be dramatically reduced when cells are incubated at 4°C (Tian et al., 2013). Also, EV uptake is not a passive process as EVs are not internalised by cells fixed with paraformaldehyde (Pan et al., 2012). EV uptake also requires a functioning cytoskeleton since EVs reduced uptake when treated with cytochalasin D which is responsible for depolymerize the actin filament network to inhibit endocytic pathways (Lamaze, Fujimoto, Yin, & Schmid, 1997). Therefore, EV uptake is an energy-requiring process and requires normal cytoskeleton activity. That also indicated EV uptake through endocytic pathway (Mulcahy et al., 2014). Based on differential dependencies, endocytosis could be classified into clathrin-mediated endocytosis and clathrin-independent endocytic pathways known as caveolin-mediated endocytosis, lipid raft-mediated endocytosis, phagocytosis, and micropinocytosis (Doherty & McMahon, 2009; Mayor & Pagano, 2007; Mulcahy et al., 2014). EV internalization utilises multiple routes involved both clathrin-dependent and clathrin-independent endocytic pathways (Mulcahy et al., 2014). Multiple endocytic pathways coexist and co-occur in EV uptake.

1.2.5.4 Intracellular fate of EVs

After different subtypes of EVs reach the recipient cells, intercellular fate of EVs is not only impacted by specific composition of EVs, but also related to the plasma membrane of target cell (van Niel et al., 2018). While EVs are internalised into the recipient cell and settled in MVB through endocytic pathway, most EVs would be targeted and degraded by lysosomes (Tian et al., 2013). However, some EVs might be capable of escaping the destination of degradation, and fuse with the limiting membrane of MVB for releasing of EV contents, or to re-secrete into extracellular space again (Figure 8) (van Niel et al., 2018). Some EVs are targeted ER to release their contents resulting in exosomal miRNA and mRNA into RNAi and translation machineries (Heusermann et al., 2016).

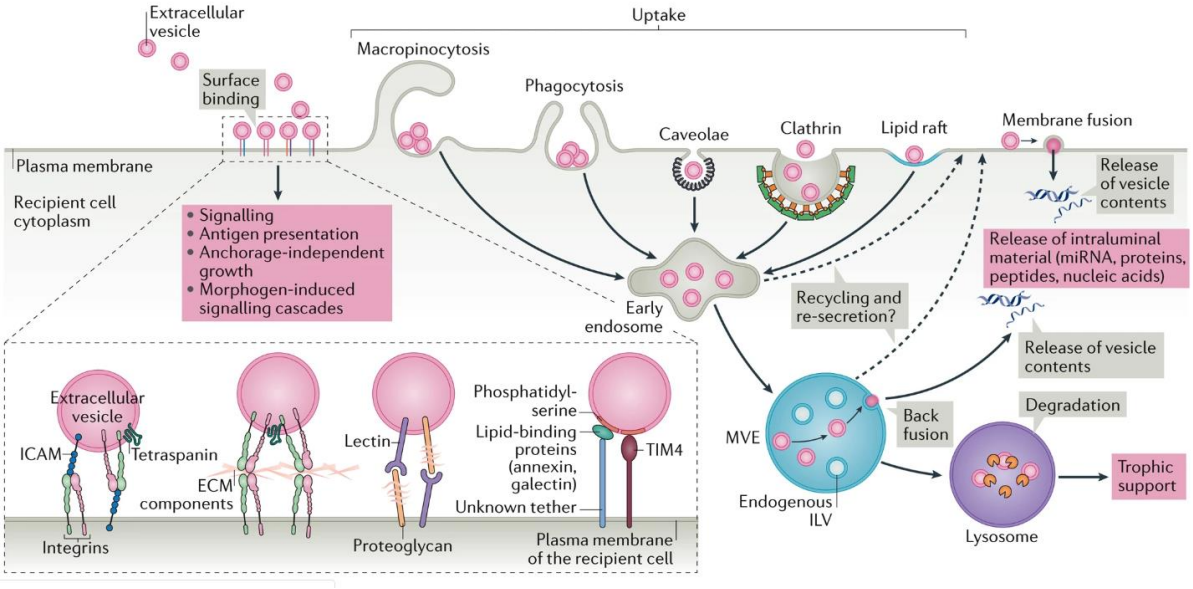


Figure 8: Intracellular fate of EVs. Adapted from (van Niel et al., 2018). Some EVs would be degraded by lysosome. Some EVs might fuse with the limiting membrane of MVB for releasing of EV contents, or to re-secrete into extracellular space again Some EVs fuse with the plasma membrane to release their contents.

However, intracellular fate of EVs is still not completely understood (Mathieu et al., 2019). Further investigations are still needed, EV labelling and tracking by using advanced live-imaging and super-resolution techniques could help us to understand more about EV uptake and intracellular fates.

1.2.5.5 The biodistribution of EVs *in vivo*

The studies of EV biodistribution and EV behaviours *in vivo* could help us gain insight into EV biological significance, exploit the EV potentials, and provide crucial clues of EV therapeutic roles. Many studies have tracked labelled EV biodistribution *in vivo* in animal models by optical imaging techniques (Gangadaran et al., 2017; Grange et al., 2014; Smyth et al., 2015). Grange and his colleagues detected labelled human MSC-derived EVs were recruited and accumulated in injured kidney (Grange et al., 2014). This demonstrated the possibility of EV uptake by injured kidney and furthermore implicates that MSC-derived EVs have beneficial effects on injured kidney recovery. *In vivo*, EVs could be rapidly taken up by liver and spleen and also along with limited uptake by the lungs and kidneys (Smyth et al., 2015). Wiklander's group reported that the EV distribution observed a similar pattern within 24 hours, but fluorescent signal distribution changed at 48 hours. And, different EV injection route, doses and different EVs derived from different cell sources variously impacted EV distribution pattern. However, EVs from different species origin did not have a change in the EV distribution (Wiklander et al., 2015).

1.2.6 EV tracking and labelling

EV tracking and imaging is an important technique to reveal biophysical property of EVs and furthermore to unveil EV therapeutic potential (Chuo, Chien, & Lai, 2018). EV labelling and imaging *in vitro* could reveal EV biophysical property for example to study the mechanism of EV secretion, EV uptake pathway, cellular localization, and furthermore the fate of EVs.

The establishment of an ideal EV labelling is important to track and image EVs. EV labelling requires stable and sufficient signal intensity with high specificity and sensitivity. There are many EV labelling techniques used in previous studies (Panagopoulou, Wark, Birch, & Gregory, 2020). For example, using free fluorescent dyes to stain total EV population non-specifically; using fluorescent vector to generate a stable cell line to release fluorescent EVs (Mittelbrunn et al., 2011; Suetsugu et al., 2013); and using immune-fluorescent method (antibodies) to stain EVs (Mondal, Ashiq, Phulpagar, Singh, & Shiras, 2019). Using fluorescent EVs released by transfected stable cell line is a more specific way to visualise EV uptake (Mulcahy et al., 2014). CD9 and CD63 enriched in EVs could be tagged with fluorescent reporter such as GFP, RFP and their derivatives to generate a stable cell line to release fluorescent EVs. These fluorescent EVs have advantages since they would not alter the behaviours of EV uptake. However, genetic labelled EVs released by the fluorescent vector transfected cells, are restricted in EV subpopulations resulting in limitation of EV utility to observe multiple EV types, and limited utility of plasma EVs (Mondal et al., 2019). Here, we will focus on labelling EVs by fluorescent dyes which has the capacity to stain whole EV population.

EV uptake could be detected and tracked the internalization through labelling EVs by using fluorescent dyes such as PKH, R18, CM-Dil, Did, SYTO, CFSE dyes (Morales-Kastresana et al., 2017). PKH, R18, CM-Dil, and Did are lipophilic dyes. Popular PKH fluorescent dyes belong to lipophilic family which constitute a highly fluorescent polar head group and a long aliphatic hydrocarbon tails to intercalate into exposed lipid bilayer to form long-term dye retention and stable fluorescence (Wallace et al., 2008).They have been widely used for EV labelling (Dominkus et al., 2018) due to their intense signal and long half-life. PKH fluorescent dyes could label EV membranes by the insertion of their aliphatic chains into the lipid bilayer. However, PKH dye easily aggregates to form micelles33, and thus associated with pelleted EVs to result in false-positive signals for EVs (Dominkus et al., 2018; Lai et al., 2015). PKH dyes could also increase EV size resulting in further effects of EV cellular uptake and intracellular localization (Dehghani, Gulvin, Flax, & Gaborski, 2019; Shang, Nienhaus, & Nienhaus, 2014). The same applies to the PKH dye, CM-Dil, SYTO dyes were also detected non-specific aggregates (Morales-Kastresana et al., 2017). Most researchers are concerned with the reliability of lipophilic dyes since the difficulty to separate labelled EVs from dye aggregates (Russell et al., 2019) and effects of EV behaviour by the presence of lipophilic dye molecules. Staining EVs by CFSE dye showed that there were non-specific aggregates and without EV size change after staining (Dehghani et al., 2019; Morales-Kastresana et al., 2017). Therefore, CFSE was considered as an optimised and suitable dye to track EVs by using microscopy and flow cytometry techniques without non-specific aggregation.

CFSE-Carboxyfluorescein Succinimidyl Ester, is a widely used fluorescent cell staining protein-binding dye (Quah, Warren, & Parish, 2007). It is non-fluorescent before going inside the cells since it presents two acetate groups. However, two acetate groups enable the compound to be highly membrane permeant and thus the dye could passively and rapidly diffuse into the cells (Figure 9). The acetate groups would be removed by intracellular esterases resulting in its stability inside cells and to be fluorescent. The succinimidyl group of CFSE covalently couples to intracellular amino groups in intracellular molecules resulting in CFSE more stable inside cells. Part of the resultants exit via the plasma membrane during the first hour of labelling (Wang, Duan, Liu, Fang, & Tan, 2005). However, some of the resultants are highly stable and they can retain within cells for long term periods which is sufficient for further analysis. CFSE seems to be an ideal dye for EV staining since stained EVs would normally take more than one hour to wash remaining dyes after staining. The one hour washing step ensures that all stained EVs are stably fluorescent.

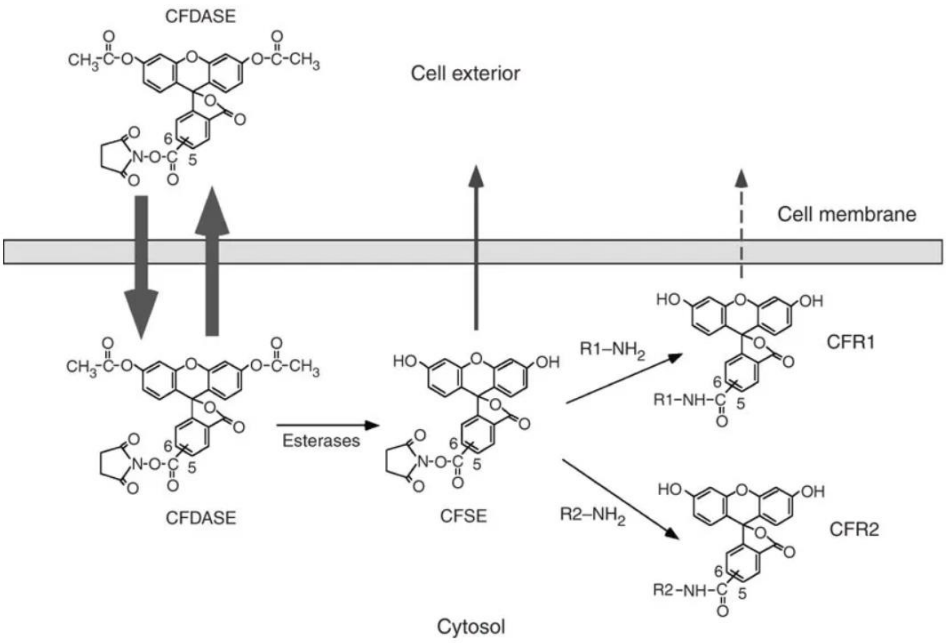


Figure 9: The mechanism of labelling cells using CFDA-SE. Adapted from (Parish, 1999).

After EV labelling, and following incubation with target cells, EV internalization *in vitro* would be visualised/detected by widely used optical tools such as confocal microscopy or flow cytometry. Confocal microscopy is widely used instrument to visualise labelled EV uptake. Confocal fluorescence microscopy provides true 3D optical resolution which is accomplished by actively suppressing any signal coming from out-of-focus planes. EVs are not capable of visualisation in an unlabelled state due to their small size. Labelled EVs emit light to enable imaging of EVs and tracking EV interaction with live cells (Panagopoulou et al., 2020). However, fluorescent microscopy has limited resolution to distinguish individual EVs less than 390 nm. This affects dynamic visualisation and localisation for individual EV analysis (Mulcahy et al., 2014). Nevertheless, it still can assess EV uptake in general, furthermore, flow cytometry is another versatile technique to detect individual labelled and unlabelled cells while the suspended cells pass through the laser beams and detectors.

1.2.7 EV isolation

1.2.7.1 The difficulty of isolation subtype EVs

EVs are bilayer lipid membrane-enclosed structures released by cells. As described before, EVs are categorized into three classes based on their biogenesis pathway: exosomes, microvesicles and apoptotic bodies (Gould & Raposo, 2013). Exosomes are ILVs formed within MVBs through endosomal pathway and released by fusion of MVBs with the plasma membrane. Their size range is from 40 nm to 120 nm with 1.11-1.19g/ml buoyant densities. Microvesicles are released from the plasma membrane by directly outward budding. They have heterogeneous diameter size ranges from 50 nm to 1000 nm. Apoptotic bodies are generated during cell apoptosis and released through outward bleb and fragmentation of the cell membrane of apoptotic cells. Their size range is from 50 nm to 2000 nm in diameter (Willms et al., 2016). Since endosomal exosomes are considered as mediator to effect recipient cells, current exosome isolation methods aim for achievement of pure exosomes. However, it is difficult to isolate pure exosomes from tissue culture supernatant or body fluids since the

formation and secretion of ILVs involve multiple mechanism resulting heterogeneous ILVs, overlapped EVs in several biophysical properties (Mateescu et al., 2017). Furthermore, there is currently no consensus on markers to distinguish exosomes from other EVs. Current isolated small EVs are heterogeneous in size, origin and molecular composition with unknown portion of exosomes (Witwer et al., 2013). The term exosome was used quite loosely in the many publications. Small EVs are often called as exosomes. In this report, the EVs pelleted over 100,000 x *g* refer to small EVs and the pellets at 10,000 x *g* refer to MVs.

1.2.7.2 Isolation methods of EVs

There are many isolation methods for small EVs—differential ultracentrifugation which is proposed as 'golden standard' and used by over 81% researchers before 2016, density gradients, polymer-based precipitation, microfiltration, size-exclusion-based chromatography (Gardiner et al., 2016) and also the immuno-isolation by different surface molecules or flow cytometric method. Many researchers are using a combination of isolation techniques which are considered as desirable to obtain purest exosomes. Small EVs can be isolated from biological fluids such as blood, saliva, urine, nasal secretions, breast milk, and cerebrospinal fluid. However, they are released by many types of cells and have a mixed cellular origin. Small EVs released by a single cell type can be achieved from conditioned culture medium (Witwer et al., 2013). Cells are normally cultured *in vitro* in the human or foetal bovine serum, which is carrying a different subset of RNAs, proteins, lipids and EVs to directly affect experimental results on following proteomics, RNA analysis and functional analysis of isolated EVs. Therefore, culture medium would be performed an overnight ultracentrifugation at 100,000 x *g* to eliminate EVs in serum. Then cells are cultured in EV-deleted medium for 24-48 hours. In below description of some EV isolation methods, we focus on the small EV isolation from the culture medium.

1.2.7.2.1 The differential ultracentrifugation

Differential ultracentrifugation is known as the 'gold standard' and is the most used method of EV isolation. It is applied by differential ultracentrifuge which involved in a series of centrifugations (Van Deun et al., 2014). Smaller particles are sequentially precipitated with sequentially increasing in speed and time of centrifugation. The pellets are discarded after each run until exosome pellets are harvested in the last run (Cvjetkovic, Lotvall, & Lasser, 2014). Cells remained in the medium and large apoptotic bodies firstly deplete by low-speed centrifugation step. The medium EVs can be pelleted by centrifugation in the 10,000-20,000 x *g* force range. Finally, the small EVs are harvested at high-speed in 100,000-120,000 x *g* (Witwer et al., 2013). The results can be impacted by many factors of differential centrifugation such as the duration, relative centrifugal force (RCF), k-factor and temperature of centrifugation, thus lead to different laboratories reporting different results. The most commonly used rotors are fixed angle rotor and swinging bucket rotor. The pellets are sediment in lower outer side of the tube in the FA rotor and the pellets are sediment at the bottom of the tube by using SW rotor (Cvjetkovic et al., 2014). Preparation of small EVs for proteomic analysis by ultracentrifugation and a followed ultracentrifugation wash was used by 37% of researchers (Gardiner et al., 2016). The EV isolation by the differential ultracentrifugation has the advantages such as low-cost and capacity of a wide range of volumes (Ramirez et al., 2018). However, it has few drawbacks such as contamination of non-vesicle particles known as protein aggregates and lipoproteins.

1.2.7.2.2 Density Gradients

Differential ultracentrifugation cannot achieve the separation of different sized EVs since the sedimentation not only depends on the spin duration and speed, as well as the density and the sedimenting distance of EVs. The contamination of extracellular proteins is also a concern under high-speed and long-time centrifugation (Witwer et al., 2013). An approach to solve the problem is to follow the differential ultracentrifugation with further purification of EVs based on EV density. Density gradients is widely used as this complementary method to remove contaminating non-vesicular particles (Van Deun et al., 2014). Sucrose and iodixanol are the most used components of density gradients. Iodixanol is better to preserve the size of vesicles in the gradient since it can form iso-osmotic solution at all densities. Density gradients ensure the membrane-enclosed vesicles floated upwards to avoid protein aggregates which cannot float into the gradient and remain in the dense fractions (Zonneveld et al., 2014). 37% researchers performed proteomics by using this method to purify EVs after the differential centrifugation (Gardiner et al., 2016). However, density gradients is quite time consuming, more risks of contamination, therefore reducing possibility for clinical setting (Ramirez et al., 2018).

1.2.7.2.3 Size-exclusion chromatography

Recently size-exclusion chromatography has become a popular isolation technique based on the size of EVs to recover purer EVs. The single step EV isolation by using size-exclusion chromatography has been performed and is considered to be a fast and easy protocol comparing with differential ultracentrifugation (Boing et al., 2014). Size-exclusion chromatography usually is known as gel filtration which smaller particles flow through the pores and elute longer time than larger particles. The advantages include the reduction of EV aggregates, efficient separation of EVs from soluble proteins, lipoproteins, protein aggregates, and high yield of intact biologically active EVs. Due to concerns such as the loss of the target EV population, deformation and breakup of large vesicles, dilution of EV samples, and contamination from filters, this method is normally combined with ultracentrifugation or other methods such as re-concentration of EVs (Witwer et al., 2013). Along with the development of the new commercial isolation kits, size exclusion chromatography has become more widely used techniques (Mateescu et al., 2017).

1.2.7.2.4 Immunoaffinity isolation

Immunoaffinity isolation of EVs is based on the characteristic surface proteins presented on the EVs. The surface proteins of EVs covalently interact with antibodies associated with beads or other matrices, and then follow physical separation through low-speed centrifugation or magnetic tools (Mathivanan, Lim, et al., 2010; Yoo et al., 2012). This method could pull-down EVs with a particular surface maker to exclude other EV population and contaminants. However, the choice of affinity reagent and ligand density highly impact on selective EV population (Mateescu et al., 2017). On the other hand, immunoaffinity method is not suitable to use with large-volume sample. Therefore, it is normally used as additional purification resulting in longer process (Ramirez et al., 2018). Its high specific to unique EV populations results in lower yields comparing with other methods (Witwer et al., 2013). Also, the harsh condition while elute EVs from beads might slightly change EV size, their surface structure and furthermore might alter EV function (Ramirez et al., 2018).

1.2.7.2.5 Polymeric precipitation

Various commercial kits based on polymeric precipitation are available for EV isolation. Polymeric precipitation mixtures are incubated with culture medium or biofluids for overnight, followed by low-speed ultracentrifugation to obtain the precipitate. A major concern for this method is that it would result in heavy contamination with proteins, lipoproteins and other biological components, which is not desirable (Raposo & Stoorvogel, 2013; Witwer et al., 2013).

1.2.8 Characterisation of EVs

After isolation, EVs can be characterised by a variety of techniques for their downstream applications. Electron microscopy, western blotting and single-particle tracking are three most widely used techniques used in characterisation of EVs (Gardiner et al., 2016).

1.2.8.1 Electron microscopy

Due to the small size of EVs, electron microscopy especially transmission electron microscopy (TEM) have been the preferred technique to directly observe the size and morphology of EVs (Raposo et al., 1996). EVs are chemically fixed, absorbed onto a filmed grid, washed, and then negatively stained before observation by TEM. TEM can also be combined with immunolabelling techniques to identify immunological epitopes on EV surface (Ramirez et al., 2018). EVs display a cup-shaped morphology under TEM since the drying step induces shrinking of subcellular structures. However, the shape of EVs is round which can be observed under cryo-EM. Cryo-EM can preserve EVs shape in their native state since physical fixation rather than chemical fixation.

1.2.8.2 The individual tracking analysis

Single-particle tracking includes nanoparticle tracking analysis (NTA), resistive pulse sensing (RPS) and flow cytometry (Gardiner et al., 2016). Based on the Brownian motion of vesicles in suspension, nanoparticle tracking analysis is the most popular quantitative method to complement with electron microscopy for identification of the EVs. Measured motion speed or diffusion coefficient can be converted to particle size through the Stokes-Einstein equation to determine the size distribution and concentration of EVs (Raposo & Stoorvogel 2013); it can be used to directly and individually visualise and count the liquid suspension of EVs in real time, and it is considered as an easy, fast existing technique to characterise EVs. Despite this being a cheap and robust method, it does not differentiate vesicles from macroprotein aggregates (Colombo et al., 2014). Dynamic light scattering technique is a similar as nanoparticle trafficking analysis technique to measure the EV Brownian motion which related to the particle size. However, instead of the trajectories of individual scattering EV is observed by nanoparticle trafficking analysis, dynamic light scattering technique analyse the EV intensity fluctuations in scattered light, and then related to the diffusion of scattering EVs (Szatanek et al.,

2017). The intensity result can be mathematically transformed to the EV size distribution with a known refractive index.

1.2.8.3 Western blotting

Western blot is usually used to confirm the presence of EV protein markers. EV commonly contains CD9, CD63, CD81, TSG101 and Alix proteins. Western blotting is employed to characterise EVs included CD9, CD81, CD63 (Zabeo et al., 2017). EVs do not contain any proteins which are originally from the nucleus, mitochondrial, endoplasmic reticulum or Golgi-apparatus (Thery, Zitvogel, & Amigorena, 2002). Therefore, the proteins from origin of nuclear, mitochondrial, endoplasmic reticulum or Golgi-apparatus can be employed as negative markers of western blotting. To investigate of EV contents, their functions or novel biomarker discovery, many downstream analyses are ongoing. Common downstream applications are proteomic, RNA, lipidomic analysis, *in vitro* and *vivo* functional analysis (Witwer et al., 2013).

1.2.8.4 Characterization of EV RNAs

There are few techniques to analyse EV RNAs such as microarray analysis, qPCR and PCR arrays and next generation sequencing (NGS) followed by bioinformatic analysis and digital droplet PCR (Ramirez et al., 2018). Microarray technology was firstly used to discover miRNAs and mRNAs in EVs, and these mRNAs resulted in protein products in target cells (Skog et al., 2008; Valadi et al., 2007). The qPCR array is a widely used, low-cost and reliable method to profile EV RNAs (Chevillet et al., 2014; Manterola et al., 2014; Ramirez et al., 2018). It could give results by simple calculation without bioinformatics. However, available probes/primers in selected platform limit new finding of EV RNAs. Digital droplet PCR (ddPCR) array could absolutely quantify RNA expression without the use of standard curve (Chen et al., 2013; Del Re et al., 2017). More recently, next generation sequencing (NGS) followed by many steps of computational analysis becomes the most comprehensive tool to identify and quantify of all RNA biotypes in the EVs (Amorim et al., 2017; Eirin et al., 2014; Ramirez et al., 2018).

1.3 MSC-derived EVs

As we descripted in Section 1.1, MSCs have been used in many clinical applications and the efficacy of MSCs were attributed, in part by secretion of EVs especially exosomes. Small EVs display important role in the regulation of normal physiological, tissue regenerative and pathological propagation processes. After small EVs are generated from MSCs, MSC-derived EVs bind to target cells through receptor ligand interaction, attach or fuse with target cell membrane to deliver their contents or be internalized through endocytosis by the target cells (Morelli et al., 2004) to modulate the recipient cells (Figure 10). MSCs are considered as prolific producers of exosomes when compared to other cell types (Kordelas et al., 2014).

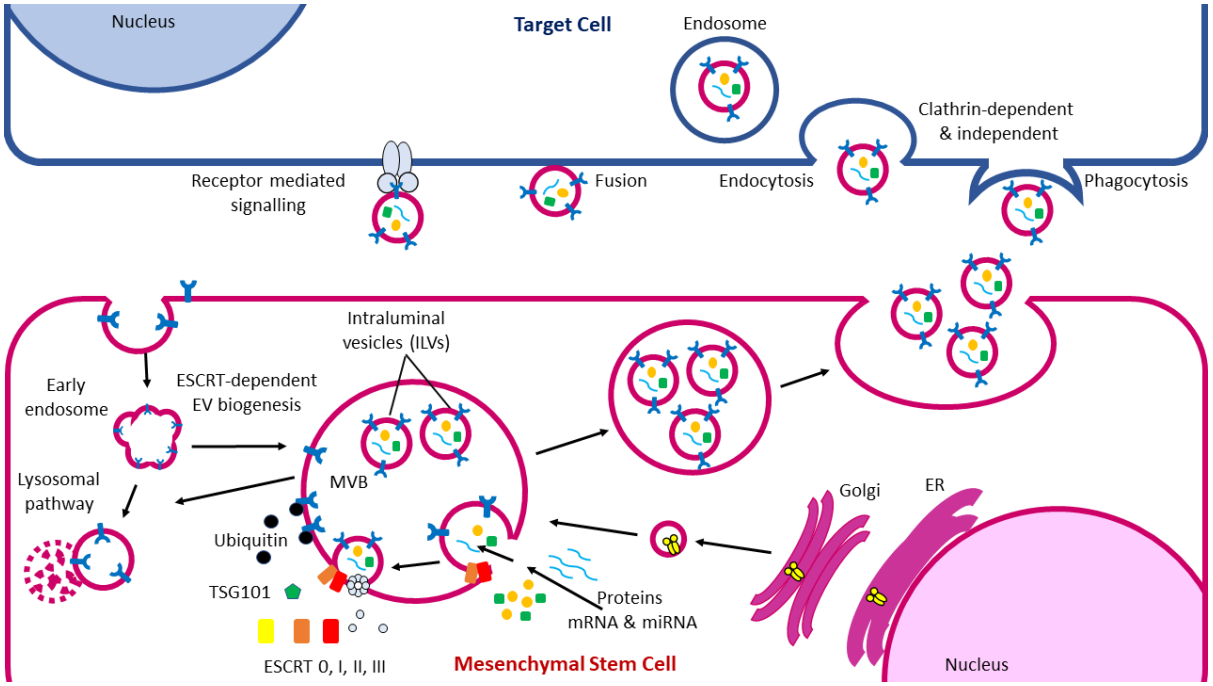


Figure10: MSC-derived EV biogenesis and uptake by the recipient cell. ILVs invaginate from the outer endosomal membrane to bud into the lumen of endosomes through ESCRT-dependent/independent machineries during the maturation of MVB from the early endosome. Matured MVB is then transported to the cell periphery and fuses with the plasma membrane to release ILVs (Exosomes). Exosomes together with microvesicles enter the target cells through signalling, fusion and endocytosis pathways to modulate the recipient cells.

Over 700 proteins have been identified to be found in MSC-derived EVs (Kim et al., 2012). These proteins reflect both features of MSCs and MSC-derived EVs. For example, 53 proteins of MSC-derived EVs were related to self-renewal genes of MSCs, and 25 proteins were differentiation genes of MSCs. In the comprehensive study by Kim et al, MSC-derived EV proteins not only included the surface markers of MSCs, but also MSC specific proteins involved in signalling pathways to facilitate self-renewal and differentiation of MSCs. Meanwhile, MSC-derived EVs contained proteins associated with EV fundamental feature such as EV biogenesis, trafficking, docking and fusion. Furthermore, a list of EV proteins such as the surface receptor--PDGFRB, EGFR, and PLAUR, signalling molecules of RAS-MAPK, RHO and CDC42 pathways, cell adhesion molecules and additional MSC antigens are associated with promotion and modulation of MSC therapeutic potential. These proteins implied a possible role of MSC-derived EVs in tissue repair and tissue regeneration. EV miRNAs were estimated to be less one copy per EV (Chevillet et al., 2014), however, EVs are continuously released and some EVs might be enriched with certain miRNAs. 171 miRNAs were discovered in MSC-derived EVs by using the NanoString platform and analysed with nSolver Sofware 3 (Ferguson et al., 2018). Abundant top 23 miRNAs could target 5481 genes to regulate many specific pathways and biological processes such as miR-130a-3p and miR-199a induce cellular proliferation, promote angiogenesis, and inhibit apoptosis. Analysis by mass spectrometry and antibody array, the proteome of purified MSC exosomes contain 938 unique gene products (http://exocarta.org). They encompass a wide range of biochemical and cellular processes including cellular communication, structure and mechanics, inflammation, exosome biogenesis, tissue repair and regeneration and metabolism.

To date, MSC-derived EVs have been used in both animal models and clinical applications in many disease areas such as cardiovascular disease, acute kidney injury, liver disease, lung disease, cutaneous wound healing, cancer suppression (Gatti et al., 2011; Lai et al., 2011; Rani et al., 2015).

Bruno and colleagues discovered the small EVs derived from bone marrow MSC were capable to recover the acute kidney injury (Bruno et al., 2009). GI MSC-derived EVs contributed to renal recovery of acute kidney injury (Ranghino et al., 2017). Ophelders et al showed MSC-derived EVs have the potential to treat preterm neonates with hypoxic-ischaemic brain injury in ovine fetuses (Ophelders et al., 2016). MSC-derived EVs could ameliorate reperfusion injury of myocardial infarction (Arslan et al., 2013). MSC-derived EVs have therapeutic function by inhibiting apoptosis and stimulating cell proliferation (Gatti et al., 2011).

MSC-derived EVs are also used for treatment of OA. A recent study compared the efficacy of MSC-derived EVs secreted from synovial membrane and induced pluripotent stem cell-derived MSCs to treat mouse osteoarthritis (Zhu et al., 2017). 8 µl exosome in PBS (1.0×10^{10}/ml) was injected into intra-articular of collagenase-induced OA mice. The result was analysed by macroscopic examination, histological analysis and IHC analysis. The treatments by injection of both source EVs demonstrated EVs attenuated OA, and iMSC-derived EVs had significant effect comparing with synovial membrane derived MSC exosomes. Zhang and colleagues firstly demonstrated intra-articular injection of 100 µg/100 µl of embryonic MSC-derived EVs to efficiently repair of osteochondral defects in rat model (Zhang et al., 2016). The results of MSC-derived EVs treated showed hyaline cartilage regeneration by the end of 12 weeks with no adverse inflammatory responses. In contrast, the defects of controls which treated with PBS just filled with fibrous and non-cartilaginous tissue. The function of MSC-derived EVs has been studied in cartilage repair by investigation MSC derived EV effects on chondrocyte survival (Zhang et al., 2018). The chondrocytes incubated with labelled MSC-derived EVs quickly endocytosed the EVs which showed that MSC-derived EVs could communicate directly with chondrocytes and enhance chondrocyte migration. MSC-derived EVs could rapidly phosphorylate AKT and ERK in chondrocytes within 1 hour to elicit cellular proliferation of chondrocytes. MSC EVs enhanced damaged cartilage regeneration through inducing proliferation, migration and matrix synthesis of chondrocytes, attenuating apoptosis and modulating immune reactivity. Adipose-derived MSC-derived EVs could repair damaged cartilage through increasing the proliferation and migration of chondrocytes (Chang Hee Woo, 2020). In their rat model, MSC-derived EVs could efficiently attenuate the development of OA. These studies demonstrate the possibility of treating chronic OA and several other clinical conditions with MSCs-EVs to address current unmet medical needs.

Overall, MSC-derived EVs have been evaluated for their therapeutic potential for the treatment of various diseases both *in vitro* and in animal models. Based on these results findings, a number of clinical trials have begun to evaluate the therapeutic potential of MSC-derived EVs for the treatment of particular diseases and the procedure similar as in Figure 11. MSC-derived EVs have improved therapy refractory graft-versus-host disease (Kordelas et al., 2014). MSC-derived EVs were isolated from allogeneic MSC cultured medium and treated into GvHD patients. Clinical GvHD symptoms were significantly improved shortly after the start of MSC-derived EVs treatment. Another clinical trial displayed efficacy outcomes using EVs derived from umbilical cord MSCs to treat chronic kidney disease(Nassar et al., 2016). These results demonstrated that MSC-derived EVs could safely improve the inflammatory immune reaction and overall kidney function in chronic kidney disease patients through MSC EV administration in two doses, the first intravenous and second intra-arterial.

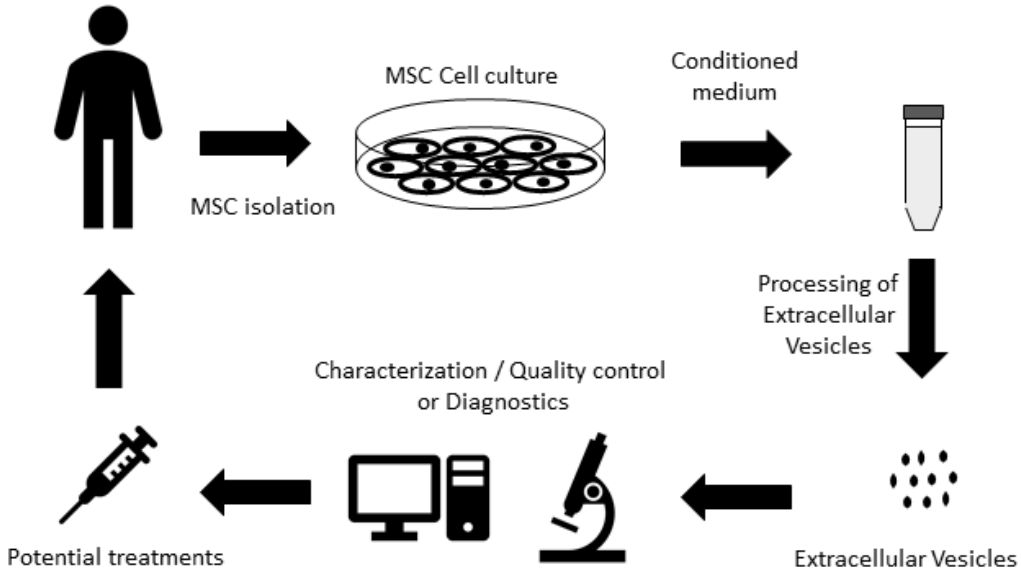


Figure 11: Workflow of MSC-derived EVs for therapeutic and diagnostic applications. MSCs can be isolated from patients from a variety of tissues. MSCs are cultured *in vitro* and the conditioned culture medium is collected and subjected to extracellular vesicle isolation and/or purification. The isolated MSC-derived EVs can be used for diagnostic purposes or undergo quality control before being used in autologous and/or allogeneic therapeutics.

MSC-derived EVs are capable of enhancing tissue repair and mediating regeneration (Balasubramanian, Rajasingh, Thangavel, Dawn, & Rajasingh, 2015) through modulating injured tissue environment, inducing angiogenesis, promoting proliferation, and preventing apoptosis (Lai et al., 2010). The use of MSC-derived EVs might serve as an alternative therapy over MSC transplantation for tissue regeneration (Kim et al., 2012) and have "off-the –shelf" therapeutic potential. Also, MSC-derived EVs clinical applications are advantageous over cell-based therapy since it has less safety concern (Baglio, Pegtel, & Baldini, 2012). Therefore, it is important to intensive study clinical therapeutic potentials of MSC-derived EVs.

1.4 Project Outline

This thesis is to study MSC derived EVs which is a rapidly developing field in the past two decades. We started a collaboration in 2018 with Magellan Stem Cells Pty Ltd to explore the therapeutic potential of MSC-derived EVs for osteoarthritis (OA) patients in 2018. Magellan Stem Cells are involved in several ethics approved clinical trials exploring cellular therapy to treat OA in clinics. Patients are given intra-articular injections of autologous MSCs in OA-affected joints and followed up for at least 12 months post-treatment for improvement in OA symptoms and structural improvement of the treated joint, assess by MRI. In this thesis, MSCs are isolated from OA patients, and MSC-derived EVs are studied with the following aims:

1. To establish an optimal method for isolation of small EVs by comparing different times of ultracentrifugation and the commercial kit.
2. To characterise MSC-derived EVs by electron microscopy, nanoparticle tracking analysis, western blot and flow cytometry.
3. To examine MSC-derived EV uptake by MSCs using confocal microscopy to discover the intracellular fate of MSC-derived EVs.

4. To identify the effect of MSC-derived EVs on MSCs properties, through analysis of specific genes by quantitative PCR.

1.5 Thesis outline

In order to achieve the aims described above, five chapters constitute this thesis to provide the current background of EVs, describe methods to optimise EV isolation and investigate EV uptake by MSCs and their effect on MSC function.

Chapter 1 introduces the background of MSCs, EVs and MSC-derived EVs, in particular the background of EVs in literature, EV biogenesis, composition, EV uptake and function, current method for EV isolation and characterisation.

Chapter 2 describes the methods used in this thesis for optimisation of EV isolation, characterisation of EVs by various techniques, investigation of EV uptake and their function by EV labelling and qPCR.

Chapter 3 presents results of EV isolation and characterisation to optimise the ideal isolation method of MSC-derived EVs.

Chapter 4 investigates the uptake of labelled EVs by MSCs, using confocal microscopy and effect of uptake on MSC properties, using qPCR technique to assess expression of three groups of genes.

Chapter 5 provides concluding discussion and proposes directions for future research.

Chapter 2

Materials and methods

This chapter describes the methods and materials used in the thesis. Firstly, cell preparation, several EV isolation methods are described, followed by the details of characterisation methods with various EV analysis techniques. Then labelling EV techniques are recorded using confocal microscopy. Finally, MSC-derived EV uptakes by MSCs through qPCR technique including RNA extraction, cDNA synthesis are described.

2.1 Tissue culture

Frozen adipose-derived MSCs from osteoarthritis patients were obtained from Magellan Stem Cell Centre. 1×10^6 MSCs were seeded into T175 Flask containing 35 mL of culture medium (5% FBS in serum free MSC Medium) in 37°C, 5% carbon dioxide and 21% oxygen humidified incubator. After confluence reached to 80-95%, MSCs were sub- cultured and 2×10^6 MSCs were seeded into T175 Flask containing 35 mL of exosome-free medium. The exosome-depleted medium was prepared by 20% FBS-containing culture medium ultracentrifuged at 100,000 x *g* and 4°C for 18 hours. Pellets were discarded to deplete the exosomes from FBS and supernatant was filtered through 0.22 µm filter. 20% FBS culture medium was then diluted to 5% FBS in culture medium. MSCs were cultured in exosome depleted medium, at 37°C, 5% CO_2 humidified incubator until approximately 90% confluence was reached (normally 48 to 72 hours). Finally, the conditioned medium was harvested for further EV isolation.

2.2 Isolation of EVs

2.2.1 Isolation EVs by the differential ultracentrifugation

This protocol was used to give a total of five samples of EVs. They are MVs, small EV 1 to 3 (number is ultracentrifuge hours) from ultracentrifugation and EVK obtained by the commercial kit (see below).

Cell culture medium was collected from flasks, mixed together and transferred to 15 mL conical centrifuge tubes. The conditioned medium was centrifuged at 300 × *g*, 4°C for 10mins and the supernatant was carefully transferred into new 15 mL conical tubes. The collected supernatant was centrifuged at 2000 × *g*, 4°C for 20 minutes and carefully transferred three quarter of supernatant into the ultracentrifuge tubes (355618, Beckman). One quarter of supernatant was transferred into 50 mL of centrifuge tube for EV isolation by using the commercial kit. The supernatant transferred in ultracentrifuge tubes was centrifuged at 10,000 × *g*, 4°C for 30 minutes by using type 70Ti rotor (Beckman) in Sorvall RC90 centrifuge. The supernatant was transferred into new ultracentrifuge tubes for small EV isolation, and the remaining pellet mixture was resuspended with 17 mL of PBS followed by centrifugation at 10,000 × *g*, 4°C for 30 minutes. Then the supernatant was carefully removed from each tube and resuspended the pellet known as MVs with 100 µL of PBS. MVs were aliquot into 0.5 mL eppendorf microcentrifuge tubes and stored in –80°C freezer for further analysis. The collected

supernatant (from 10,000 × g) was centrifuged at 118,000 × *g*, 4°C for 70 minutes, 120 minutes, and 180 minutes separately. Then the supernatant was carefully removed and left a few millimetres above the pellet. The reminded mixture was resuspended with 17 mL of PBS and centrifuged at 118,000 × *g*, 4°C for 70 minutes, 2 hours, and 3 hours respectively. Finally, the supernatant was carefully removed from each tube and resuspended the pellet known as small EVs with 100 µL of PBS. Small EVs were aliquot into 0.5 mL eppendorf microcentrifuge tubes and stored in –80°C freezer for further analysis.

2.2.2 Isolation EVs by the commercial kit

EVs were isolated using Total Exosome Isolation Kit (Cat#4478359, Life Technologies) according to the manufacturer's instructions. The supernatant was collected into 50 mL centrifuge tube from the centrifuge 2000 × *g* step and identical with the supernatant subjected to the ultracentrifugation. Half the volume of the Total Exosome Isolation reagent was added into supernatant and mixed well by vortexing. After overnight incubation at 4°C fridge, mixture was transferred into ultracentrifuge tubes and centrifuged at 10,000 × *g*, 4°C for 60 minutes. The supernatant was carefully removed and resuspended the pellet known as EVs by 100 µL of PBS. EVs were transferred into 0.5 mL Eppendorf microcentrifuge tubes and stored in –80°C freezer for further analysis.

2.3 Nanoparticle trafficking analysis (NTA)

Nanoparticle tracking analysis for each sample was performed in Latrobe University using Nanosight NS300 instrument (Malvern Technologies, Malvern, UK). Each sample was diluted 1 to 20 by PBS which was filtered through 0.22 µm filter. Diluted sample was filled up into sample syringe and then put sample syringe into automatic screw infusion. Camera level was adjusted from 8 to 10, adjusted focus till observed the particle moving and capture. This was performed three times, 30 seconds to create a document.

2.4 Total protein concentration

The total protein concentration was obtained by using Pierce™ BCA Protein Assay Kit (Cat#23225, ThermoFisher Scientific). The procedure set out by the manufacturer's instructions were followed. First a serial of diluted BSA standards were prepared from vial A to J with BSA concentration 750, 50,400,325, 250, 200, 125, 100, 50, 25, 0 µg/mL respectively. The working reagent were made by mixing 50 parts of BCA Reagent A with 1 part of BCA Reagent B. One group of samples were diluted by adding the same volume of nuclease-free water. 25 µL of each standard and each sample duplicates were pipetted into a microplate well. 200 µL of the working reagent was added to each well and mix plate thoroughly on a plate shaker for 30 seconds. The microplate was covered and incubated at 37°C for 30 minutes. The total protein concentration was determined based on the measured absorbance at 562 nm on a plate reader.

2.5 SDS-PAGE and Western blot analysis

Five samples were subjected to western blot analysis. MSCs were used as positive control and exosome-free medium as negative control. 1×10^6 MSC pellet resuspended with 500 µL of PBS and exosome-free medium was four times diluted. All samples added 0.25 volume of Bolt LDS Sample

Buffer were loaded into the gel (Bolt™ 4-12% Bis-Tris Plus gels, Life Technologies) alongside a molecular weight marker (SeeBlue® Plus2 Pre-Stained Protein Standard) by using Mini Gel Tank (CAT#A25977, Life Technologies). The gel was run at 120 V for 45 minutes to separate the proteins. The gel unit was disassembled, cut and removed the gel removed with a blade, then transferred the gel into a tray contained with 10 mL of Coomassie blue staining buffer (Bulldog) and incubate on rocker for 1 hour (SDS-PAGE). Then the gel was imaged in ChemiDoc™ XRS+ (BIO-RAD). Blotting was performed on the 0.45 µm pure nitrocellulose membrane (BIO-RAD) and proteins were transferred from gel to membrane by using Trans-Blot® Turbo™ Transfer System (DIO-RAD). Then the membrane was blocked in 5% skimmed milk in TBS-T for 1 hour on the rocker. The membrane was washed three times by PBS and incubated with 1/2000 dilution of primary antibody (in 0.02% Tween20, 1% BSA in TBS) on the rocker for overnight at 4°C. Primary antibodies were included CD9 (Cat#AHS0902, Life Technologies), CD63 (mouse anti-CD63, Cat#10628, Life Technologies), CD81 (Cat#MA513548, Life Technologies) and Calnexin (SC-80645, SANTA CRUZ). The membrane was washed three times by PBS and incubated in a 1/2000 dilution of secondary antibody (goat anti-mouse HRP conjugate in 0.02% Tween20, 1% BSA in TBS) for 1 hour. Again, the membrane was washed three times by PBS, put membrane on plastic film and spread 2 mL of substrate solution prepared by mixing equal parts of the Stable Peroxide Solution and the Luminol/Enhancer Solution (SuperSignal West Femto Maximum Sensitivity Substrate, Cat#34095, Life Technologies) for 5 minutes. The membrane was placed on the Whatman filter paper with 90 degree to drain excess reagent and placed in a clear plastic wrap. Then membrane was imaged in ChemiDoc™ XRS+ (BIO-RAD).

2.6 Electron microscopy

Scanning electron microscopy (SEM) was used to detect the morphology of EVs. The preparation of EV to avoid aggregation was a key step to visualise EVs under scanning electron microscopy. TEM sample preparation was employed to fix sample for SEM. The EV1 sample was firstly fixed on the EM Grid (GHCAU300, ProSciTech). Briefly, the EV1 sample was mixed with equal volumes of 4% paraformaldehyde, 5 µL of solution was deposited onto the EM grids and absorbed for 20 minutes. The EM grid was then washed the membrane side of EM grid onto 100 µL of PBS and followed by transfer into 50 µL of the 1% glutaraldehyde solution and incubated for 5 minutes. EM grid was washed eight times in 100 µL of distilled water for 2 minutes each. Finally, EM grid was stained onto 10 µL of UAR-EMS Uranyl Acetate replacement stain (22405, Lot#161012-14) for 15 minutes. EVs on EM grid were imaged by scanning electron microscopy (SUPRATM 40VP, ZEISS).

2.7 Flow cytometry

2.7.1 FACS (Fluorescent Activated Cells Sorting) for EVs

EVs could not be directly analysed by conventional flow cytometry due to their small size which are smaller than that of the resolution limits of imaging techniques. Magnetic Dynabeads were employed for EV surface protein analysis using flow cytometry. EVs were absorbed onto larger magnetic Dynabeads (4.5 µm in diameter) coated with human CD63 antibody and separated using Dynabeads® magnetic separation technology. Subsequent fluorescent antibody labelling to detect specific EV surface proteins was detected via flow cytometer. The procedure was performed using the Exosome-Human CD63 Isolation/Detection Reagent (Cat#10606D. Invitrogen), according to the manufacturer's

instruction. Firstly, 20 µL of anti-human CD63 coated magnetic beads were washed in 200 µL of isolation buffer (1% BSA in PBS). EV samples were added to the magnetic beads and incubated overnight at 4°C with mixing on a shaker, then washed twice and resuspended in 300 µL of isolation buffer. This was split into 3 X 100 µL and incubated for 60 minutes at room temperature with 1/100 dilution of primary antibodies; mouse anti-CD9 (Cat#AHS0902, Life Technologies), mouse anti-CD63 (Cat#10628, Life Technologies) or mouse anti-CD81 (Cat#MA513548, Life Technologies), respectively. The bead-bound EVs were washed twice, resuspended in 100 µL of isolation buffer with 1 /100 dilution of goat anti-mouse secondary antibody conjugated with AF488 (ab150113, Abcam) and incubated for 30 minutes at room temperature. After wash and resuspension, the bead-bound exosomes were analysed by flow cytometry (Attune NxT, Life Technologies). Magnetic beads alone acted as negative control.

2.7.2 MSC FACS

2×10^6 MSCs were undergone the normal incubation procedure of flow cytometry and acted as positive control for EV FACS. Briefly, three tubes of 0.5×10^6 MSCs resuspended in 500 µL of FACS buffer (1% BSA and 0.1%EDTA in PBS) were incubated for 30 minutes at 4°C with 1/500 dilution of primary antibodies, CD9, CD63 or CD81, respectively. After wash and resuspension, MSCs were incubated with 1/500 dilution of goat anti-mouse secondary antibody AF488 conjugate. Washed and resuspended labelled MSCs, unlabelled MSCs at the same concentration and bead-bound exosomes were subjected to flow cytometry to analyse surface markers.

2.8 Labelling EVs by using CFSE

SH MSC (derived from young sport injured osteoarthritis patient) culture medium was collected from T175 flask contained 40 mL of culture medium once MSCs reached to 90% confluence. SH MSC-derived EVs were isolated by serially centrifuging at 300 X *g* for 10 minutes, 2000 X *g* for 20 minutes, 10,000 X *g* for 30 minutes, 118,000 X *g* for 70 minutes and followed washing EV pellets with 16 mL of 20 mM Hepes buffer. Then fresh isolated EV pellets were resuspended by 100 µL of 20 mM Hepes buffer for each ultracentrifuge tube. Prepare CellTrace™ stock solution immediately prior to use by adding 18µL of DMSO to one vial of CellTrace™ CFSE dye (CAT#34554). The stock concentration is 5 mM. 1µL stock solution of CFSE was added into 250 µL of freshly isolated EVs in Hepes to reach 20 µM working solution. The mixture was incubated for 1 hours at 37°C, mixed by flicking and protected from light. The stained EVs were resuspended by 16 mL Hepes buffer (20mM, pH7.0), centrifuged at 118,000 × g, 4°C for 70 minutes and resuspended EVs with the previous volume of Hepes buffer. 200µL of the washed stained EVs was loaded into the attached SH (Younger age) and LBC (Older age osteoarthritis patient) MSCs in 12-well plate which $4x10^4$ MSCs were seeded with 1.5 mL culture medium in prior day. 12-well plate was incubated for 24 hours at 37°C. SH MSCs were stained with CFSE as positive control. The procedure of MSC labelling was to grow SH MSCs to the desired density in 12-well plate with 1.5 mL of culture medium. The CellTrace™ DMSO stock solution was diluted in pre-warmed (37°C) PBS to the 1 µM concentration of CFSE loading solution. The culture medium of MSCs was removed, replaced with CFSE loading solution, and incubated MSCs for 20 minutes at 37°C. MSCs were washed twice with culture medium and incubated with pre-warmed culture medium for overnight. Then MSCs were detached by Trypsin, harvested, and detected by flow cytometry. MSCs served as negative control. 100 µL of the stained EVs was loaded into the attached SH and LBC MSCs in 8

chamber culture slides ($4x10^4$ MSCs seeded and incubated for 24 hours), incubated for overnight at 37°C. For experiments where frozen EVs were used, the same staining procedure to load into 12-well plate and 8 chamber culture slides. Finally, MSCs in 12-well plate were detached by Trypsin, harvested and detected by flow cytometry. SH MSCs without stained EVs or CFSE dye as negative control. MSCs in 8 chamber culture slides were analysed using confocal microscopy.

2.9 Analysis of EV function by qPCR

2.9.1 MSC samples

For this experiment, two cell lines belonging to two different OA patients of different age, cell line, SH-MSC from a 27-year-old female and another cell lines BJ, from a male patient of age 70 years old were used for the EV uptake from one cell line to another cell line. A cell count of 0.5 X 10^6 SH-MSCs were seeded into two T25 flasks and 0.75 X 10^6 BJ-MSCs. MSCs were seeded into two T25 flasks. 800 µL of freshly harvested EVs isolated as previously described method from SH MSC conditioned medium were transferred into one each of SH MSC and BJ MSC T25 flask. All flasks were incubated at 37°C, 5% CO_2 humidified incubator for 3 days. MSCs were cultured to over 80% fluency. MSC pellets were harvested by trypsin. Four MSC samples named as SH (SH MSCs), SHE (SH MSCs incubated with EVs), BJ (BJ MSCs) and BJE (BJ MSCs incubated with EVs) were stored in -20°C freezer for later RNA extraction.

2.9.2 RNA extraction

RNAs of four samples were extracted using Qiagen RNeasy Mini Kit (Cat. # 74104). The protocol was performed by following the manufacturer's instruction. Briefly, 350 µl of buffer RLT mixture was added into the tubes contained SH, SHE, BJ, BJE MSC pellets and centrifuged for 3 minutes at highest speed. Each homogenized lysate was transferred into fresh tubes, added same volume of 70% ethanol, and mixed well by pipetting. Immediately afterwards the 700 µl of the sample in each tube was transferred to the1.5 ml of RNeasy Mini spin column placed in a 2 ml collection tube. Then all the spin columns were centrifuged for 15 s at 8,000 x *g* and the flow-through discarded. The 700 µl of Buffer RW1 was added to each RNeasy spin column and centrifuged for 15 seconds at 8,000 x *g*. 500 µl RPE buffer was added into each column and centrifuged for 15 seconds at 8,000 X *g*. The flow-through was discarded. The 500 µl of Buffer RPE was added to each RNeasy spin column and centrifuged for 2 minutes at 8,000 x *g*. After discarding the flow-through, the RNeasy spin column was placed in a new 1.5 ml collection tube. 50 µl of RNase-free water was directly added to each spin column membrane and centrifuged for 1 minute at 10,000 x *g* to elute RNAs. 50 µl of RNAs purified for each sample in each tube were aliquoted at 7 µl and 25 µl into new centrifuge tubes. RNA samples were stored in -80°C freezer for later cDNA synthesis. The concentration of the extracted RNAs was determined by using NanoDrop 2000 Spectrophotometer (Thermo Scientific).

2.9.3 cDNA synthesis

To determine the gene expression in each sample, the extracted RNAs were subjected to the two-step qRT-PCR which involved creating double-stranded cDNA and followed by PCR reaction. cDNA was synthesised from the extracted single-stranded RNAs by reverse transcriptase reaction. The Oligo dT

primers method in Tetro cDNA Synthesis Kit was employed. Firstly, the priming premix was prepared which contained 1µl of Primer (Oligo (dT)18), 1 µl of 10 mM dNTP mix, 1 µl of Ribosafe RNase inhibitor, 1 µl of Tetro Reverse transcriptase (200 u/µl) and 4 µl of 5×RT Buffer for each sample. Each 8 µl mixture was transferred into 4 PCR tubes. According to the concentration of RNAs, 5 µg RNAs of SH, SHE, BJ, BJE were added into each PCR tube and brought to 23 µl with DEPC-Treated water. Samples were incubated using one PCR cycle (45°C for 30min, 85°C for 5 minutes). The synthesised cDNAs were analysed by using NanoDrop 2000 Spectrophotometer (Thermo Scientific). The synthesised cDNAs were stored in -80°C freezer for further analysis.

2.9.4 qPCR

The specific primer sequences used in gene expression analysis was chosen from the published article by Dudakovic and colleagues (Dudakovic et al., 2014). The specific genes were listed below in Table 3.

Table 3: The details of specific genes.

Gene marker type	Name of gene primer	Abbrev	Accession number	Forward and reverse sequences	Length of PCR products (bps)
House Keeping	Glyceraldehyde-3-phosphate dehydrogenase	GAPDH	NM_002046.5	F: ATGTTCGTCATGGGTGTGAA R: TGTGGTCATGAGTCCTTCCA	144
Pluripotent gene	POU class 5 homeobox	POU5F1	NM_002701.5	F: GCAATTTGCCAAGCTCCTGAA R: AAGCTAAGCTGCAGAGCCTCAAAG	141
	Nanog homeobox	NONAG	NM_024865.3	F: CAACTGGCCGAAGAATAGCAATG R: TGGTTGCTCCAGGTTGAATTGTT	159
	Kruppel like factor 4	KLF4	NM_004235.5	F: AAGAGTTCCCATCTCAAGGCACA R: GGGCGAATTTCCATCCACAG	91
Osteogenic gene	Runt related transcription factor 2	RUNX2	NM_004348.3	F: ATGTGTTTGTTTCAGCAGCA R: TCCCTAAAGTCACTCGGTATGTGTA	195
	Collagen type I alpha 1 chain	COL1A1	NM_000088	F: GCTACCCAACTTGCCTTCATG R: TGCAGTGGTAGGTGATGTTCTGA	168
Chondrogenic gene	SRY-box 9	SOX9	NM_000346.3	F: TGTATCACTGAGTCATTTGCAGTGT R: AAGGTCTGTCAGTGGGCTGAT	187
	Collagen type II alpha 1 chain	COL2A1	NM_001844.4	F: TGAAGGTTTTCTGCAACATGGA R: TTGGGAACGTTTGCTGGATT	67
Adipogenic gene	Peroxisome proliferator activated receptor gamma	PPARG	NM_005037.5	F: TGGAATTAGATGACAGCGACTTGG R: CTGGAGCAGCTTGGCAAACA	182
CD markers gene	Thy-1 cell surface antigen	THY1 (CD90)	NM_006288.4	F: ATGAAGGTCCTCTACTTATCCGC R: GCACTGTGACGTTCTGGGA	112
	5'-nucleotidase ecto	NT5E (CD73)	NM_001204813.1	F: AAGGACTGATCGAGCCACTC R: GGAAGTGTATCCAACGATTCCCA	161
	Endoglin	ENG (CD105)	NM_000118.3	F: TGCACTTGGCCTACAATTCCA R: AGCTGCCCACTCAAGGATCT	107
	Protein tyrosine phosphatase receptor type C	PTPRC (CD45)	NM_002838.4	F: ACAGCCAGCACCTTTCCTAC R: GTGCAGGTAAGGCAGCAGA	88
	CD14 molecule	CD14	NM_000591.3	F: CAACCTAGAGCCGTTTCTAAAGC R: GCGCCTACCAGTAGCTGAG	135

The chosen primers were checked on the website http://primer3.ut.ee/. The specific gene primers were purchased from Sigma-Aldrich Company. The sequence for each specific gene was obtained from website https://www.ncbi.nlm.nih.gov through FASTA NCBI Reference Sequence. The length of PCR product for each gene was determined using https://www.ncbi.nlm.nih.gov/tools/primer-blast/ by entering FASTA sequence, forward primer and reverse primer. GAPDH encoded the protein which catalysed an important energy-yielding step in carbohydrate metabolism and known as a popular housekeeping gene. GAPDH gene was chosen as reference gene for its high-level stable expression in the cells.

qRT-PCR was performed by using SsoAdvancedTM universal SYBR Green Supermix kit (ThermoFisher Scientific). Triplicates were made for each sample (SH, SHE, BJ, BJE) with each specific primer and GAPDH primer as reference gene. The reaction Mix preparation and Thermal Cycling Protocol was followed according to SsoAdvancedTM universal SYBR Green Supermix kit. The assay master mix for each specific primer was prepared and 19 µl aliquots were dispensed into the wells of the PCR plate. Each 19 µl of aliquot contained of 10 µl SsoAdvancedTM universal SYBR Green Supermix, 0.5 µl of forward primers, 0.5 µl of reverse primer, and 8 µl of Nuclease-free water. The cDNA templates of each sample were diluted 1:10 with nuclease-Free water according to the cDNAs concentration. Each 1 µl of 1:10 solution for each sample was transferred into the relevant wells of PCR plate. PCR plate was sealed with film and centrifuged at 300 x *g* for 1 minute to mix components thoroughly. Bio-Rad CFX96TM system was used to program thermal cycling protocol which performed initial denaturing at 95°C for 30 seconds, 40 cycles of denaturing at 95°C for 10 seconds, annealing and extension for 30 seconds in gradient temperature to suit each primer (The annealing temperature for each primer was Tm minus 5 which Tm was obtained in Sigma-Aldrich technical Datasheet) and Melt-Curve Analysis from 65°C to 95°C with 0.5°C increment. The cycle threshold value of each plate well was used to calculate gene expression using the relative quantification $2^{-\Delta\Delta CT}$ method.

Chapter 3

Optimisation of EV isolation method

This chapter develops and assesses a suitable MSC-derived EV isolation method. The aims of this chapter are to optimise isolation method and investigate:

1. Protocols to isolate EV samples based on current available equipment in Swinburne.
2. Visualisation of MSC-derived EV by the scanning electron microscopy to identify EV size.
3. Evaluation of MSC-derived EV size distribution by nanoparticle trafficking analysis.
4. Determination of MSC-derived EV total protein concentration.
5. Characterisation of MSC-derived EV protein markers using western blot and FACS technique.
6. Comparison of results obtained from above techniques to determine an ideal MSC-derived EV isolation method.

An introduction to existing EV isolation methods is given in Section 1.2.7. Based on current available equipment in Swinburne, four MSC-derived EV samples are obtained and characterised by scanning electron microscopy (SEM), nanoparticle trafficking analysis, western blot and FACS analysis. Based on the results, 1 hour of differential ultracentrifugation was optimal to isolate high quality MSC-derived EVs. This optimised method would help further clinical research into MSC-derived EVs, with a low-cost high-quality EV preparation.

3.1 MSC derived EV isolation and characterisation

The main aim of this chapter is to optimize the isolation method of MSC-derived EVs. The first step is to isolate few EV samples. Since differential centrifugation is the benchmark for EV isolation, three EV samples EV1, EV2, EV3 were isolated from the conditioned medium by performing differential ultracentrifugation with three different time points 1, 2, 3 hours of centrifugation and EVK obtained by commercial kit (Figure 12). The details are described in Section 2.2.

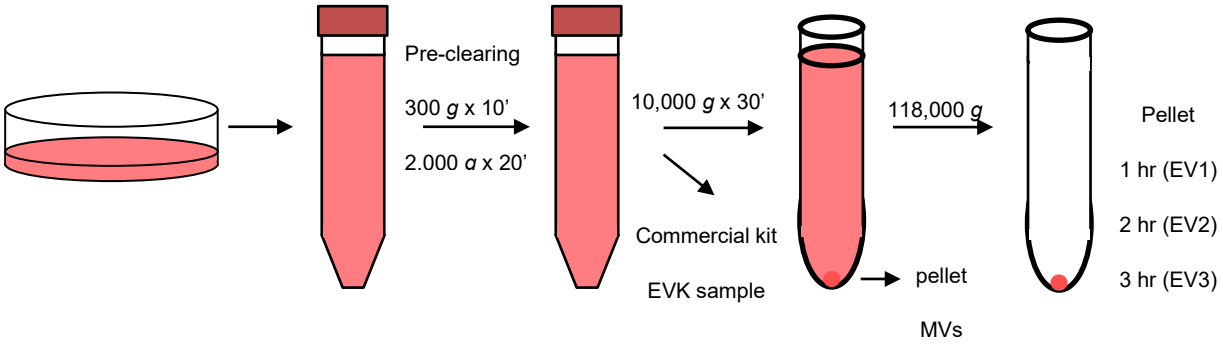


Figure 12. Sample Preparation flow chart. Cells were cultured in exosome-free medium for 48-72 hours and then the conditioned medium was harvested. A series of initial pre-clearing low speed centrifugation steps were performed to remove cells and larger debris. The sample was then split to be used for EV isolation using a commercial kit (EVK), and the remaining supernatant was further centrifuged at 10,000 x *g* for 20 minutes. The resulting pellet was collected for processing as microvesicles (MVs). The supernatant was centrifuged at 118,000 x *g* for either 1, 2 or 3 hours and the resulting pelleted EVs processed as EV1, EV2 or EV3, respectively.

Following isolation of the EV1, EV2, EV3, EVK and microvesicles (MVs), a number of characterisations were performed. We performed SEM, NTA and total protein assay to confirm the quality of MSC-derived EVs. Then western blot and flow cytometry techniques were employed to characterise MSC-derived EV protein markers to examine quantity of MSC-derived EVs.

3.1.1 Scanning electron microscopy

EVs were too small to be seen under a regular microscope. To visualise nanoparticle EVs, scanning electron microscopy was employed to take images of small EVs which were fixed and immobilized on ultrathin holey carbon film grid. According to the procedure of fixation EVs on grid which included many washing steps, it was difficult to compare the difference between the samples. Only EV1 sample was examined by SEM. MSC derived EV images (Figure 13) demonstrated variable size of small EVs. Some of EVs were found to aggregate together. The majority of non-aggregated EVs were less than 120 nm. SEM result confirmed the isolated EVs with expected size differential ultracentrifugation for 70 minutes.

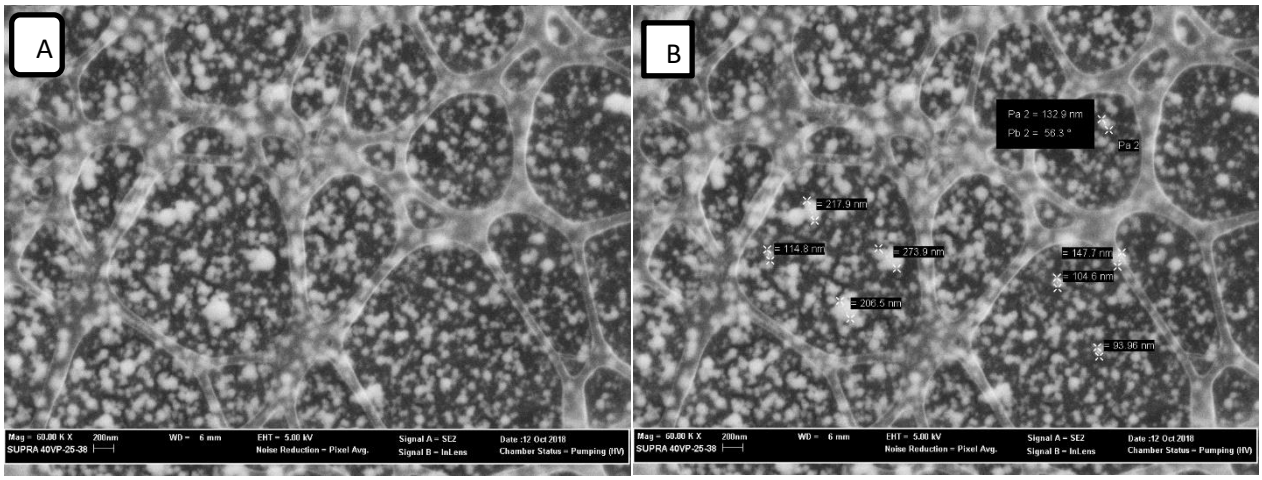


Figure 13: MSC derived EV images by SEM. Left (Image A) was original. Right (Image B) was same image as A but few EVs are labelled with measurement.

3.1.2 Nanoparticle trafficking analysis

The particle concentration and size distribution of the 5 EV samples (include MVs) was analysed. NanoSight NS300 instrument was employed to examine EVs in each PBS-resuspended EV sample. The result (Figure 14A) showed that the highest particle concentration was EV2 sample ($1.16x10^9$ particle/mL), followed by EVK ($7.79x10^8$ particle/mL), EV1 ($5.21x10^8$ particle/mL), EV3 ($4.69x10^8$ particle/mL), and the lowest particle concentration was MVs ($8.2x10^7$ particle/mL). The size distribution of each sample is shown in Figure 14B. EV1 and EV3 had a relatively similar size distribution with single peak, whereas EV2 and EVK had a broader double peak of smaller particles. The size distribution of MVs had a few broader peaks of smaller particles along with lowest particle concentration.

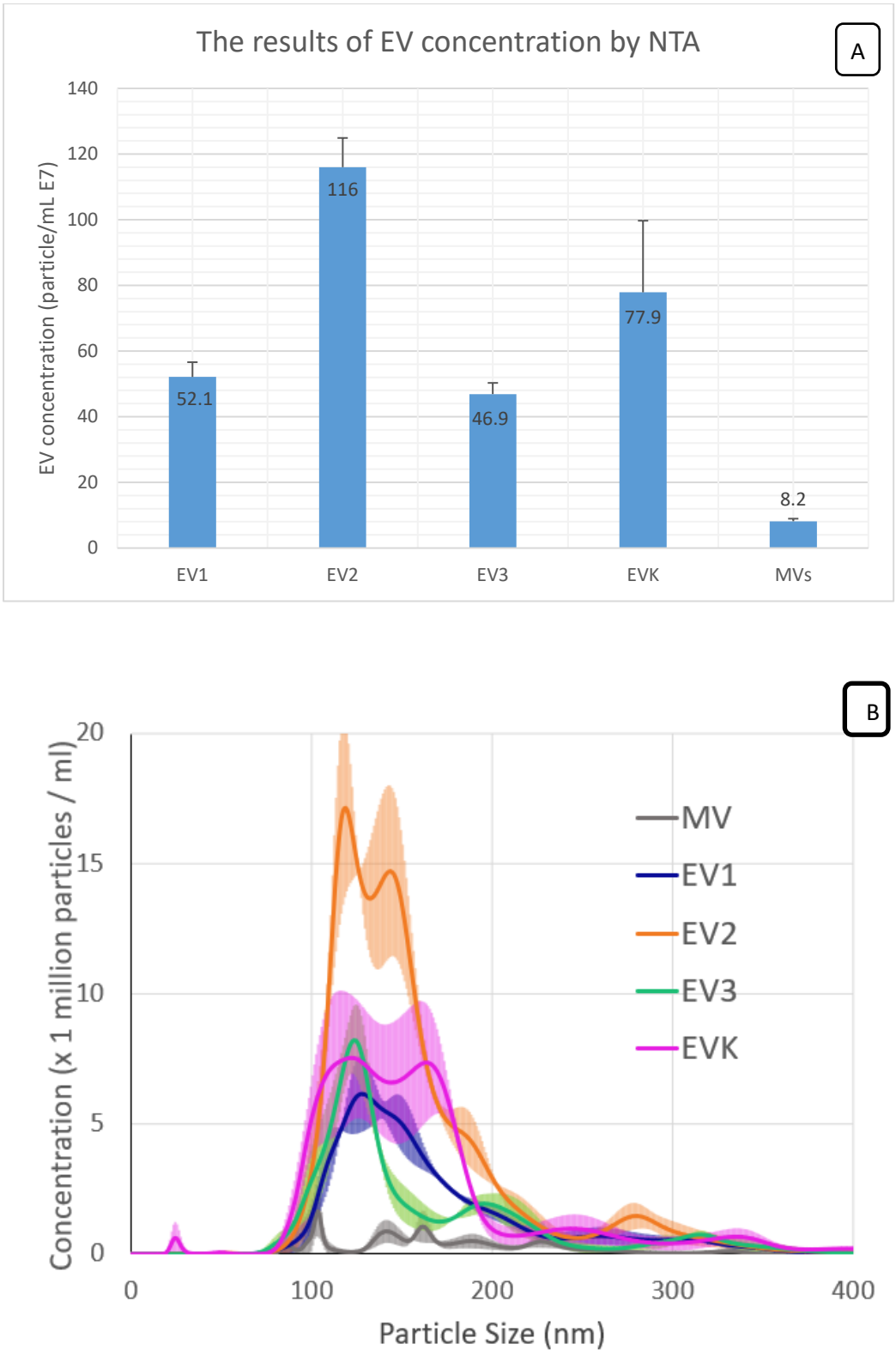


Figure 14: Comparison of the EV particle concentration (A) and size distribution (B) by using NTA. EV1-centrifuged for 70 minutes. EV2-centrifuged for 2 hours. EV3-centrifuged for 3 hours. EVK-isolated by the commercial kit. MVs isolated by low speed (10,000 x *g*).

3.1.3 Total protein concentration

Total protein concentration was examined by using Pierce™ BCA Protein Assay Kit. The results were averaged from two performances and showed in Figure 15.

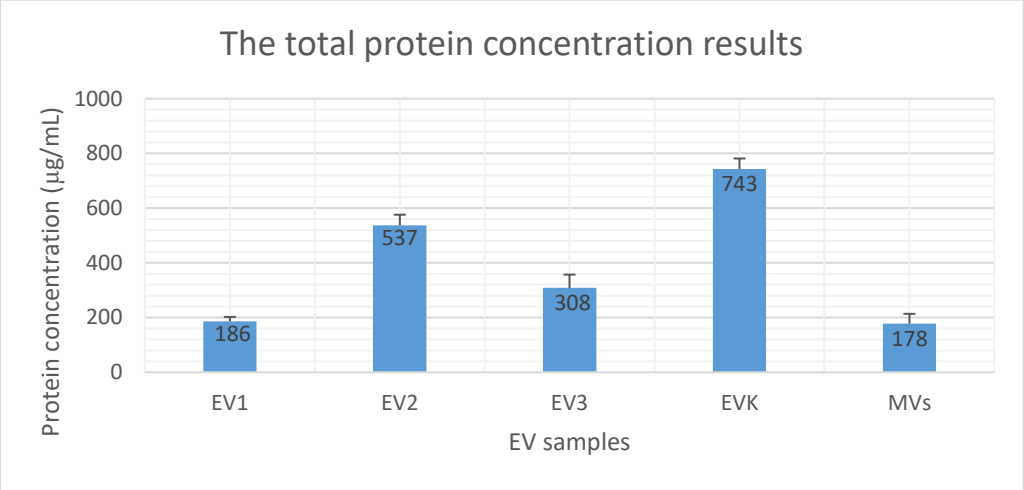


Figure 15: The results of EV protein concentration by BCA protein assay. EV1-centrifuged for 70 minutes. EV2-centrifuged for 2 hours. EV3-centrifuged for 3 hours. EVK-isolated by the commercial kit. MVs isolated by low speed (10,000 x *g*).

Sample EVK had the highest total protein concentration (743 µg/mL), and sequentially EV2 (537 µg/mL), EV3 (308 µg/mL), EV1 (186 µg/mL) and lastly MVs (178 µg/mL). Based on the results of particle concentration in section 3.1.3 and protein concentration, the protein amount in a single EV could be calculated and shown in Figure 16.

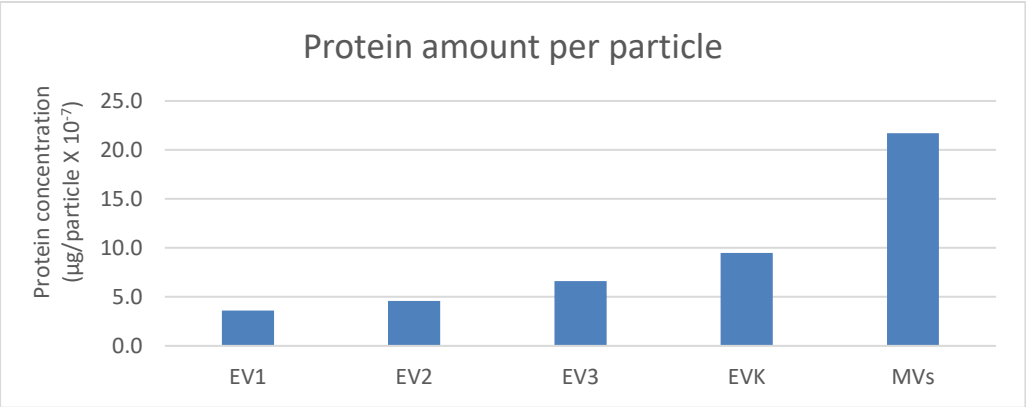


Figure 16: Protein amount per particle generated from the results of protein concentration and particle concentration. EV1-centrifuged for 70 minutes. EV2-centrifuged for 2 hours. EV3-centrifuged for 3 hours. EVK-isolated by the commercial kit. MVs isolated by low speed (10,000 x *g*).

According to the results of protein amount per particle, EVs in MVs had the highest protein amount and followed by EVK, EV3, EV2, and the lowest protein amount per particle was EV1. Leaving aside MVs which would be larger size than other EV samples, 4 samples EV1, EV2, EV3 and EVK should have similar protein value per particle since they were isolated from identical cell culture medium. EV sample with higher protein amount in a single particle might indicate the sample was contaminated

with non-EV proteins, protein aggregates or some lipoproteins. This would be assessed and made clearer by characterisation of EV protein markers.

3.1.4 SDS-PAGE and Western Blot

EVs commonly contain CD9, CD63, CD81, TSG101 and Alix proteins, and do not contain any proteins originally from nuclear, mitochondrial, endoplasmic reticulum or Golgi-apparatus organelles (Thery et al., 2002; Zabeo et al., 2017). Western blot technique was performed to detect three positive EV protein markers CD9, CD63, CD81 and one negative protein Calnexin, the endoplasmic reticulum protein which was not expected to be present in EVs. MSCs, exosome-free medium and five samples were subjected to SDS-PAGE and Western blot for demonstration and detection of proteins. All samples were measured by loading into gel wells with same volume of samples (12 µL of each sample was added 4 µL of Bolt LDS Sample Buffer) or by loading same amounts of proteins (≈1.5 µg) for each sample according to the results of BCA protein assay. The results of SDS-PAGE are shown in figure 17.

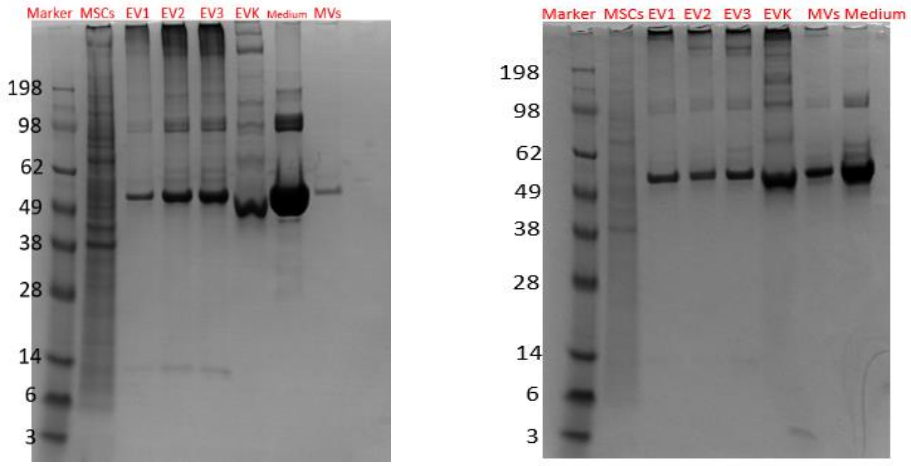


Figure 17: Comparison two SDS-PAGE results. Left image is SDS-PAGE by loading same volume of samples. Right image is SDS-PAGE by loading same mass of proteins (≈1.5 µg) based on BCA protein assy.

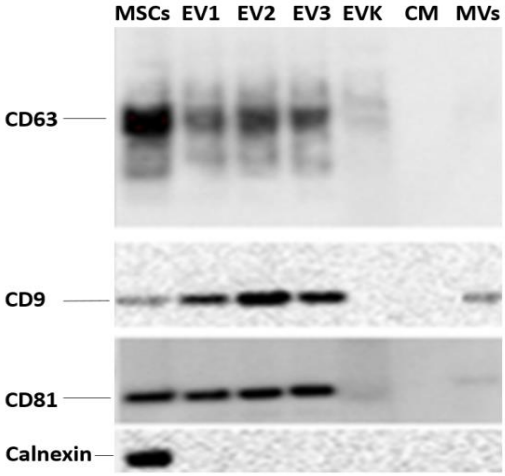


Figure 18: Comparison between two Western blot results of EVs. The left image showed the western blot result by loading the same volume of samples. The right showed the result by loading same amounts of proteins based on BCA protein assy. MSCs was the sample of MSCs. EV1 to 3 were EV samples isolated by differential ultracentrifugation at 70 minutes, 2, and 3 hours. EVK was the sample isolated using the commercial kit. CM was the diluted conditioned cell culture medium. MVs was the sample of macrovesicles. MSCs was the positive control and CM was the negative control.

CD9, CD63, CD81 are members of tetraspanin family which are cell surface glycoproteins with four transmembrane domains. The predicted molecular mass of the encoded protein is 24-27kDa. The highly glycosylated CD63 antigen displays a broad band with a molecular mass ranging from 30 to 60kDa in Western Blot (Metzelaar et al., 1991).

First, five EV samples of the same volume were into gel wells. The results of Western blot (Figure 18 left) showed detected CD9, CD63, CD81 with expected protein size/size range in MSCs, EV1, EV2, EV3 samples. EV2 sample was found as strong bands of CD9 and CD63. EVK and MVs sample had demonstrated by faint bands of CD9, CD63 and CD81. Based on these results, it might be a better choice to isolate MSC-derived EVs by differential ultracentrifugation for two hours. To confirm this, another experiment of Western blot was performed—loading same amounts of proteins based on the results of BCA protein assay. The result (Figure 18 right) showed the strongest signals of CD9, CD63, and CD81 in EV1 sample. Comparing two results of Western blot, despite stronger bands of EV2 sample by loading same volume of EVs, EV1 sample was detected as having the strongest signals of CD9, CD63 and CD81 by loading same amounts of proteins. This indicated that the concentration/purification of small EVs in EV1 sample was higher than other samples. EV2 and EV3 samples might have been contaminated by non-EV proteins, protein aggregates or some lipoproteins. Negative control Calnexin was detected on the MSCs sample only. All four EV samples and MVs were negative for Calnexin which indicated there was no contamination from endoplasmic reticulum.
Faint bands of EVK sample in western blot, highest protein concentration in protein assay indicated excessive contamination of soluble proteins in EVK sample. From the results in this experiment, this commercial kit was not recommended for the EV isolation from our adipose derived MSC cell lines.

3.1.5 FACS

Since small EVs were too small to be detected by normal flow cytometry, Dynabeads® magnetic separation technology with bound anti-CD63 antibodies was employed to purify and cluster only CD63-positive EVs. Subsequent fluorescent antibody labelling was the used to detect EV surface proteins, CD63, CD9 and CD81. Normal FACS analysis of the same markers on MSCs acted as the positive control. MSC FACS results are shown in Figure 19A. Three protein markers CD9, CD63 and CD81 were detected on MSCs by flow cytometry, and CD81 was detected as the strongest signal and CD63 was detected as the weakest signal on MSCs.

For flow cytometry analysis of MSC-derived EVs, magnetic Dynabeads were used for flow cytometry analysis to confirm EV surface proteins CD9, CD63 and CD81. All EV samples (EV1, EV2, EV3, EVK and MVs) were detected the surface markers CD9, CD63 and CD81 as seen in Figure 19B, C, D. When Compared with the Western blot results which detected weak CD9, CD63, CD81 signals in EVK and MVs samples, all EV samples (EV1, EV2, EV3, EVK and MVs) detected surface markers CD9, CD63 and CD81. The reason for different results between FACS and Western blot is that the Dynabeads® magnetic separation technology was employed for EV FACS. Magnetic Dynabeads were coated with human CD63 antibody and only captured $CD63^+$ EVs. After magnetic sorting steps, detected EVs were purified CD63 EVs. This is also responsible for a stronger CD63 signals in EV FACS results than MSC FACS. All EV samples were detected with a similar intensity of CD9 and CD63 proteins. Interestingly the detection of CD81 demonstrated a weaker signal in EV2 and EVK samples compared with other

samples, not as CD9 and CD63 were detected similar signals in all of EV samples. It may be a possibility that less CD81+/CD63+ EVs in EV2 and EVK samples comparing with the other samples. CD81 could be detected only in small EVs but CD9 and CD63 were detected in both small and large EVs (Tkach, Kowal, & Thery, 2018). Given that EVs produced a strong signal of CD81 and CD63 this might indicate the higher percentage of exosomes in EV samples. It was unclear why MV sample was detected strong signal of CD81 at this stage. This may be caused by different types of cells. We studied MSC-derived EVs whereas Tkach and colleagues studied human primary dendritic cells, and MSCs might express CD81 but not in dendritic cells. According to the FACS results of EV samples, EV1 gave more stable and reliable results.

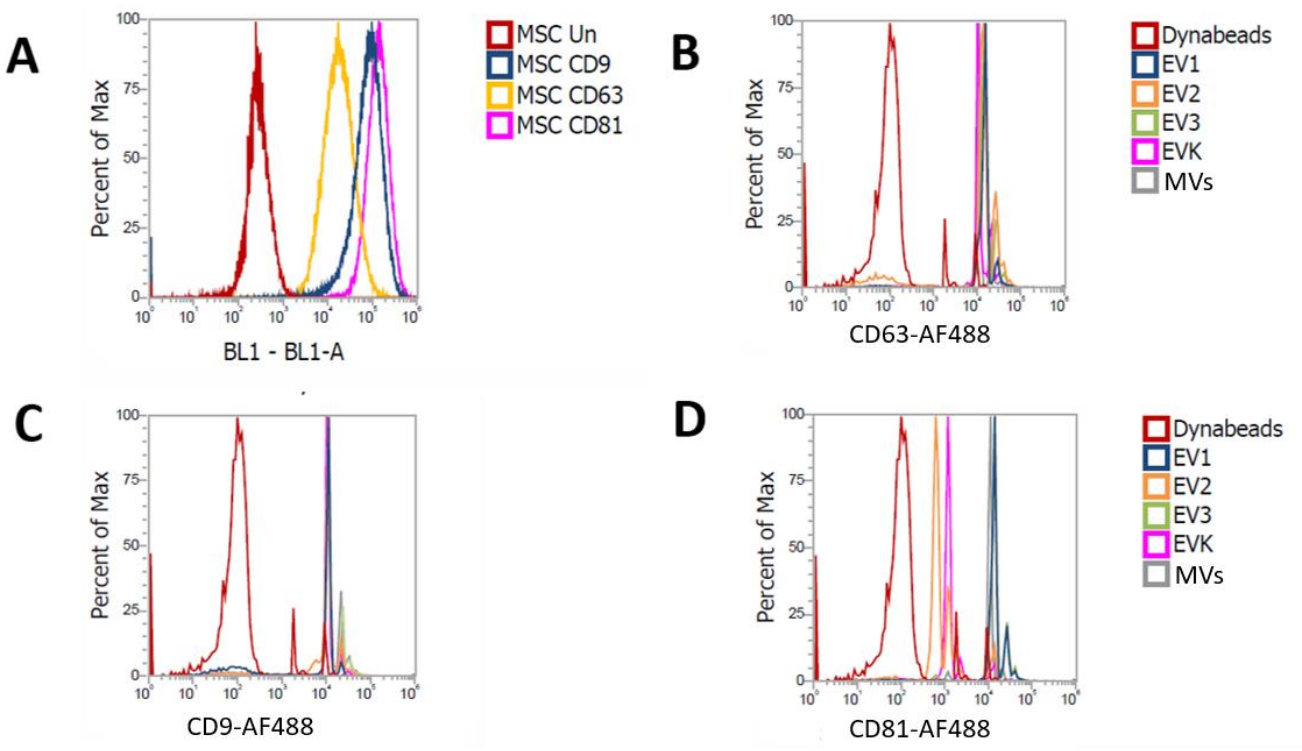


Figure 19: Detection of surface protein markers by FACS. **A**: Detection of MSC surface protein markers by flow cytometry, acting as positive control for detection on EVs. MSC Un – Unstained MSCs. MSC CD9—MSCs stained with anti-CD9. MSC CD63—MSCs stained with anti-CD63. MSC CD81—MSCs stained with anti- CD81. **B-D**: Detection of EV surface protein markers on EVs captured by anti-CD63 Dynabeads, labelled by anti CD63 (B) anti-CD9 (C) or anti-CD81 antibodies, anti-mouse AF488 secondary antibody and detected by FACS. Unbound Dynabeads acted as negative controls.

3.2 Discussion and Conclusion

In this chapter, we isolated four EV samples: EV1, EV2, EV3, MVs by differential ultracentrifugation with different duration and one EV sample isolated by commercial kit (EVK). Samples were quantified and characterised by NTA, BCA protein assay, Western blot, and FACS techniques. A protocol for detection of EV surface protein markers by flow cytometry was developed. The other protocol for visualisation of EVs by scanning electron microscopy was developed as well. Here, we would focus on four samples—EV1, EV2, EV3 and EVK. MVs were also analysed and presented here for researchers who interest in MVs.

To verify and provide an alternative measure of the size of EVs, scanning electron microscopy was employed to visualise EVs. A sample of EVs was fixed and immobilized on ultrathin holey carbon film grid. An example SEM image of EV1 is shown in Figure 13. The image shows a range of small EVs, some EVs appeared to be aggregated, possibly due to an artefact of fixation. The EM imaging indicated that the diameter of most non-aggregated EVs was less than 120 nm. The SEM result confirmed expected approximate size distribution measured by NTA. Comparing the results of SEM and NTA, it indicates EV sizes from SEM were slightly smaller than measured by NTA. This may be explained by previous that found that EV shrinkage during fixation (Dragovic et al., 2011).

Size distribution of EVs was measured by nanoparticle trafficking analysis using a NanoSight NS300 instrument. The results (Figure 14, A and B) show that EV1 and EV3 had a relatively similar size distribution with single peak, whereas EV2 and EVK had a broader double peak of smaller particles. MVs displayed a wider range of particle size, which is not unexpected as MVs were isolated at 10,000 x *g*, conditions that will favour the pelleting of larger vesicles. For the small peaks of smaller particles (size less than 50nm) in EV2 and EVK, an important question was whether they were membraned vesicle or non-membraned particles, which may be determined in protein analysis. Our results of EV size distribution were consistent with the previous studies which 90-160 nm modal size EVs from cell culture supernatants (Gardiner, Ferreira, Dragovic, Redman, & Sargent, 2013).

The results show EVK sample contained the highest protein concentration, with faint band intensity of EV protein detection in Western blot. These results suggest that excessive contamination of proteins in EVK sample. This is in agreement with Deun and colleagues' results (Van Deun et al., 2014). They examined two commercial kits which showed co-precipitate non-exosomal impurities. This commercial kit might not be ideal for the EV isolation from adipose derived MSCs. When comparing between samples EV1, EV2, and EV3, EV2 sample was given the highest particle concentration and highest total protein concentration, however, EV2 detected the weakest signal of EV protein in Western blot by loading same amounts of proteins. This might be due to the EV2 sample containing large protein aggregation. Density gradient such as iodixanol (OptiPrepTM) can be applied to obtain more pure small EVs (Théry, Amigorena, Raposo, & Clayton, 2006). It might be more reliable to compare EV protein concentration after the performance of purification process by density gradient or even size exclusion. However, it should be noted that increasing processes results in not only loss of EVs but also increasing more risks of contamination which are more concerned in clinical setting. This comparison indicated that whilst the yield of total protein and EV markers is highest in EV2, the relative purity of EV markers is highest in EV1. The longer time of ultracentrifugation times for EV2 and EV3 samples may result in greater contamination by non-EV proteins, protein aggregates or some lipoproteins. Theoretically, EV3 sample should have had the most protein due to the longer centrifugation time, however, this was a repeated observation over a number of experiments.

From the results of the Western blot analysis the EV1 sample contained the highest purity of small EVs, supported by results for EV markers by FACS. EV1 sample was obtained by the 1 hour of differential ultracentrifugation, which is less time consuming than EV2 and EV3 samples. Overall, the isolation method for EV1 could be considered an optimal balance of concentration and purity of EVs. It provides a reliable source of MSC-derived EVs for study EV uptake experiments to investigate intracellular fate of EVs, and EV functional analysis *in vitro* or *in vivo*, especially for clinical purpose which require minimal sample processing. However, for investigation of the contents of MSC-derived

EVs, novel biomarker discovery, and many downstream analyses such as proteomic, RNA, lipidomic analysis, etc. may require purer EVs and need further purification.

In summary, both high quality and quantity of EVs are required to study various biological functions of these exciting nanoparticles especially in medical research. Here I present an optimised and simple method for the isolation of EVS from adipose tissue derived MSCs. It was shown that the isolation of EV samples obtained by low-cost differential ultracentrifugation produces samples with better purity when compared to a commercial kit. Based on these results, 1 hour of differential ultracentrifugation was optimal to isolate high quality MSC-derived EVs as demonstrated by the SEM, NTA, and FACS assays. This optimised method would help further clinical research into MSC-derived EVs, with a low-cost high-quality EV preparation.

Chapter 4

Analysis of EV uptake and function

EVs have been described as playing important biological roles, in particular MSC-derived EVs which possess significant therapeutic potentials. EVs could bind to target cells through receptor ligand interaction, attach or fuse with target cell membrane to deliver their contents or be internalized through endocytosis by the target cells: all possible means to modulate recipient cells (Morelli et al., 2004). EV uptake pathways are heterogeneous just like the EVs. Furthermore, different cell types take up the heterogeneous EVs through different pathways, involving highly specific processes (Mulcahy et al., 2014). The theoretic details of EV internalisation pathways are described in Section 1.2.5. EV uptake by the recipient cells is the first essential initiative step to elicit their therapeutic potential on target cells. This chapter firstly will focus on establishment of EV labelling to visualise EV uptake based on the current available equipment in Swinburne University. Then the effect of EV uptake on MSC phenotype is investigated using the qRT-PCR technique. The aims of this chapter are:

1. To develop an EV labelling protocol for visualisation of EV uptake.
2. Investigation of donor MSC-derived EV uptake by recipient MSCs using confocal microscope and FACS techniques.
3. Examination of the effect of two types of EV storage conditions: freshly isolated EVs versus EVs stored at -80°C.
4. Investigation of effects of donor MSC-derived EV uptake on the phenotype of recipient MSCs, through qPCR technique.

EVs were isolated, based on the results in Chapter 3, in which 70 minutes of differential ultracentrifugation was found to be optimal for isolation of high quality MSC-derived EVs. In this chapter, MSC-derived EVs were isolated using differential ultracentrifugation from an MSCs cell line which are derived from adipose tissue of a young patient with osteoarthritis from a sustained sport injury (SH MSCs). These SH MSC-derived EVs were used to investigate their uptake and function on LBC and BJ MSCs cell lines, obtained from older osteoarthritis patients. All the MSCs cell lines were obtained from our collaborator Magellan Stem Cell Centre.

In this chapter, section 4.1 describes the uptake of SH MSC-derived EVs, either freshly isolated or stored at -80°C, by their parental MSCs or LBC MSCs. Section 4.4 then describes the effects of the uptake of SH MSC-derived EVs on the gene expression properties of parental MSCs and BJ MSCs. This is followed by discussion and conclusion in Section 4.3.

4.1 EV uptake analysis

Many techniques of EV tracking and imaging have been developed and introduced in Section 1.2.6 of this thesis. The aim here was to label EVs with fluorescent dyes that stain the whole EV population. Based on comparison of reports from previous studies that used fluorescent dyes to stain EVs (Dehghani et al., 2019; Morales-Kastresana et al., 2017), carboxyfluorescein succinimidyl ester CFSE was chosen as it has no non-specific aggregates and does not change the EV size after staining. Furthermore, CFSE is ideal for EV labelling as it can support a stable and strong signal intensity with

high specificity and sensitivity. Therefore, MSC-derived EVs were stained by CFSE dye to visualise MSC derived EV uptake.

After incubation of recipient MSCs with the stained donor MSC-derived EVs, EV internalisation was visualised and detected by confocal microscopy and flow cytometry, respectively. The detail of the method is described in Section 2.8. The diagram below (Figure 20) shows workflow of visualisation of SH MSC-derived EV uptake by SH MSCs and LBC MSCs.

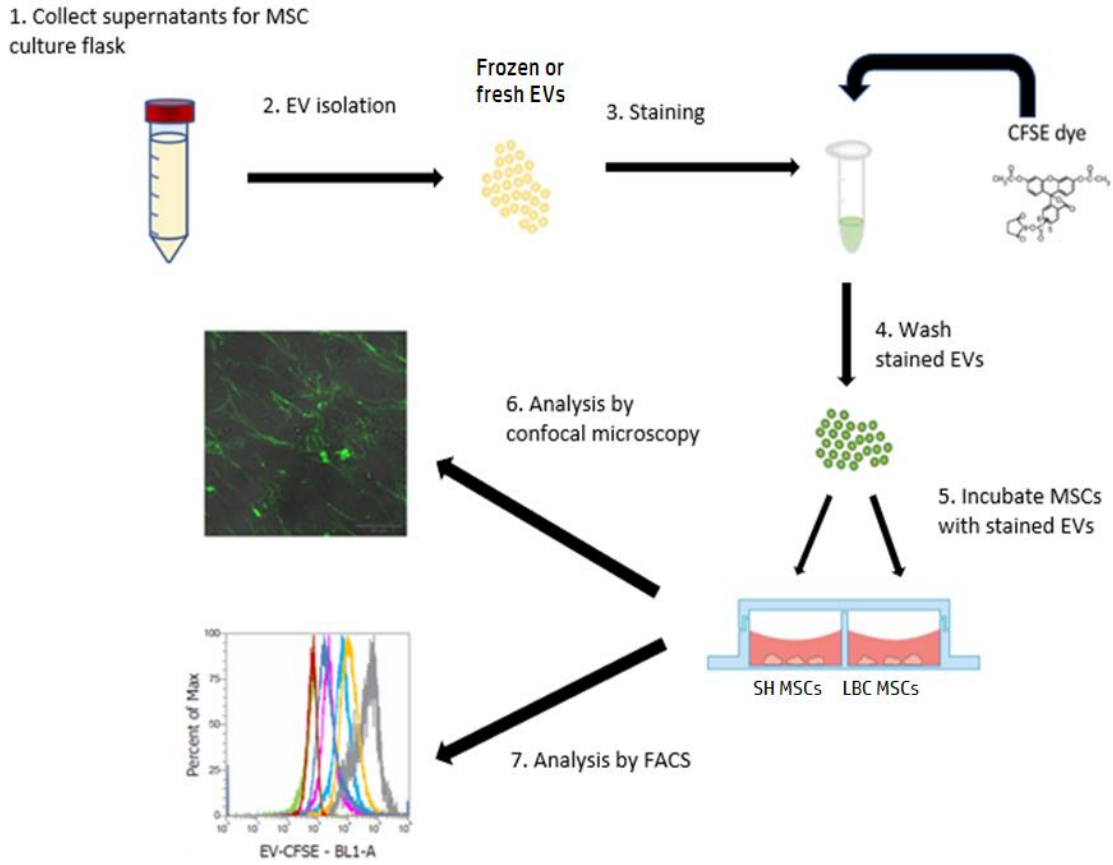


Figure 20: Workflow of MSC derived EV uptake analysis. 1. Conditioned medium was collected from MSC culture flask, with MSCs at approximately 90% confluency. 2. MSC-EVs were isolated by previous optimised isolation method. 3. Previously frozen or fresh MSC-EVs were stained with CFSE for one hour. 4. Stained MSC-EVs were washed by ultracentrifugation for one hour at 118,000 x *g*. 5. Cultured SH MSCs and LBC MSCs in 12-well plates and culture chamber slides were incubated with stained MSC-EVs for overnight. 6. MSCs in culture chamber slides were imaged by confocal microscopy to detect cellular internalization of stained MSC-EVs by confocal microscopy. 7. MSC-EV uptakes was analysed by FACS.

In brief, SH MSC-derived EVs were isolated by the previously optimized isolation method, which involves serial centrifugation at 300 X *g* for 10 minutes, 2000 X *g* for 20 minutes, 10,000 X *g* for 30 minutes, 118,000 X *g* for 70 minutes, followed by washing of the EV pellets with 16 mL of 20mM Hepes buffer. Afterwards, freshly isolated SH MSC-derived EVs and previously frozen SH MSC-derived EVs were stained with CFSE. This was followed with wash steps to eliminate free CFSE dye, via an extra ultracentrifugation step. Stained MSC-derived EVs were incubated overnight with adherent SH MSCs and LBC MSCs, that had been cultured to 90% confluency in 12-well plate and 8 culture chamber slides for overnight. MSCs in 12-well plate were detached by trypsin and analysed by flow cytometry. MSCs in 8 culture chamber slides were analysed by confocal microscopy. CFSE-labelled SH MSCs served as positive control.

In this section, we not only investigated whether MSC-derived EVs could be internalised, but also compare SH MSC-derived EV uptake between SH MSCs (EV parent cells) and LBC MSCs. In additionally the differences in uptake between fresh isolated MSC-derived EVs and frozen MSC-derived EVs was compared. A previous study showed that storage of neutrophilic granulocyte EVs at -80°C for up 28 days had no effect on EV number or size but partially reduced antibacterial function (Lorincz et al., 2014). Here, we investigated the effects of EV storage at -80 °C on EV uptake by MSCs.

4.1.1 EV uptake confirmation by confocal microscopy

SH MSC-derived EVs were labelled with CFSE, washed by an extra ultracentrifugation at 118,000 × *g*, 4°C for 70 minutes, and incubated with SH MSCs or LBC MSCs in 8-chamber slides overnight (17 hours). Figure 21 shows the results of MSC-derived EV uptake detected by confocal microscopy.

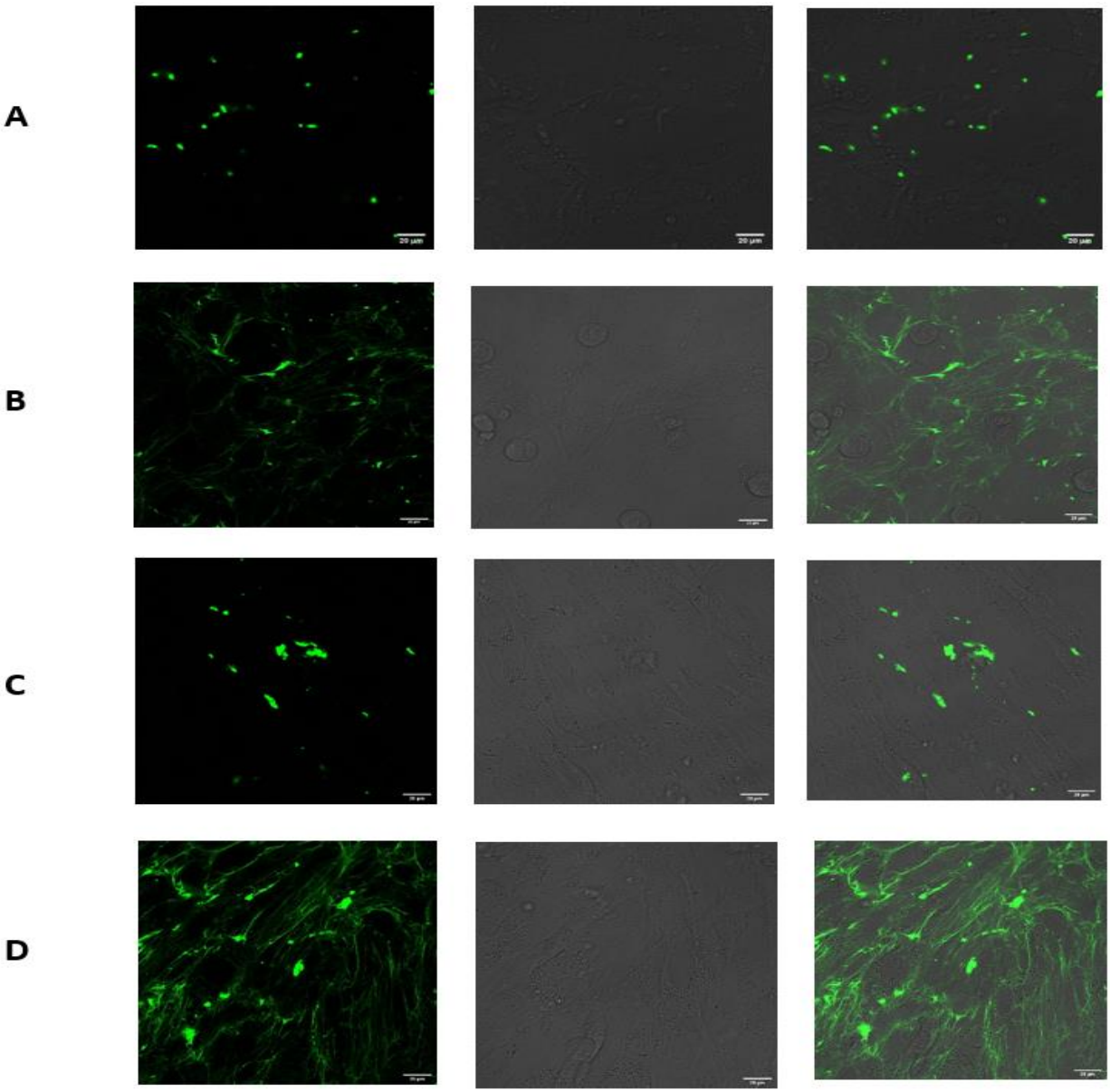


Figure 21: Confocal images of MSCs after overnight incubation with MSC-EVs labelled with CFSE. A: frozen SH MSC-EVs into SH MSCs. B: fresh SH MSC-EVs into SH MSCs. C: frozen SH MSC-EVs into LBC MSCs. D: fresh SH MSC-EVs into LBC MSCs. SH is a younger OA patient. LBC is an older OA patient. Left images--CFSE staining. Middle images--Bright field. Right images—merged. Magnification: 60X

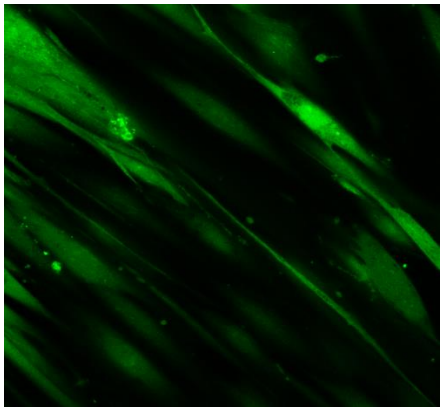


Figure 22: Image of CFSE-labelled SH MSCs, which serves as a positive control. Magnification: 60X

From the confocal microscopy results, CFSE-labelled donor MSC-derived EVs are effectively taken up by both cell lines, the donor and the recipient cells, SH MSCs and LBC MSCs, respectively. In particular, the fluorescence signal from the freshly isolated CFSE labelled MSC-derived EVs appear to be more distributed throughout the cells than from frozen EVs. It is difficult to distinguish the regions of interest inside imaged cells when viewed in 2D. However, the confocal images and 3D reconstruction video from Z-stacks (attached in appendix), it could be observed that the EVs are taken up by MSCs and the staining was localised to the cytoplasm and in cell periphery. The uptake of frozen EVs was observed distinct localised foci in the cells, similar to the results in most previous studies (Escrevente, Keller, Altevogt, & Costa, 2011). The CFSE staining following incubation with frozen EVs were distinct foci throughout the cells. Surprisingly, uptake of fresh EV was quite different to those of previous published studies for which labelled EVs mostly localised to the perinuclear region (Durak-Kozica, Baster, Kubat, & Stepien, 2018). Our fluorescent images showed fresh MSC-derived EVs are localised in the cytoplasm and cell periphery, giving a much stronger fluorescent intensity than frozen EVs. Furthermore, the fluorescence in the cell periphery appears with fibre-like structures. In addition, LBC MSCs uptake of SH MSC-derived EVs demonstrated stronger fluorescent intensity than uptake into SH MSCs. This suggests that SH MSC-derived EVs are more efficiently taken up by LBC MSCs than by the EV parental MSCs. Consequently, it was difficult to distinguish if EVs localised in the cell periphery or the plasma membrane in SH MSCs.

If EVs are in the plasma membrane, it is unknown whether EVs are cycled through the endocytic pathway, then exocytic pathway to dock to the plasma membrane and ready to be released into extracellular space and therefore recycled as EVs again (after 17 hours incubation). Alternatively, EVs could be fused with the plasma membrane, offload their contents and some of the remaining fluorescent components remain localised in the plasma membrane. A previous study of dynamic EV internalization and trafficking, which observed the cultured cells in real time for 3 hours, demonstrated EVs were endocytosed into cells, diffused in local microenvironments of the cytoplasm, and then were actively transported along actin filaments or microtubules (Tian et al., 2013). In an additional study it was shown that labelled EVs were taken up by the recipient MSCs, co-localised with MSC surface markers were ready to re-release after 24 hours incubation with MSCs (Dabrowska et al., 2018). The mechanisms for the interactions between EVs and cells are still elusive and many questions are still remain unanswered (Chuo et al., 2018). For example, what is the fate of EVs after they are endocytosed by individual cells, whether they fuse with endo/lysosomal membranes to reach the cytoplasm, to the ER, to be degraded in lysosomes, or to be recycled to generate new EVs? How are

EV proteins and RNAs offloaded into cells? Here, since the stronger fluorescent intensity is in the cell periphery, it may indicate that after MSC-derived EVs are internalised to MSCs, EV cargo is unloaded, some components of EVs might undergo EV biogenetic pathway to dock to the plasma membrane and ready to release to extracellular space again.

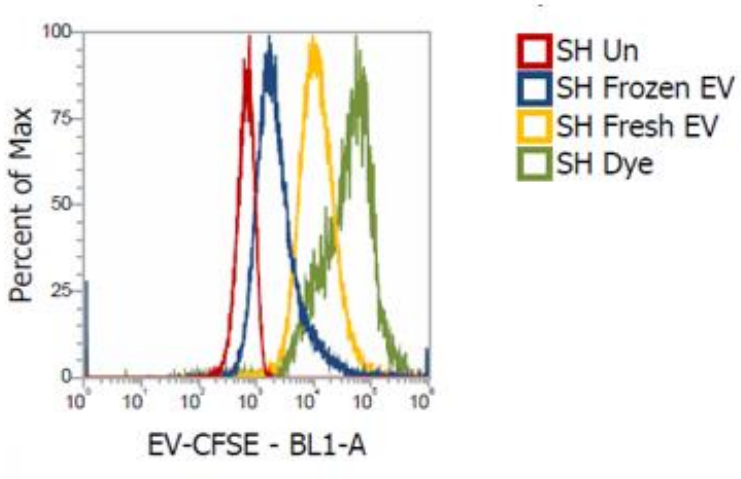
Overall, confocal microscopy results confirm MSC-derived EV internalization by both different MSCs as well as parental MSCs. Furthermore, freshly isolated MSC-derived EVs are more efficiently taken up by MSCs. Some fibre like strong fluorescence signal are detected and localised to the cell periphery/plasma membrane which suggests that the fate of MSC-derived EVs may be to be recycled and ready to be re-released.

4.1.2 EV uptake detection by FACS

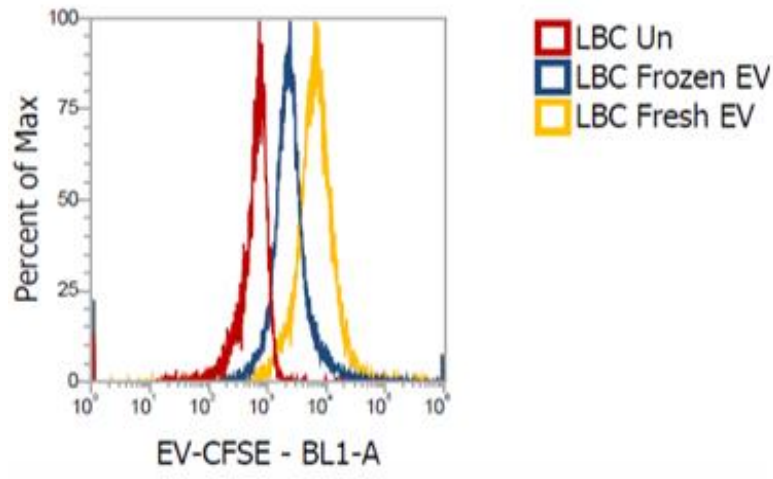
After confirmation of MSC-derived EV uptake by confocal microscopy in Section 4.1.1, FACS (Fluorescence-activated cell sorting) technique was used to further demonstrate MSC-derived EV uptake. FACS is a specialized type of flow cytometry by which individual cell is illuminated by lasers to generated cell size, granularity and composition by the detection of forward and side scatter. It is one of the important techniques used to confirm EV uptake across a cell population.

Freshly isolated or -80°C stored SH MSC-derived EVs are labelled by CFSE, washed by ultracentrifugation at 118,000 × *g*, 4°C for 70 minutes, and incubated with SH MSCs or LBC MSCs in 12-well plate till MSCs reach to 90% confluency (40 hours) to gain enough cells. MSCs are detached by trypsin, resuspended by FACS buffer (1% BSA and 0.1%EDTA in PBS) and subjected to flow cytometry. MSCs incubated with labelled MSC-derived EVs would become fluorescent if labelled MSC-derived EVs are taken up and then enable to be detected by flow cytometry. The uptake of CFSE-labelled MSC-derived EVs is evident in increasing CFSE fluorescent signal on MSCs. MSCs without incubation with labelled EVs serve as negative control and SH MSCs stained with CFSE serve as positive control (Figure 23).

A



B



C

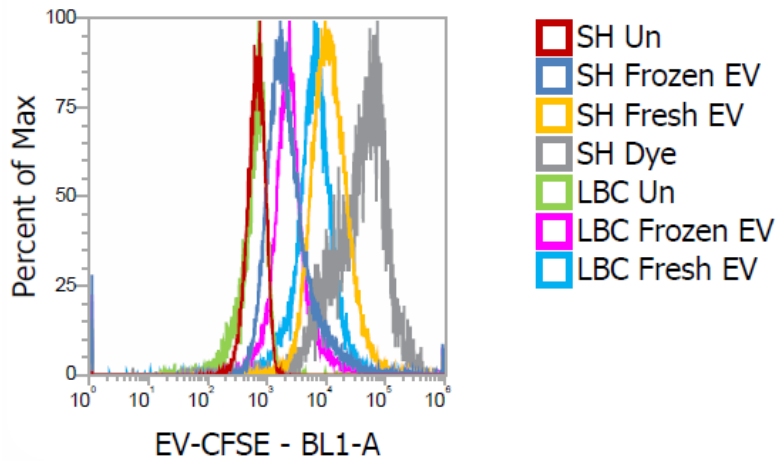


Figure 23: Detection of CFSE-labelled EV uptake by FACS. MSCs were incubated with stained MSC-EVs for overnight. A: Detection of SH MSCs taking up CFSE-stained MSC-EVs. B: Detection of LBC MSCs taking up CFSE-stained MSC-EVs. C: Merging A and B. SH Un: Unstained SH MSCs served as negative control. SH Frozen EV: SH MSCs incubated with frozen MSC-EVs. SH Fresh EV: SH MSCs incubated with fresh MSC-EVs. SH Dye: CFSE-stained SH MSCs served as positive control. LBC Un-Unstained LBC MSCs served as negative control. LBC Frozen EV: LBC MSCs incubated with frozen MSC-EVs. LBC Fresh EV: LBC MSCs incubated with fresh MSC-EVs. SH is a younger OA patient. LBC is an older OA patient.

According to the flow cytometry result, both SH MSCs and LBC MSCs incubated with either freshly isolated or frozen CFSE labelled EVs were positive for CFSE fluorescence which further confirms that both frozen and fresh EVs could be taken up by MSCs. However, both SH MSCs and LBC MSCs incubated with fresh EVs have a much stronger fluorescent signals than the same cells incubated with frozen EVs. The FACS results support the confocal microscopy. However, there is some discrepancy with LBC MSCs incubated with fresh EVs which have a weaker signal when compared with SH MSCs with fresh EVs. One thing to note is that the FACS is performed much later than confocal microscopy as the cells are cultured for 23 hours to gain enough MSCs for flow cytometry analysis. Since the interaction mechanism between EVs and cells is dynamic, some fluorescence in LBC MSCs might be released into extracellular space faster than in SH MSCs leading to weaker detection on LBC MSCs than SH MSCs. Nevertheless, both LBC and SH MSCs incubated with freshly isolated EVs have fluorescence detected in over 95% population which is quite promising.

In summary, in this section we use confocal microscopy and FACS techniques to analyse CFSE labelled fresh and frozen SH MSC-derived EVs uptake by MSCs and their parental cells. Both frozen and fresh EVs could be taken up by MSCs, while it was observed that fresh EV uptake gives rise to a stronger signal than frozen EVs. These findings in this section suggest that fresh EVs are more bioactive than frozen EVs, EVs stored at -80°C alter EV uptake and lost some of their bioactivity. Therefore, using fresh EVs is recommended for further EV functional analysis.

4.2 qPCR analysis of EV function

In the previous section above, we used confocal microscopy and FACS to analyse CFSE labelled fresh and frozen MSC-derived EVs uptake by MSCs and their parent cells. Both frozen and fresh EVs could be taken up by MSCs, we also observed that fresh EVs appear to be more efficiently taken up to MSCs than frozen EVs. Based on these results, fresh MSC-derived EVs are used to study EV function using qPCR technique which is an extensively used technique for studying gene expression. The main aim of this section is to examine whether young age MSC-derived EVs could alter or affect gene expression in the recipient cells by analysing pluripotent, differentiation MSC marker gene expression.

The protocol is described in section 2.9. In brief, SH (young, sport injured osteoarthritis patient) MSC-derived EVs are isolated by serially centrifugation at 300 X *g* for 10 minutes, 2000 X *g* for 20 minutes, 10,000 X *g* for 30 minutes, 118,000 X *g* for 70 minutes and followed washing EV pellets with 16 mL of 20mM Hepes buffer by an extra centrifugation 118,000 X *g* for 70 minutes. Then fresh isolated SH MSC-derived EVs are incubated with parental SH MSCs and BJ (old osteoarthritis patient) MSCs to compare the influence of MSC-derived EVs on MSC gene expression. Two-step qPCR technique is employed to analyse relative gene expression of the three groups of genes. Figure 24 simply shows the workflow of experiment. SH MSC-derived EVs are isolated from SH MSC culture medium once the cultured cells reach to 90% confluence. Then either SH MSCs or BJ MSCs are incubated with or without freshly isolated SH MSC-derived EVs for 3 days. MSCs incubate without SH MSC-derived EVs serve as controls (calibrators). There are four MSC samples named as SH (SH MSCs), SHE (SH MSCs incubated with fresh isolated SH MSC-derived EVs), BJ (BJ MSCs) and BJE (BJ MSCs incubated with fresh isolated SH MSC-derived EVs). Total RNAs are extracted from four MSC samples, cDNAs are synthesised by one cycle of PCR, and then cDNA templates are subjected to quantitative real time PCR to analyse relative gene expression.

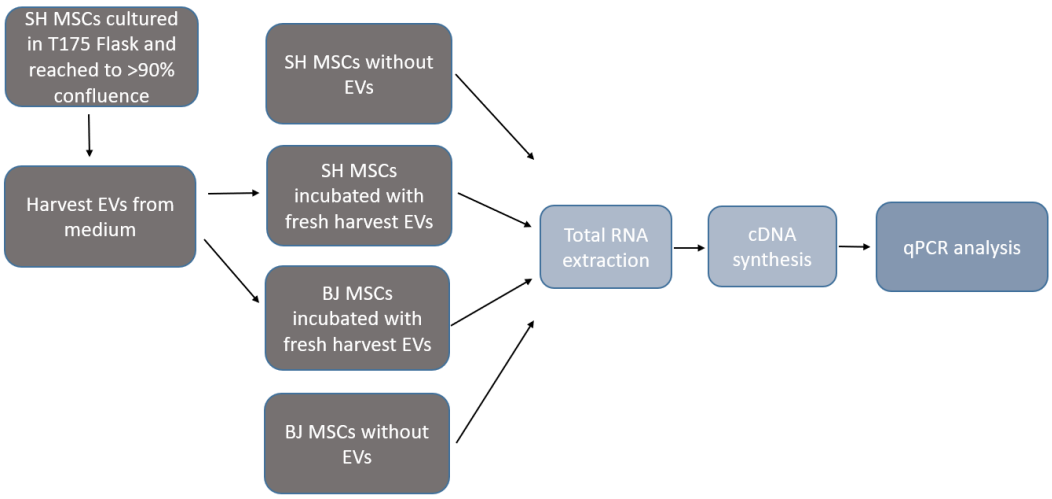


Figure 24: Workflow of qPCR to analyse MSC-EV effects on SH MSCs and BJ MSCs. Total RNAs were extracted from 4 samples which were SH MSCs and BJ MSCs incubated with or without SH MSC-EVs. qPCR technique was used to quantitatively analyse SH MSC-EV effects on MSCs.

Unlike conventional PCR which qualitatively detects the end-point PCR amplification by gel electrophoresis, real time PCR (qRT-PCR) could quantitatively measure amounts of amplified products during the reaction progresses by analysis with fluorescence detection modules at each cycle. The threshold cycle (C_T) is the point which amplified products started to yield a detectable fluorescent signal and is used as an index of initial amounts of templates in the exponential phase of reaction. Relative quantification $2^{-\Delta\Delta CT}$ method is used to analyse relative amount of cDNA templates. The reference gene also known as housekeeping gene is crucial for normalization of qRT-PCR. Since GAPDH gene is a comparable stable gene (Ragni et al. 2013), GAPDH gene served as reference gene. The SH sample and BJ sample served as calibrators (controls). The target gene expression in all other samples is expressed as increase or decrease relative to the calibrators. To calculate relative gene expression, C_T (target, test), C_T (target, calibrator), C_T (GAPDH, test), C_T (GAPDH, calibrator) obtained from qRT-PCR, then ΔΔCT was calculated as:

$$\Delta C_T(\text{test}) = C_T(\text{target, test}) - C_T(\text{GAPDH, test})$$

$$\Delta C_T(\text{calibrator}) = C_T(\text{target, calibrator}) - C_T(\text{GAPDH, calibrator})$$

$$\Delta\Delta C_T = \Delta C_T(\text{test}) - \Delta C_T(\text{calibrator})$$

Finally, target gene expression is calculated as $2^{-\Delta\Delta CT}$. This analysis results in relative quantification, which is a ratio that is the relative fold difference of the target nucleic acid in the test and calibrator sample. The results display relative amounts of cDNA templates in the test sample compared with calibrator. Since cDNAs are synthesised from RNAs by one cycle from mRNA, the gene expression analysis results present the relative amount of mRNA templates in the test sample. The graph is plotted for each target gene expression. The standard deviation of each sample is calculated using error propagation according to each C_T standard deviation generated from C_T of the triplicate samples.

As introduced in Chapter 1, MSCs are multipotent stem cells which have three minimal criteria: MSCs must be plastic adherent in tissue culture flasks maintained in standard culture conditions; over 95% of MSC population must express CD105, CD73 and CD90 and lack expression (≤2%) of CD45, CD34,

CD14 or CD11b, CD79α or CD19 and HLA class II; MSCs must be able to differentiate into osteoblasts, adipocytes and chondrocytes in *vitro* under standard differentiating conditions (Dominici et al., 2006). Therefore, three groups of genes were analysed; pluripotent genes, differentiation genes and MSC surface marker genes which are important to define MSCs. These three groups of genes are organized and analysed in this chapter. The results are presented in below Sections. Original data were obtained in triplicates thus each C_T value had themselves' standard deviation derived from triplicates. The error bars showed in graphs were calculated according to the standard deviations derived from triplicates. The one-way ANOVA was used to analyse statistics of the results. Value of $P<0.05$ was considered statistically significant. ANOVA analysis was worked out by Data Analysis tool in Excel. All relative gene expression displayed in graph; their P values were less than 0.05 which meant their statistically significant.

4.2.1 Pluripotent genes: POU5F1 (Oct-4), NANOG, KLF4

Figure 25 shows qPCR result of pluripotent gene expression in four samples. SHE and BJE are MSCs incubated with SH MSC-derived EVs to work out the effects on SH (young age) MSCs and BJ (old age) MSCs by these EVs. SH and BJ samples without incubation of MSC-derived EVs serve as controls. SH MSC-derived EVs are observed to have various effects on SH MSC pluripotent gene expression. This includes an increase in KLF4 gene expression; while POU5F1 and NANOG gene expression decreased when compared to the SH sample. Interestingly, all three pluripotent genes are expressed at higher level in BJE sample than in BJ sample.

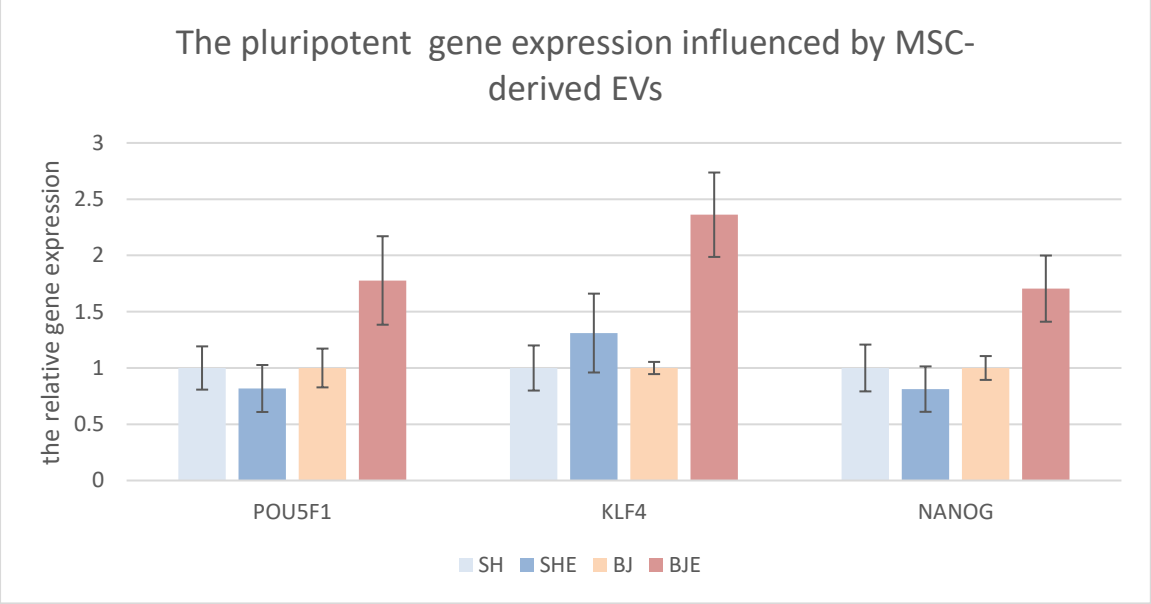


Figure 25: Influence of SH MSC-derived EVs on the pluripotent gene expression of SH MSCs and BJ MSCs. n=3. Error bars represent SD. Statistically significant difference, $P<0.05$.

POU class 5 homeobox 1 (POU5F1) gene is also known as Oct-4 gene that encodes a transcription factor containing a POU homeodomain involved in embryonic development and stem cell pluripotency. Nanog homeobox (Nanog) gene encodes a DNA binding homeobox transcription factor involved in stem cell proliferation, renewal, pluripotency, and also can block stem cell differentiation.

Kruppel like factor 4 (KLF4) gene encodes Kruppel family transcription factors involved in the regulation of proliferation, differentiation, and mediated of the tumor suppressor gene p53 ("Gene," 2017). Oct-4 and KLF4 genes are two of four transcription factors to reprogram the somatic cell to induced pluripotent stem cells (Takahashi & Yamanaka, 2006). The roles of Oct-4 and NANOG are to maintain MSCs properties, keeping MSCs in proliferative and undifferentiated states (Tsai, Su, Huang, Yew, & Hung, 2012). Therefore, these three pluripotent genes are important for maintenance of MSC stemness. The higher-level of pluripotent gene expression could indicate more pluripotent capacity of MSCs. As these three genes in BJE sample are stimulated, higher level of expression by SH MSC-derived EVs result in more pluripotent of BJ MSCs, SH MSC-derived EVs are capable of the beneficial effects on BJ MSCs. Our results concur with a recent study which the pluripotency markers Nanog and Oct4 significantly increased expression while old age MSCs treated by young age MSC-derived EVs (Fafian-Labora et al., 2020). In contrast to BJE sample, SHE pluripotent genes have no significant changes when comparing with SH sample especially when standard errors is considered. This could be expected as it could be hypothesised that MSC-derived EVs might not have too much impact on the parental MSCs.

4.2.2 Differentiational genes: Osteogenic genes (RUNX2, COL1A1), Chondrogenic genes (SOX9, COL2A1), Odipogenic gene (PPARG)

Next we investigated the genes associated with MSC differentiation. Runt related transcription factor 2 (RUNX2) and Collagen type I alpha 1 chain (COL1A1) gene expression are osteogenic pathway genes to be examined for osteogenic differentiation potential of MSCs. Chondrogenic genes SRY-box 9 (SOX9) and Collagen type II alpha 1 chain (COL2A1) gene are chondrogenic pathway genes. COL2A1 gene encoded alpha 1 chain of type II collagen – a fibrillar collagen found in cartilage. SOX9 gene is master regulator of chondrogenesis (Robins et al. 2005). Adipogenic gene Peroxisome Proliferator Activated Receptor gamma (PPARG) gene encodes a member of the peroxisome proliferator-activated receptor subfamily of nuclear receptor gamma which is a master regulator of adipogenesis (Aguilar et al., 2010). Those five differentiational genes could represent the differentiation capacity of MSCs.

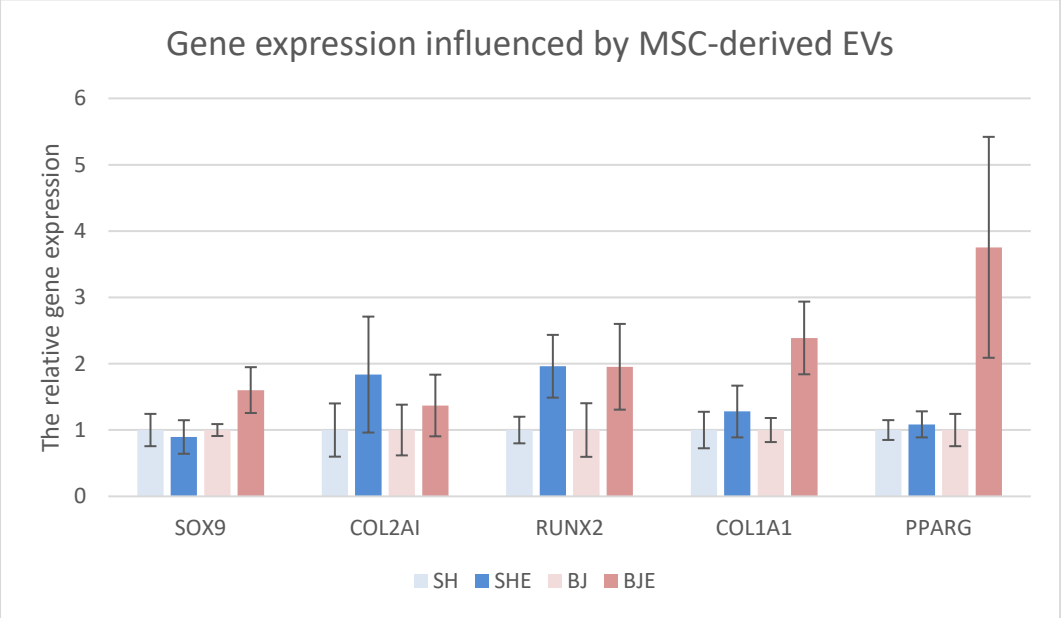


Figure 26: Influence of SH MSC-derived EVs on the differential gene expression of SH MSCs and BJ MSCs. n=3. Error bars represent SD. Statistically significant difference, $P<0.05$.

Figure 26 shows the results of differentiational gene expression in the various samples. Under the influence of SH MSC-derived EVs, five differentiational genes in SHE sample do not significantly express higher level especially for COL1A1 and PPARG genes. SOX9 gene expression is observed to decrease when compared with SH sample. Therefore, SH MSC-derived EVs have no significant effect on the 5 differentiation genes analysed on the parental SH MSCs. When analysing the SHE samples, all 5 differentiation genes of BJE sample express higher level than in BJ sample. This indicates that SH MSC-derived EVs are taken up by, and have an impact on BJ MSCs, leading to increased differentiation capacity of BJ MSCs. Therefore, SH MSC-derived EVs appear to have a beneficial effect on differentiation genes in BJ MSCs. Interesting observation here is the host cells' own EV did not impact the gene expression of its own cells but significantly altered that of the donor MSCs. This could have impact in donor derived EV therapy in the future.

4.2.3 MSC surface marker genes

Finally, we investigated the effects of EVs on MSC surface marker genes. The cell surface marker genes Thy-1 cell surface antigen (THY1/CD90) gene encodes a cell surface glycoprotein and member of the immunoglobulin superfamily protein which involved in cell adhesion and cell communication. Ecto-5'-nucleotidase (NT5E/CD73) encodes a plasma membrane protein which catalysed the conversion of extracellular nucleotides to membrane-permeable nucleosides and acted as a determinant of lymphocyte differentiation. Endoglin (ENG/CD105) gene encoded a homodimeric transmembrane protein. These three important positive markers are recommended by ISCT and must be expressed in MSCs.

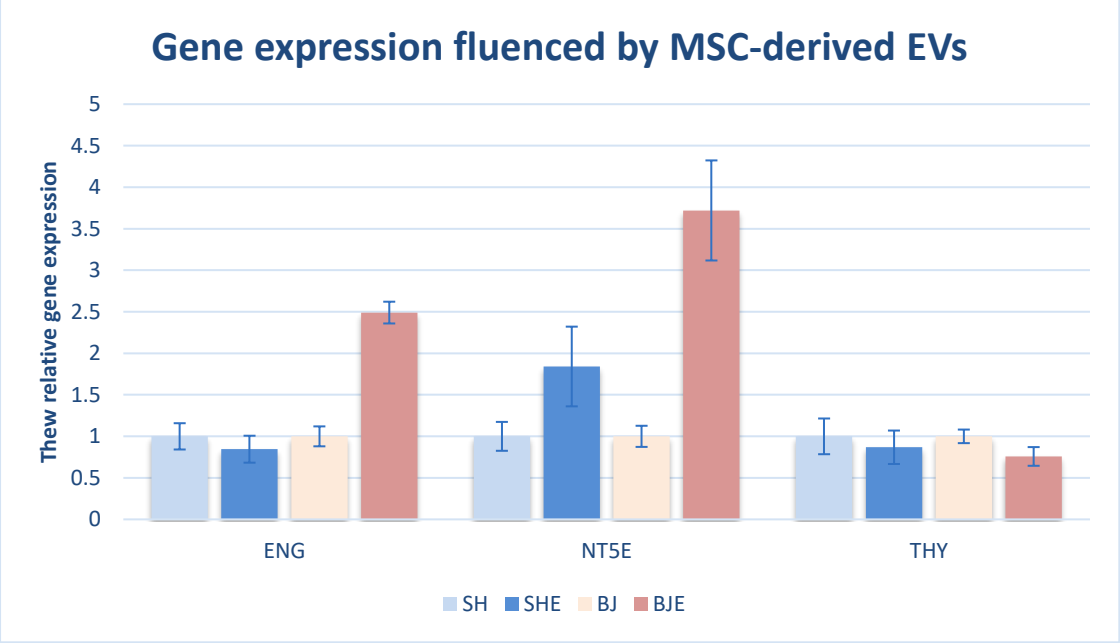


Figure 27: Influence of SH MSC-derived EVs on the surface marker gene expression of SH MSCs and BJ MSCs. n=3. Error bars represent SD. Statistically significant difference, $P<0.05$.

From the result (Figure 27), CD73 gene expression in SHE sample increases but CD90 and CD105 gene expression slightly decrease when compared with SH sample. This indicates that SH MSC- derived EVs have little effect on SH MSCs. In contrast to SHE sample, SH MSC-derived EVs lead to an increase in CD105 and CD73 genes in BJ MSCs. The relative gene expression of CD105 and CD73 in BJE sample is

expressed 2 to 3- fold higher levels than in BJ sample. Surprisingly, CD90 gene expression decreases in BJE sample. CD90 is one of the main immunophenotypical markers of MSCs. A recent study demonstrated CD90 of MSCs associated with osteogenic and adipogenic differentiation. Knockdown CD90 of MSCs could lower the stemness guard of MSCs to enhance further osteogenic and adipogenic differentiation (Moraes et al., 2016). However, it is not clear why SH MSC-derived EVs lead to less expression of CD 90 in BJ MSCs. This may need further investigation in the future, such as confirmation of MSC surface markers by flow cytometry.

In summary, SH (young age) MSC-derived EVs are taken up by BJ (old age) MSCs and appear to have beneficial effects on BJ MSCs by increasing their pluripotent, differential gene and MSC surface marker gene expression except CD90 gene. This suggests that MSC-derived EVs could act as paracrine factors to influence BJ MSCs. SH MSC-derived EVs have limited impact on SH MSCs but are not significant as seen for BJ MSCs. Taken together, these results suggest that the MSCs from which the EVs are derived might not have a significant effect on themselves, while the same EVs appear to significantly affect other MSC cell lines.

4.3 Discussion and conclusion

In this chapter, MSC-derived EV labelling protocol is initially developed. Then confocal microscopy and FACS techniques are used to analyse labelled fresh and frozen SH (young age, sport injured osteoarthritis patient) MSC-derived EVs uptake by LBC (old age osteoarthritis patient) MSCs as well as their parental MSCs. Both frozen and fresh EVs could be taken up by MSCs, while fresh EV uptake results in a stronger signal than frozen EVs. This suggests that fresh EVs were more easily and efficiently taken up into MSCs than frozen EVs and that EVs stored at -80°C may alter EV bioactivity. Therefore, freshly isolated MSC-derived EVs were used for further EV functional analysis using qPCR technique. We found that freshly isolated MSC-derived EVs from a young age patient have a beneficial impact on old age MSCs through comparison of three groups of genes.

In section 4.1.1, SH (young age osteoarthritis patient) MSC-derived EVs are detected to be taken up by both SH MSCs and LBC (old age osteoarthritis patient) MSCs. The most significant finding is that fresh isolated MSC-derived EVs can be more efficiently taken up and lead to much stronger fluorescence signal which indicates freshly isolated EVs are more bioactive than frozen MSC EVs. The stronger fluorescent intensity seen in cells following incubation with fresh EVs mostly locate in cytoplasm and cell periphery. This suggests that after MSC-derived EVs are internalised to MSCs, EV cargo is unloaded into cytoplasm, some components of EVs might undergo EV biogenetic pathway to dock to the plasma membrane and ready to release to extracellular space again. This is consistent with Sylwia Dabrowska *et al* study which discovered labelled EVs were taken up by MSCs, co-localised with MSC surface markers and ready to release after 24 hours incubation with MSCs (Dabrowska et al., 2018). Due to the limitation of the current equipment in Swinburne University and the time-consuming nature involved in fresh EV isolation and labelling (6-8 hours), MSC images of confocal microscopy are taken after 17 hours incubation with labelled EVs. EV internalization and trafficking are dynamic and the interaction mechanisms between EVs and cells is still elusive (Chuo et al., 2018). It would be ideal to observe EV uptake starting from EV addition and using live-cell real-time imaging

to examine the fate of MSC-derived EVs entering MSCs. Additionally, future work could also include the staining of nucleus and cytoskeleton with the MSC and would help to localise internalised EVs.

Section 4.1.2 further confirms EV uptake using FACS technique. The results of FACS are similar to the results of confocal microscopy for which both parental MSCs and LBC MSCs could take up MSC-derived EVs, and fresh isolated EV uptake results in a stronger signal than frozen EVs. Both LBC and SH MSCs incubated with fresh EVs leads to fluorescent signal detected in over 95% of the cell population. However, LBC MSCs incubated with fresh isolated EVs have a weaker signal compared with SH MSCs incubated with fresh EVs. These differences in the results may be caused by the different detection time in the two different techniques. Confocal microscopy is taken after 17 hours incubation and FACS is performed after 40 hours incubation to gain enough MSCs for flow cytometry analysis. The labelled MSC-derived EVs and their components in LBC MSCs may be more bioactive and could be released into extracellular space faster than in SH MSCs leading to weaker detection on LBC MSCs than SH MSCs. Further study is needed to verify this hypothesis.

In order to investigate MSC-derived EV beneficial effects on MSCs, MSCs incubated with freshly isolated MSC-derived EVs are examined using qPCR technique. Three groups of genes which include; pluripotent genes included POU class 5 homeobox 1 (POU5F1) gene known as Oct-4 gene, Nanog homeobox (Nanog) gene, Kruppel like factor 4 (KLF4) gene, differentiational genes included Runt related transcription factor 2 (RUNX2), Collagen type I alpha 1 chain (COL1A1) gene, chondrogenic genes SRY-box 9 (SOX9) and Collagen type II alpha 1 chain (COL2A1) gene, COL2A1 gene, SOX9 gene, adipogenic gene Peroxisome Proliferator Activated Receptor gamma (PPARG) gene; cell surface marker genes Thy-1 cell surface antigen (THY1/CD90) gene, Ecto-5'-nucleotidase (NT5E/CD73), Endoglin (ENG/CD105) gene were analysed. The results showed that SH (young age) MSC-derived EVs have beneficial effects on BJ (old age) MSCs by increasing their pluripotent, differential gene and MSC surface marker gene expression except CD90 gene. Further analysis could be performed in the future to verify some of these results. For example, flow cytometry to verify MSC surface markers, MSC differentiation capacity could be verified by differentiation assays of MSCs could differentiate into adipogenic, osteogenic and chondrogenic lineages. These three groups of genes are all representative of the bio-property of MSCs. The results of gene expression analysis showed that SH (young age) MSC-derived EVs have beneficial effects on BJ (old age) MSCs by increasing their pluripotent, differential gene and MSC surface marker gene expression (except for CD90 gene). Pluripotency genes as well as differentiation genes concurrently increased expression in the BJ-MSC when incubated with SH MSC-derived EVs. Therefore SH MSC-derived EVs not only impacted BJ-MSCs to be more pluripotent but also to increase differentiation capacity of BJ-MSCs. These results are quite promising and suggest that MSC-derived EVs could act as paracrine factors to influence other MSCs. Furthermore, suggest that they may have therapeutic potential. MSC-derived EVs have been used for OA treatment which described in Section 1.3. Numerous studies in *vitro* and *vivo* demonstrate the possibility of treating chronic conditions with MSC-derived EVs to address current unmet medical needs (Zhu et al., 2017; Zhang et al., 2016; Chang Hee Woo, 2020; Zhang et al., 2018; Kordelas et al., 2014; Ranghino et al., 2017; Ophelders et al., 2016).

Taking these results into account, it is predicted that MSC-derived EVs could be used in the OA treatment. This project is a collaboration with Magellan Stem Cell Centre which has several clinical trials exploring cellular therapy to treat OA in clinics. The patients are injected with autologous MSCs in the OA joints by intra-articular injection and are then followed up after at least 12 months post

treatment for improvement in OA symptoms and structural improvement by an MRI of the treated joint. The clinical results are promising. However, there are some OA patients with little improvement. Therefore, Magellan wanted to investigate MSC-derived EVs based on clinical outcomes. We planned these MSC-derived EVs to be extensively characterised and compared using next generation sequencing and proteomics techniques to understand the genetics and protein chemistry of the EVs aimed for discovery of the possible OA therapeutic biomarkers**.** Unfortunately, our project had to be halted due to Magellan did not want to share the clinical data. In spite of this unfortunate disagreement, depending on the obtained qPCR result which young age MSC-derived EVs are capable of the beneficial effects on old age MSCs, Magellan probably may try using young age MSC-derived EVs to treat OA patient, such as mixing young age MSC-derived EVs with autologous MSCs together to inject into the OA joints.

In summary, we used confocal microscopy and FACS techniques to analyse labelled fresh and frozen SH MSC-derived EVs uptake by MSCs and their parent cells. Both frozen and fresh EVs could be taken up by MSCs successfully and fresh EV uptake resulted in stronger signal. This suggests that the fresh EVs are more bioactive and efficiently taken up by MSCs. Therefore, EVs stored at -80°C appear to alter EV bioactivity. The freshly isolated EVs are used to determine their effects on gene expression. The result of qPCR suggests that young age MSC-derived EVs have beneficial effects on old age MSCs by increasing their pluripotent and the differentiation capacity. Taken together, these results suggest that EVs have therapeutic potential and may be used in ‘cell-free’ therapy.

Chapter 5

Conclusion and Future Directions

In the last two decades, the EV field has rapidly developed because of their important roles in biological homeostasis, cell to cell communication and pathological propagation. In addition, MSCs are the most used cell type in clinical applications in the past last three decades, they are considered as prolific producers of EVs when compared with other cell types and have become attractive candidates in regenerative therapeutics. The therapeutic potential of MSC-derived EVs has previously been examined in both animal models and various clinical applications in many disease areas. Their potential as diagnostic tools, drug delivery vehicles and vaccine has also been studied. This project was a collaboration with Magellan Stem Cell which involved in clinical treatment of OA by using autologous adipose-derived MSCs. This research was motivated by the question of the variability in clinical outcomes of OA treatment, where MSC-derived EV might play key roles for OA treatment. This thesis developed the optimal isolation method of MSC-derived EVs and investigated EV uptake and beneficial effects on MSCs in gene expression level to shed light on the possibility of EV therapeutic advantage.

There are many challenges in the EV field such as their heterogeneity, their nano-size and their isolation methods. EV nano-size elicits analytic difficulty using the limited current technical equipment. In this thesis, firstly, we optimised MSC-derived EV isolation method in Chapter 3. Four MSC-derived EV samples were isolated by differential ultracentrifugation and one MSC derived EV sample was isolated by the commercial kit using the same parental cell line. They were analysed and compared by NTA, SEM, BCA protein assay, western blot, flow cytometry to assess the purity both qualitatively and quantitatively and their concentration. We concluded that 70 minutes ultracentrifugation is enough to isolate high quality MSC-derived EVs. Although MSC-derived EVs could be purified by other further purification methods such as density gradient or size exclusion chromatography, it should be noted that increased sample processing results in both a reduction in EV yield and also increases the risks of contamination, which is a concern in a clinical setting. For investigation of the contents of MSC-derived EVs, novel biomarker discovery, and many downstream analyses such as proteomic, RNA, lipidomic analysis may require purer EVs and need further purification. The motivation for this project was related to a clinical setting; therefore, MSC-derived EVs were purified by an extra ultracentrifugation and further purification was not performed.

In Chapter 4, the investigation of MSC-derived EV uptake by two MSCs cell lines were firstly described. MSC EVs derived from young age, sport injured OA patient were isolated by differential ultracentrifugation method which was developed in Chapter 3. Both MSCs derived from young age, sport injured OA patient and MSCs derived from old age OA patient were incubated with fresh isolated and/or -80°C frozen MSC-derived EVs labelled with CFSE dye, and then analysed by confocal microscopy and flow cytometry. The results confirmed that both fresh isolated and frozen MSC-derived EVs could be taken up by MSCs and even their parental MSCs, while the fresh isolated MSC-

derived EV appear to be more bioactive since fresh EVs have given much stronger fluorescent signal than frozen EVs. The experimental result from this chapter suggested that using freshly isolated MSC-derived EVs was the best condition for further MSC-derived EV functional analysis.

MSC-derived EV functional effects on MSCs were examined through comparison of MSCs incubated with or without MSC-derived EVs by qPCR technique. Freshly isolated MSC EVs derived from young age, sport injured OA patient were isolated by differential ultracentrifugation method. The EVs were then added to tissue culture of MSC cell lines derived from old age OA patient and the EV parental MSCs. The effects of the EVs on the cell lines were determined by qPCR. Three groups of genes which are included pluripotent genes, differentiational genes and MSC cell surface marker genes were investigated and compared. This comparison highlighted that young aged MSC-derived EVs have significant effects on old aged MSCs by increasing their pluripotent, differential gene and MSC surface marker gene expression except CD90 gene. These *in vitro* experimental results suggested that MSC-derived EVs could potentially have therapeutic potential as they are able to alter gene expression in the recipient cells.

Future directions

The experiments in this thesis set out to optimise a suitable isolation method of MSC-derived EVs. This project started in 2018, at which point the differential ultracentrifugation is considered as 'golden standard' EV isolation method and commonly used in EV field. Therefore, in this thesis three EV samples were isolated by differential ultracentrifugation with three different ultracentrifuge durations to investigate the influence of ultracentrifuge time on quality and quantity of MSC-derived EVs. These three EV samples compared with another EV sample which extracted using a commercial kit. EV isolation by the differential ultracentrifugation has the advantage of being low-cost, however there are some limitations such as being time consuming, contamination of non-vesicle particles known as protein aggregates and lipoproteins. Due to rapid the rapid increase in interests in the EV field, various alternate EV isolation techniques are constantly being developed. Size exclusion chromatography (SEC) technique which use qEV SEC columns has become a more attractive isolation technique since it has the advantage of separation EVs from protein contaminants. Furthermore, other EV isolation techniques have also emerged and developed in recent years for example flow field-flow fractionation, ion-exchange, electrophoresis and dielectrophoresis. Future work could involve the evaluation of this different isolation techniques or a combination of current available techniques. This may lead to the identification of the best option for MSC-derived EV isolation method.

One of the challenges in the project is the nano-size of EVs making it difficult for visualisation studies. The interaction between EVs and receptor cells is also not well understood. A Combination of confocal microscopy and flow cytometry techniques were used to investigate MSC-derived EV uptake *in vitro* in Chapter 4, and EV uptake was confirmed. However, it was a challenged to localise the intracellular location of the labelled EVs. Future work could explore the use of different fluorescence dyes or antibodies to label MSC nucleus, cytoskeletons or organelles which can improve the localisation of stained EVs and their cargo. Live-cell confocal microscopy in real time may improve the tracking of initial EV uptake which will help discover the details of the interaction between MSCs and MSC-derived EVs, and furthermore identify the intercellular fate of EVs *in vitro*. Some new approaching modern

optical imaging techniques such as super-resolution microscopy in real time could improve tracking labelled EVs to unveil EV internalisation mechanisms *in vitro*. Since the EV interaction mechanism *in vivo* currently remains elusive, future studies could be directed towards the investigation of EV biodistribution and EV behaviours *in vivo* to gain insight into EV biological significance, exploit the EV therapeutic potentials.

In this thesis, the experimental results demonstrated that freshly isolated EVs were more bioactive than EVs stored in -80°C condition. However, using freshly isolated EVs would definitely limit the utility of EVs in various widespread application of EV-based therapies. The best storage method to preserve the function of EVs will need to be developed. Many studies have investigated to investigate the storage of EVs in various condition (Bosch et al., 2016; Y. R. Cheng, Zeng, Han, & Xia, 2019; El Baradie et al., 2020; Frank et al., 2018; Lorincz et al., 2014; Maroto et al., 2017; Richter, Fuhrmann, & Fuhrmann, 2019). Lyophilization with two lyoprotectants trehalose and PVP40 could improve the stability of freeze-dried products and gave a promising result to preserve EV bioactivity (El Baradie et al., 2020). To date, -80°C storage condition is most widely used. However, from our results it decreases EV bioactivity and therefore it may not be suitable for EV therapeutic use. Furthermore, EV transport and handling may be a critical barrier in their clinical use in the future. Further research investigating the EV bioactivity following multiple storage conditions should be explored to develop an ideal EV storage strategy.

Another interesting set of experimental results in this thesis demonstrated that freshly isolated EVs derived from young aged MSCs could increase pluripotent and differential capacity of old aged MSCs. Although these are promising findings at the gene expression level. The results are from individual samples due to limitation of obtaining samples. In the future, groups of samples (young age group, old age group) should be used to determine the beneficial effects of young age MSC-derived EVs on old age MSCs.

MSC-derived EVs have been studied in both animal models and various clinical applications in many disease areas, tested as potential diagnostic tools, antitumor therapeutics, drug delivery vehicles and vaccines. Therapeutic potential of MSC-derived EVs in regenerative application has been highlighted in previous studies. Clinical applications of MSC-derived EVs are advantageous over MSC cell-based therapy, as they have lower immunogenicity, capacity to cross biological barriers, and less safety concerns, such as the possibility of MSC differentiation or tumor generation. However, EVs derived from heterogenetic MSCs which include tri-, bi, and unipotent populations are highly heterogeneous and results in variable outcomes. Some EV clinical studies have been terminated without publication. More research of EV therapeutic potential in animal model and preclinical application are needed. One difference between the use of cell therapies and EVs is that MSC-derived EVs have relatively short half-life and therefore would require subsequent doses to retain sufficient therapeutic levels. Using alternate delivery methods such as bioengineered scaffolds, PEI, encapsulation with PEG hydrogels, or GelMA aimed to maintain the sustained release of the MSC-derived EVs may be good option for future research in EV regenerative application.

Overall, EV potential benefits described in this thesis would encourage further to explore and discover the therapeutic potential of MSC-derived EVs. We believe one day MSC-derived EVs can become an “off-the-shelf” cell-free therapeutic.

Appendix

The movies of CFSE-labelled MSC derived EV uptake by MSCs


EV labelling Media1 frozen EVs in SH MS


SH MSCs with fresh SH MSC EVs.avi


LBC MSCs with frozen EVs.avi

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Appendix II

Publication 1

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New approaches to treat Osteoarthritis with Mesenchymal Stem Cells

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Abstract

Osteoarthritis is one of the most common chronic health problems that causes disability and chronic pain with reduced mobility in the world and is a progressive degenerative disease in weight bearing joints such as the knee. The pathology of the joint resulting from OA includes loss of cartilage volume and cartilage lesions leading to inflammation of the articular joint structures, its incidence and progression are associated with variety of risk factors. Most of the current treatments focus on symptom management such as physical and occupational therapies, pharmacological intervention for pain management, and surgical intervention with limited success and do not address nor halt the progression of the disease. In this review we will describe the current treatments options for OA and the exciting new translational medical research currently underway utilising mesenchymal stem cells for OA therapy.

Osteoarthritis

Osteoarthritis is pathology of articular joints most commonly associated with defects in cartilage such as osteochondral defects and is one of the most common chronic disabling diseases affecting people worldwide. It can cause severe limitation of daily activities that can seriously affect the quality of life. Approximately, 9.6% of men and 18% of women who are over 60 years old have symptomatic osteoarthritis worldwide [1, 2]. The musculoskeletal condition is characterized by degenerative articular cartilage that leads to thinning of cartilage (Figure 1) resulting in the bone contact, eventually leading to the symptoms of stiffness, pain and limitation of movement. The major risk factors for OA are older age, obesity, previous injury, sports related injury, occupational overuse and genetic background [3, 4]. As the elderly population and obesity is increasing around the world, OA has become more widely spread causing a substantial health and economic burden globally [5]. It is estimated that associated costs of OA has a socioeconomic burden between 1.0 to 2.5% of gross domestic product in developed countries [6].

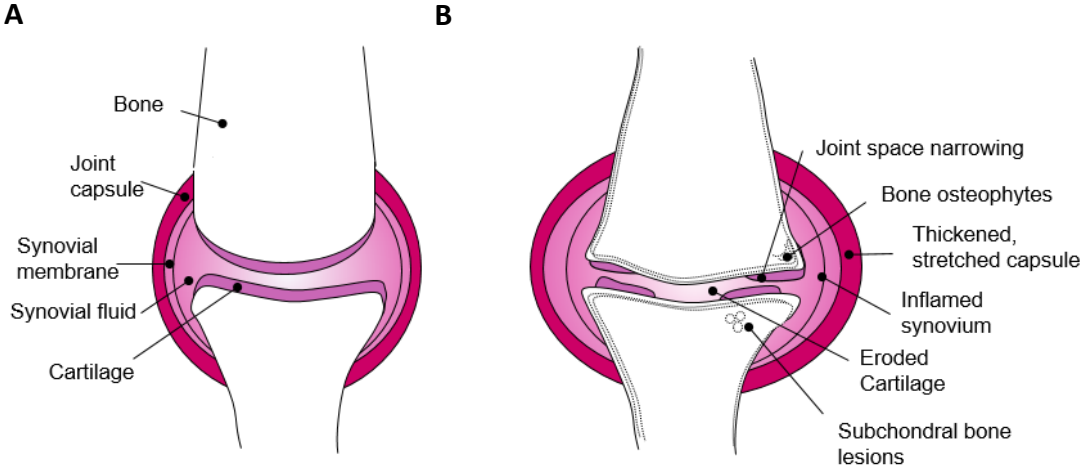

Figure 1: Diagram of the synovial knee joint in (A) a healthy individual and (B) with mild osteoarthritis.

OA is usually associated with the synovial joints (diarthroses) also known as the freely moveable joints [7]. The normal synovial joint (Figure 1) is formed by two bones' ends covered with a thin layer of smooth, firm articular cartilage, a capsule filled with the synovial fluid, ligaments, tendons, muscles, blood vessels and nerves [8].

Those structural components form a functional unit with their mechanical interaction. The changes in any component lead to the anabolic or catabolic responses in other components [9]. The abnormality in the synovial joint tissues such as articular cartilage, subchondral bone, ligaments, menisci, synovium, peripheral nerves and muscles can cause stress in the joint, and eventually result in degeneration of articular cartilage resulting in OA [7, 10].

Articular cartilage is a special type of connective tissue which is non-neural, non-lymphatic, nonvascular and therefore restricted in self repair. Articular cartilage is a metabolically active tissue, and its architecture and biochemical composition are regulated, developed and repaired by chondrocytes. Chondrocytes are the only cell type in the articular cartilage [9]. Nutrition is supported by the synovial fluid and subchondral bone by diffusion through regular joint movement. The movement of the synovial joint forces the synovial fluid in and out of the articular cartilage to deliver nutrients and dispose of waste products for cartilage [11]. The proximal subchondral bone provides nutrients such as glucose, oxygen and water to cartilage by perfusion from their dense vessels in the subchondral region [12]. Therefore, cartilage, subchondral bone and synovium interact with each other and play key roles in pathogenesis of OA when there are abnormal mechanics involving the entire articular joint [13, 14].

OA is also associated with the physiological imbalance of degradation and synthesis by chondrocytes resulting in alterations in the composition of the cartilage matrix [15]. In the early stages of OA, the quiescent chondrocytes become activated to remodel the contents of cartilage matrix [16], the water content increases and loss of glycosaminoglycan in the cartilage lead to the changes of the cartilage mechanical properties at this hypertrophic anabolic phase [17, 18]. After failure of these early compensating attempts, chondrocytes become catabolic and undergo senescence and apoptosis, and ultimately results in the progressive degeneration of articular cartilage [19] which is considered as an irreversible state of OA [18, 20]. Furthermore, fibrillations (microscopic cracks) in the superficial zone are formed, as well as deep fissures, bone marrow lesions and delamination in the cartilage [21]. In addition to the progressive degraded articular cartilage, subchondral bone interacts with cartilage

through various signalling mechanisms be presented and associated with the increased pain and dysfunction [18], due to peripheral and central pain sensitization [16].

Current Pharmacological Treatment of Osteoarthritis and their limitations:

As mentioned above, the degeneration of the articular cartilage remains the most significant structural changes seen in OA, resulting in severe pain and reduced mobility [16, 22]. The innate ability to heal the degenerated cartilage is limited by the avascular nature of cartilage, posing a significant challenge in the treatment of OA. Currently, there is no cure for this debilitating condition and most of the treatments focus on the symptom management including 3 main modalities as outlined in Figure 2 [23]. These are, firstly, physical and occupational therapies such as weight loss or assistive devices for load bearing joints. Secondly, pharmacological intervention for pain management by Nonsteroidal anti-inflammatory drugs (NSAIDs), opioids, viscosupplements, or corticosteroids injection. Third option is surgical intervention-arthroscopy, micro fracture, or finally total joint replacement.

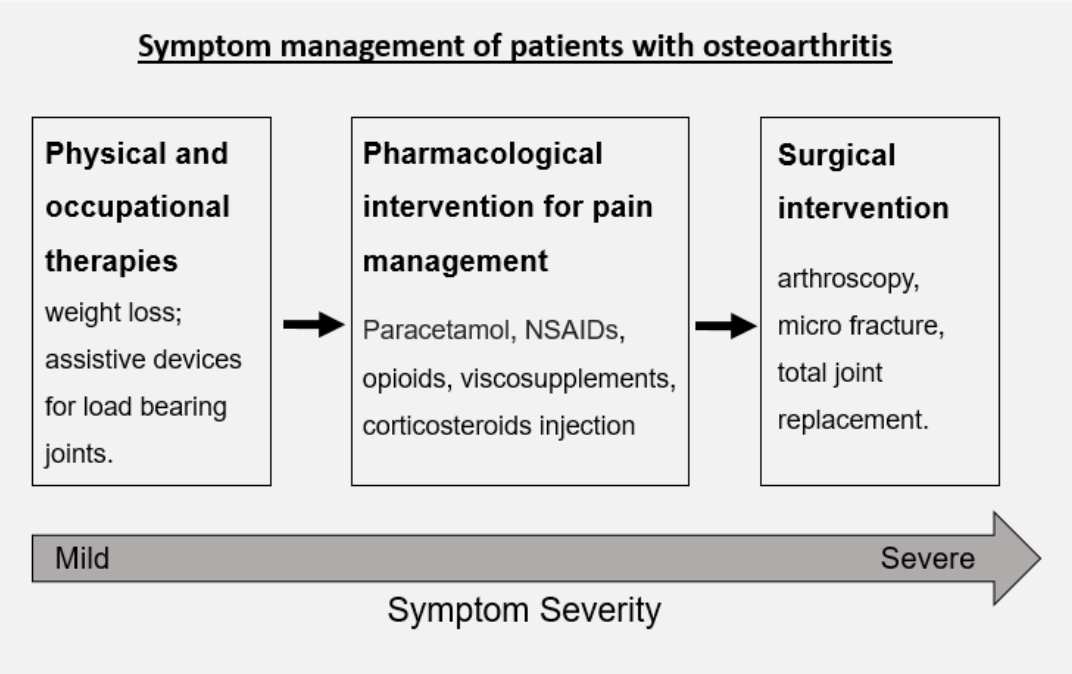

Figure 2: Current recommended treatment modalities for the osteoarthritis (Source: Arden et al., 2014)

The Arthritis Foundation (www.arthitis.org) recommends that OA patients undertake self- education in managing the condition and encourages losing weight for overweight and obese patients. This entails diet and exercise to reduce and manage healthy weight, however, due to pain and physical limitation resulting from the OA, exercise is hard to implement and sustain. Joint targeted physical therapy has shown to improve the pain and function; however, there is no long-term improvement. Assistive device is designed to provide the mechanical support to the joint structure in the patients with OA causing instabilities in the joint and also to distribute the load bearing for relief in pain and improve function, but these only have limited success.

Currently, the primary strategy of OA pharmacological management is mainly to relieve pain, improve function and manage the OA process [22, 23]. Pharmacological treatment is used for patients with mild to moderate pain and medications such as NSAID, opioids and corticosteroid are used routinely to alleviate the pain, however, there is no long term relief and these pharmacological agents have unwanted side effects [24].

Acetaminophen (Paracetamol) used to be the first-line pharmacologic management to treat mild to moderate OA pain. However, it became an inconclusive recommendation due to lack of compelling evidence [15]. Furthermore, using acetaminophen was associated with risks such as gastrointestinal (GI) adverse events and multi-organ failure [17] with minimal short term benefit [21]. Despite it being less effective than NSAIDs and since some patients have adverse effects with NSAIDs, it is still used by some patients but is recommended with conservative doses and treatment duration [15].

Non-steroidal Anti-inflammatory Drugs (NSAIDs) are a big family of drugs including oral NSAIDs such as ibuprofen, aspirin, naproxen, COX-2 inhibitors and topical NSAIDs such as diclofenac formed as cream, patches, gels or solution. Issues with oral NSAIDs include adverse gastrointestinal (GI) effects and need to be taken in conjunction with the GI protectant [25]. Furthermore, they are associated with potential toxicity especially in elderly patients [22]. The oral COX-2 inhibitors can reduce GI side effects but cause other adverse effects such as the risk of cardiovascular events. The use of topical NSAIDs eliminate the GI side effects of the oral NSAIDs but can be less effective [26], and have been associated with dermatological adverse events [27].

Opioids can be used for pain relief when patients cannot use NSAIDs and acetaminophen due to their associated side effects. However they have limited long-term efficacy [27] and are associated with adverse effects such as respiratory depression, opioid use disorder and overdose [28]. In meta-analysis of trials, patients who received opioid therapy were four times more likely to drop out due to adverse effects as compared to patients receiving placebo and their long term use is not recommended [27]. There are a wide range of medicines aimed at pain relief and improvement of quality of life for patients with OA. However currently there are no pharmacological agents that can prevent, halt or reverse the onset of OA. These studies highlight lack of effective pharmacological solutions for the OA sufferers.

Surgical Intervention to treat OA

Surgical interventions are recommended when the progression of OA has resulted in severe damage to the joint, severity pain and function deteriorates and cannot be managed with any other options. The initial surgical option to restore the structural stability such as joint debridement by arthrotomy or arthroscopy to remove loose cartilage, fragments of meniscus, shaving of the cartilage and removing osteophytes have shown to result in limited pain and function relief [29]. Arthroscopy remains the most performed surgery in the developed world by orthopaedic surgeons to help with the mechanical movement of the affected stiff knee. A blinded controlled clinical trial on the arthroscopy for the debridement and lavage with a placebo showed, there is no pain relief achieved after the surgery when compared with the placebo [30].

Joint replacement is considered the final option provided to OA patients when the condition progresses to the most severe. Surgical procedures for the replacement of hip and knees are extremely painful and require a long period of time for rehabilitation. Furthermore, total knee replacement has shown adverse outcomes such as pulmonary embolism, infections and surgery related deaths in some cases [31].

Cellular therapy and regenerative medicine for OA

In more recent times, many regenerative techniques have been used such as autologous chondrocyte transplantation (ACT) for focal damage of cartilage,

microfracture and mosaicplasty. The ACT technique in addressing confined cartilage damage involves the transplantation of chondrocytes that are harvested from non-weight bearing cartilage from the patient [32] but does not addressed generalized OA . This method has some concerns since it not only causes donor site morbidity, but also chondrocyte dedifferentiation in the transplanted site leading to the expression of type I collagen rather than type II collagen that may result in fibrocartilage rather than the desired hyaline like cartilage [33]. Another common surgical technique is the microfracture, which triggers the migration of bone marrow cells to the articular surface through stimulating the inflammatory response by drilling holes in the subchondral plate at the chondral defect site. The purpose of this technique is to provide an enriched environment for tissue regeneration [34]. However, the resultant tissue is again fibrocartilage containing type I collagen or hybrid repair cartilage tissue not the normal hyaline cartilage (type II collagen). Furthermore, the observed subchondral bone overgrowth (25%-49%) might limit durability and the long-term outcome of the microfracture [35]. Finally, the mosaicplasty procedure is similar to the ACT technique and involves the use of autologous osteochondral grafts, however, the results are disappointingly minimal and only offers short-term benefits [19].

The above demonstrates that the current treatment for the OA is only focused on symptom management and none of these options addresses or halts the progression of the disease or offers long term benefits. Hence, there is an unmet medical demand for the treatment for OA suffers that can halt the progression of the disease and to provide long term relief from the symptoms of OA. Cellular therapy has provided a real promise to combat this debilitating degenerative condition and can provide disease modifying long term benefit. Tremendous efforts have been made in the pre-clinical and now in the clinical trials evaluating the regenerative potential of the adult stem cells, especially mesenchymal stem cells (MSCs) to repair the structural damages of the joint space, cartilage degeneration and the inflammation.

Mesenchymal Stem Cells Therapy for OA- A new therapeutic Paradigm for OA

Modern medicine is exploring the regenerative potential of cellular therapy to address the currently unmet medical needs of various degenerative conditions such as OA. Cellular therapy has been extensively invested in, exploring a new paradigm

for the treatment of many degenerative conditions including degenerative disc disease (DDD) and the osteoarthritis of the joints amongst many other conditions. For this reason, this review will focus on the therapeutic properties of MSCs to treat OA. The disease modifying potential of cellular therapy such as the use of adult stem cells for regeneration of the damaged tissues has been hailed as a breakthrough in the 21st century and provides an exciting promise to chronic degenerative conditions. Currently, there are over 500 clinical trials registered on the ClinicalTrial.gov, exploring the safety and efficacy of the adult stem cells e.g., pluripotent stem cells, umbilical cord derived stem cell, placental stem cells, and mesenchymal stem cells, to treat OA. Of these, mesenchymal stem cells have been a leading choice for many medical researchers around the world with over 352 registered clinical trials [36]. In the clinical studies, MSCs are isolated from the patient either from bone marrow or adipose tissues, purified and administered as intraarticular injection in the affected joint under ultrasound guidance (Figure 3). MSCs are described to exert their therapeutic effects by homing to the injured site when injected locally to the joint for a short period of times and disappears and believed to be secreting a myriad of growth factors and cytokines to initiate the repair process, as discussed below.

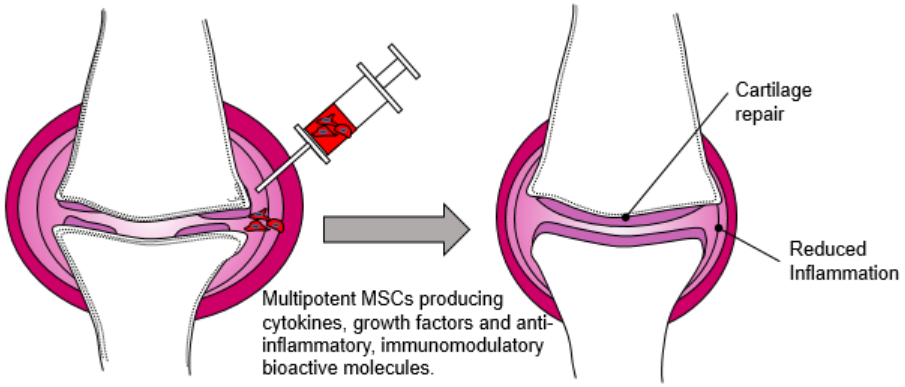

Figure 3: Model of regeneration of a knee joint before and after treatment with MSCs

In 1974, Friedenstein and colleagues first described stromal precursors derived from bone marrow that were able to form plastic-adherent fibroblast colonies in the monolayer culture and their differentiation characteristics [37]. The term Mesenchymal Stem Cell has been in use since it firstly was coined by Caplan in 1991 [38]. MSCs are ubiquitous throughout the musculoskeletal system in human body and are classified as self-renewing, postnatal, multipotent stem cells that can be differentiated in to the all tissue types of skeletal system and the connective tissues such as bone, fat, cartilage and muscle [39]. MSCs produce a vast array of cytokines, growth factors and anti-inflammatory bioactive molecules [40]. MSCs are heterogeneous, clonogenic and relatively easily isolated from various tissues and can be cultured expended *in vitro* due to their plastic adherence property and have fibroblast like morphology under the microscope [41]. Multipotent MSCs are originally derived from embryonic tissue - mesenchyme which is developed from the mesoderm and can be isolated *in vitro* from various sources including bone marrow, periosteum, trabecular bone, adipose tissue, synovium, skeletal tissues and deciduous teeth [42]. *In vivo*, the main role of MSCs is believed to be for self-repair and maintain the tissue homeostasis [43]. The resident MSCs are distributed into the tissues at various stages of maturation and involved in tissue regeneration [44].

Originally, MSCs were isolated from bone marrow but more recently they have been successfully isolated from various other tissues such as adipose tissue [45], brain, muscle tissue [46], skin [47], and teeth [48]. Moreover, MSCs can also be derived from different organs and tissues included spleen, liver, kidney, lung, thymus, pancreas, blood vessels and could be proliferated *in vitro* [49].

Since human MSCs are heterogeneous and can be obtained from many sources, different methods of isolation, expansion and different approaches to characterize the cells, this has caused the difficulty of comparing study outcomes. The Mesenchymal Stem Cell Committee of the International Society for Cellular Therapy (ISCT) has provided three minimal criteria to define MSCs for laboratory-based investigation and pre-clinical studies in 2006. First, MSCs must be plastic-adherent in the tissue culture flasks. Second, more than 95% of MSCs population must express CD105, CD73 and CD90 and lack expression (less than 2% population) of CD45, CD34, CD14 or CD11b, CD79a or CD19 and HLA class II. Third, MSCs must be able

to differentiate into osteoblasts, adipocytes and chondroblasts *in vitro* with standard differentiation conditions [50].

The 'stemness' of MSCs is maintained by the Leukemia inhibitory factor (LIF), fibroblast growth factors (FGFs), mammalian homologues of Drosophila wingless (Wnts), among other growth factors and cytokines [51]. The intra-populations of MSCs are functionally heterogeneous regarding their multilineage differentiation potentials. The tri-potent clones of MSCs (able to differentiate into three cell types e.g. osteoblasts, adipocytes and chondroblasts) display the highest rate of proliferation and a lower rate of apoptosis compared with the bi-potent (only two cell types) and uni-potent (only one cell type) clones [52, 53]. The proliferation capacity of MSCs is affected by both the cell seeding density [54], and also decreases as cells progress toward terminal differentiations [55]. The long-term expansion of MSCs might impact the composition, function and therapeutic potency of MSCs populations [56]. Furthermore, culture conditions such as culture media and oxygen tension has a major impact on gene expression, proteome and cellular organization [57-59].

The differentiation process of MSCs is tightly controlled and involves the activities of various transcription factors, cytokines, growth factors and extracellular matrix molecules [55]. The differentiation efficiency is also correlated with patients age whereby isolated cells from younger patients showed higher differentiation capacity in culture [60]. A number of biomarkers are used to determine differentiation towards adipogenic, chondrogenic and osteogenic lineages. The biomarkers for adipogenic differentiation are adiponectin, C/EBPα, FABP4, leptin, peroxisome proliferate receptor gamma (PPARγ); for chondrogenic differentiation are aggrecan collagen type II and Sox 9; while alkaline phosphatase, bone sialoprotein, osteocalcin, osterix, runx2 are biomarker for osteogenic differentiation [61-64].

MSCs have shown disease modifying effects in bone and cartilage defects, as discussed previously. Because of the multipotent properties of MSCs, they have also generated significant clinical interest in cardiovascular, neural and orthopedic therapeutic application. Moreover, the anti-inflammatory and anti-fibrotic properties of the MSCs make them the ideal candidate for the regenerative medicine. These cells are able to suppress the growth of activated T-cells and help regulate the production

of regulatory T-Cells (Treg) [36, 65]. The investigation of the anti-inflammatory properties of MSCs is well advanced and there is a number of advanced phase clinical trials for the treatment of Graft versus host diseases (GVHD) and Chron's disease [36]. Furthermore, the therapeutic effects of MSCs have been studied extensively focusing on the immuno-modulatory properties and the paracrine activity by secreting a wide variety of cytokines and growth factors that are attributed to the angiogenic and regenerative potential in the damaged tissues [36]. More recently, studies have shown that MSC paracrine effects are mediated by secretion of extracellular vesicles such as exosomes [66, 67]. The use of MSC exosomes might serve as an alternative therapy over MSC transplantation for tissue regeneration [68]. A recent study reported the efficacy of MSC exosomes secreted from synovial membrane and induced pluripotent stem cell-derived MSCs to treat mouse osteoarthritis whereby both source exosomes demonstrated exosomes attenuated OA [69].

MSC based treatment of OA has a lower risk to the patient and a variety of sources such as adipose tissue, bone marrow and synovium [70]. These autologous cells can be harvested from patients by either liposuction or aspirated from bone marrow. The adipose-derived MSCs are preferred by patients as compared to MSCs aspirated from bone marrow because comparatively MSCs are more abundant in adipose tissues than in bone marrow aspirate. However, bone marrow-derived MSCs may have higher chondrogenic potential than adipose-derived MSCs [71]. Furthermore, synovial tissues obtained from the surgical removal of subsynovial tissue, non-cartilaginous area of medial condyle of femur, has also become an attractive source of MSCs in treatment of OA [9] [70].

Current Clinical trials and case series investigating MSCs to treat OA:

A proof of concept clinical trial conducted in Korea, showed promising safety and efficacy results of the adipose derived MSCs to treat OA. Patients showed reduced pain, improved function of the joint and in the high dose patient cohort, and regeneration of hyaline-like cartilage suggesting the disease modifying effects of MSCs when injected into the affected joint [72]. Another pilot study by Orozco demonstrated significant improvement in the pain and functional improvement of up to 65% to 78% in chronic OA patients when treated with the bone marrow derived MSCs,

as compared with the conventional treatment methods [73]. The cartilage mapping by T2 MRI showed evidence of improvement in the good cartilage quality i.e. hyaline like cartilage and significant decrease in the poor cartilage i.e. fibrocartilage. The same group conducted a pilot clinical trial examining the safety and efficacy of MSC as a novel treatment of intervertebral disc disorder [74]. After 1 year follow up, the primary end point of pain and functional improvement was met in approximately 85% of the cases and no adverse event was observed, the water content was significantly improved in the treated disc and patient reported significant improvement in quality of life index [74]. Furthermore, Phase I Dose-escalation trial to treat severe OA of the knee by using adipose-derived MSCs to treat patients with symptomatic and severe OA of knee with single-articular injection of autologous adipose-derived MSCs also showed significant improvement in patients after a six month of follow up [75]. These results showed that the treatment was safe and well tolerated by all patients.

Adipose derived MSCs to treat patients with joint disease also act as a precursor to treat degenerative OA. Osteochondritis dissecans is a joint disorder pertaining to articular cartilage and chondral defects resulting in damage to the articular cartilage and underlying bone. Adipose derived MSCs have been reported to have disease modifying effects in a clinical case series published recently [76]. This study showed regeneration of the lost cartilage and significant reduction of pain and improvement in mobility (Figure 4) [76].

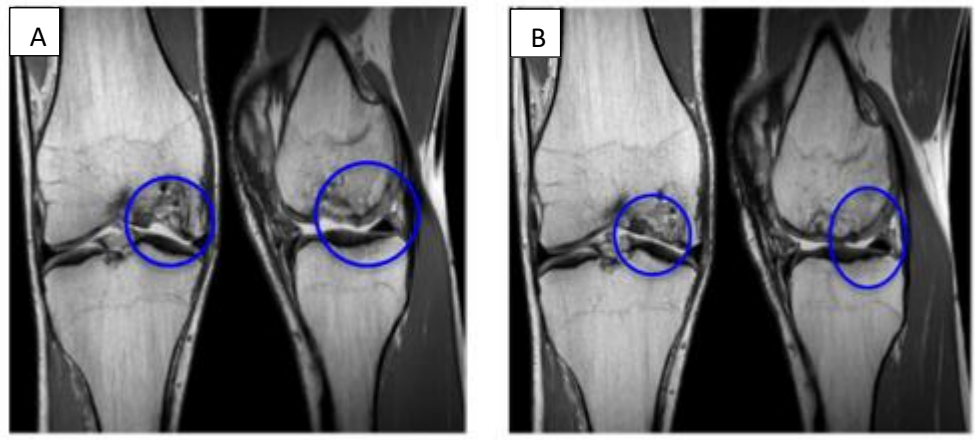

Figure 4: (A) Pre-Treatment Proton Density (PD) weighted Coronal and Sagittal MRI images of the knee showing the isolated chondral defect involving the central weight bearing area of the medial femoral condyle (B) Post-Treatment PD weighted Coronal and Sagittal MRI imaging at 18 months indicating articular cartilage regeneration at the site of the osteochondral defect [76].

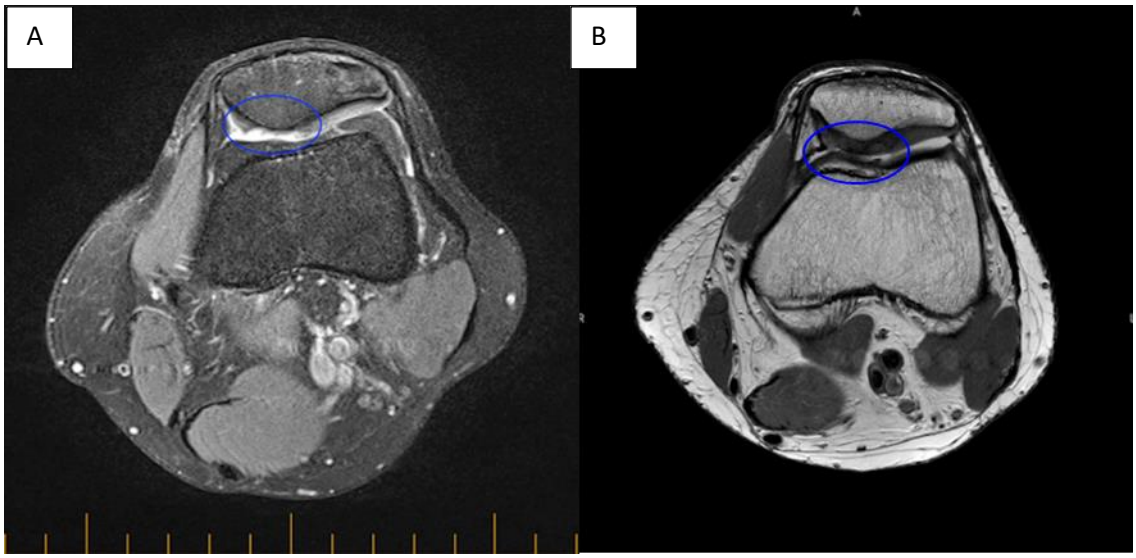

Figure 5: (A) Pretreatment proton density fat-suppressed axial MRI of the knee showing the isolated chondral defect involving the medial facet of the patella.(B) Post-treatment proton density axial MRI indicating articular cartilage regeneration at the site of the chondral defect with smooth integration with the surrounding joint surface [77].

These evidence based clinical outcomes strengthen the model for treatment of OA with MSCs (Figures 4 & 5). The results of these trials provide an exciting and promising long-term relief for OA patients and herald a new paradigm for the treatment of chronic and debilitating OA and as well as other degenerative condition. Intriguingly, several hundred clinical trials globally have been registered in past 10 years but only a handful of results from these trials are published. Therefore, there is a need for more clinical trials data to be released from the completed trials to further support and develop this novel model of treatment.

Autologous versus allogeneic MSCs for therapy:

The choice between autologous and allogeneic MSCs treatment is another aspect that will need further supportive data. Due to the immune -privileged aspect of MSCs [65], allogeneic stem cell treatment shows more promise and is likely to attract more attention as an ‘off the shelf’ product. However, long term safety and efficacy data are warranted. The mechanism involved in modulating the host immune system is believed to be facilitated by the ability of MSCs to influence immune cells’ cytokines

secretion. MSCs influence mature dendritic type 1 cells to decrease secretion of Tissue Necrosis Factor- alpha (TNF-a), and instruct mature dendritic cells type 2 to increase the anti-inflammatory cytokine IL-10. MSCs can direct T helper cells to decrease secretion of Interferon gamma (IFN-y), T helper cells 2 to increase IL-4 production and help reduce production of IFN- y from the Natural Killer (NK) cells [78-80]. When co-cultured with immune cells, MSCs also enhanced the production of Prostaglandin E2 (PGE2), therefore MSCs are able to modulate the immune system by alteration of cytokines production in the host [78-80]. In a comparative study with autologous and allogenic MSCs, in which 5 patients each receiving MSCs the result revealed similar level of favourable benefits to the quality of life improvement in patients with ischemic cardiomyopathy and no immune rejection in the allogeneic group [81]. In a canine study, when autologous and allogeneic MSCS transplants were compared in spinal cord injury, both types of cells exhibited therapeutic benefits and transplanted cells were observed in the injured tissue for up to 4 weeks and no immune reactions or adverse effects were reported [82]. Given the safety reports of the allogeneic MSCs therapy and the surgery related complications involved in autologous treatment, MSCs derived from a donor for allogenic therapy provides a better and more affordable treatment option.

Future direction:

With the approval of Prochymal, an adult stem cells therapy to treat graft versus host disease (GvHD) in children, in Canada and New Zealand, it heralds a new era for cellular therapy to address the unmet medical conditions of previously untreatable diseases. The translational medical research currently underway targeting MSCs for OA therapy in the clinical trials database is promising, however, they need careful evaluation of the outcome data. The results require focus primarily on the safety and then on the efficacy. Furthermore, the various stages of clinical trials currently registered needs their outcome data published for the wider scientific community to consider and to evaluate the robustness of the therapy. The large number of MSCs trials indicates the promise of these cells, however, there is considerable paucity of the published clinical trial data and therefore it is early to envisage the extent of their therapeutic application.

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Publication 2

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Mesenchymal stem cell derived extracellular vesicles and their therapeutic potential

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Abstract

Extracellular vesicles (EVs) are cell-derived membrane-bound nanoparticles, which act as shuttles, delivering a range of biomolecules to diverse target cells. They play an important role in maintenance of biophysiological homeostasis, cellular, physiological and pathological processes. EVs have significant diagnostic and therapeutic potential and have been studied both *in vitro* and *in vivo* in many fields. Mesenchymal stem cells (MSCs) are multipotent cells with many therapeutic applications and have also gained much attention as prolific producers of EVs. MSC-derived EVs are being explored as a therapeutic alternative to MSCs since they may have similar therapeutic effects but cell-free. They have applications in regenerative medicine and tissue engineering, and most importantly, confer several advantages over cells such as lower immunogenicity, capacity to cross biological barriers and less safety concerns. In this review, we introduce the biogenesis of EVs, including exosomes and microvesicles. We then turn more specifically to investigations of MSC-derived EVs. We highlight the great therapeutic potential of MSC-derived EVs and applications in regenerative medicine and tissue engineering.

Extracellular Vesicles

Extracellular vesicles (EVs) bearing nucleic acids, proteins and lipids can be released into the extracellular space from eukaryotic cells, as well as from some prokaryotic cells [1]. These released EVs are lipid bilayer-bound nanoparticles and are found in many biological fluids such as serum, cerebrospinal fluid, saliva, urine, nasal secretions, and breast milk. They can also be collected in cell culture medium. Originally EVs were regarded as cellular waste [2], and nowadays EVs are known playing important biological roles in cellular homeostasis and the spreading of biomolecules to neighbouring cells and tissues. Transported biomolecules can contribute to normal physiology or disease states or could be therapeutics to be delivered to damaged cells and tissues. For these reasons EVs show significant potential in biotechnology [3-5]. Many different names have been used for extracellular vesicles, following several independent discoveries, which has led to confusing nomenclature. As the extracellular vesicle field has grown tremendously over the past few decades, the International Society for Extracellular Vesicles (ISEV) was launched in 2011, with the aim of advancing extracellular vesicle research globally. The term “extracellular vesicles” (EVs) was introduced by ISEV to describe preparations of vesicles isolated from biofluids and cell cultures [3]. Based on their size and biogenesis, EVs could be classified into three main sub-classes: exosomes (40-120nm), microvesicles (50-1000nm) and apoptotic bodies (500-2000nm) [6]. Both microvesicles and apoptotic bodies are directly shed from the plasma membrane, but via different cellular processes, whereas exosomes are generated by the endocytic pathway and are originally considered to play a particularly important role in cell to cell communication [7].

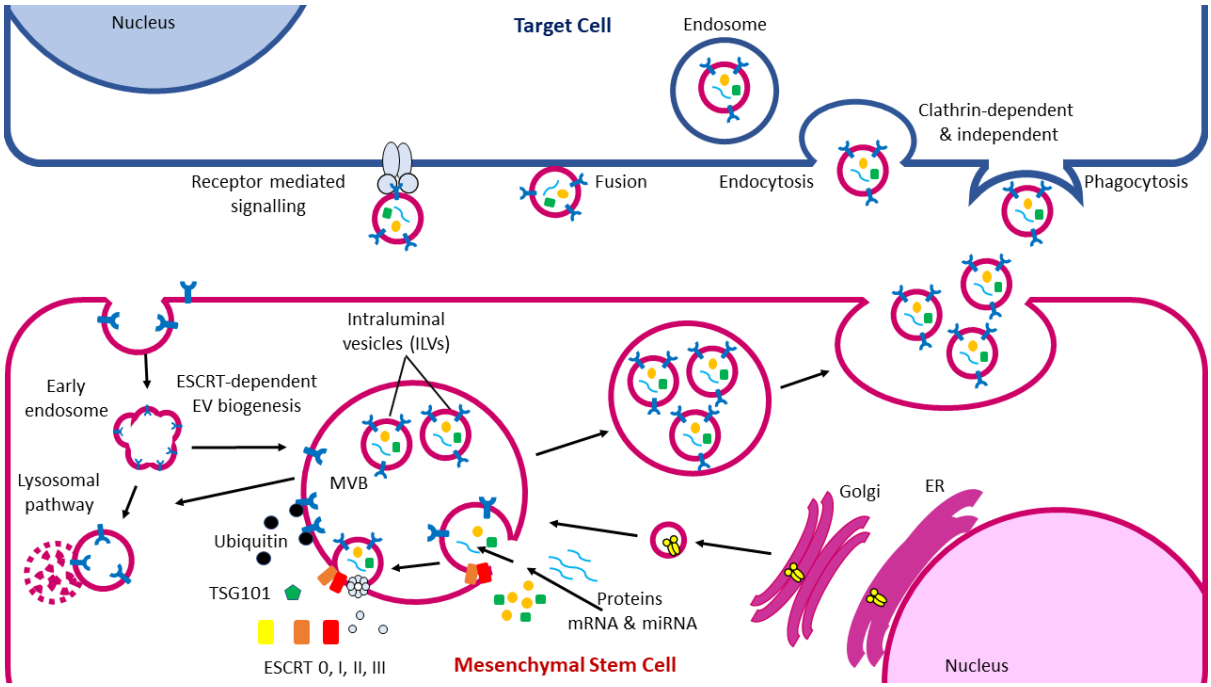

Figure1: Extracellular vesicle biogenesis; ILVs invaginate from the outer endosomal membrane to bud into the lumen of endosomes through ESCRT-dependent/independent machineries during the maturation of MVB from the early endosome. Matured MVB is then transported to the cell periphery and fuses with the plasma membrane to release ILVs (Exosomes). Exosomes together with microvesicles enter the target cells through signalling, fusion and endocytosis pathways.

Exosomes

The term exosome was first used to describe membrane nanovesicles released from mammalian reticulocytes through the endosomal pathway in the 1980s [8-10]. Exosomes were originally thought to be waste products released by cells. In the subsequent decades, further research identified that exosomes have an important function as transport vehicles and can act to stimulate immune suppression of tumor growth [11, 12]. One of the important discoveries in the field was the presence of nucleic acids-mRNA and miRNA in exosomes and hence the ability to alter specific gene expression and protein translation in recipient cells [13]. Today exosomes are recognised to play an important role in intercellular communication through transfer of proteins, lipids and nucleic acids into recipient cells [6, 14, 15] (Figure 1).

Exosome biogenesis

Many cellular processes are involved in the generation of exosomes. These include the production of microvesicular bodies (MVBs) and formation of intraluminal vesicles (ILVs) during early endosomal maturation into MVBs. This is followed by trafficking and fusion of MVBs with the plasma membrane, releasing ILVs extracellularly as exosomes [16]. Several cellular mechanisms are involved in the formation of ILVs and maturation of MVBs, including the Endosomal Sorting Complex Required for Transport (ESCRT) which involves both ESCRT-dependent and ESCRT-independent transport mechanisms, described below.

The best-described mechanism for formation of ILVs is ESCRT-dependent machinery [17, 18]. ILVs are formed from early endosomes by the inward budding of the limiting membrane and then scission of the narrow neck to release the bud into the endosomal lumen as a vesicle. ESCRT proteins sort ubiquitinated proteins into these buds [19]. The role of the four ESCRT complexes ESCRT-0, ESCRT-I, ESCRT-II, ESCRT-III in formation of ILVs in the interior of MVBs was well-described in the early 2000s [20-22]. The ESCRT-dependent mechanism starts from the interaction of the ESCRT-0 complex with ubiquitylated proteins, which are organized by clathrin into specialized endosomal subdomains [23]. Then direct interaction between ESCRT-0 with TSG101 of ESCRT-I complex, recruits ESCRT-I ESCRT-II and start inward budding of the ILVs into lumen of the MVBs.

The ESCRT-I/ESCRT-II system is one core part of the ESCRT machinery, which functions as one branch of the ESCRT pathway to feed into ESCRT-III and Vps4 scission machinery [19]. ESCRT-II recruits the ESCRT-III complex to develop a curved membrane-binding surface and line tubules extended away from cytoplasm [24]. ESCRT-III also recruits associated protein Alix for recruitment of deubiquitinating enzyme Doa4 [25]. Finally, ESCRT associated proteins Vps4 and Vta1 cleave the ILV into free vesicles and disassemble ESCRT complexes [17]. Some ESCRT components and accessory proteins such as TSG101, HRS and ALIX are retained in the ILVs and become important protein markers of exosomes. However, it is not clear whether they are specific markers for exosomes since ESCRT-I/II/III and their accessory molecules are associated with various other budding and membrane scission processes, such as microvesicle release, wound repair on the plasma membrane, neuron pruning, membrane abscission in cytokinesis, nucleus envelope reformation, cellular autophagy processes etc [19]. Alternatively, ESCRT-0 has been specifically implicated in exosome secretion, and not yet descripted in plasma membrane budding and scission

processes. Therefore, ESCRT-0 components might be more specific markers to demonstrate endosomal origin [26].

Interestingly, ILVs can still form in MVBs via ESCRT-independent mechanisms [27]. Many studies suggest that ESCRT-independent mechanisms are involved in ILV formation and exosome biogenesis. The ESCRT-independent mechanisms involve lipids (ceramide, cholesterol and PLD2), tetraspanins, syntenins or heat-shock proteins [23, 28-31]. For example, the depletion of the ESCRT subunits such as Hrs, TSG101, Alix or Vps4, exosomes enriched in proteinlipid protein (PLP) and CD63 were still secreted through ceramide-dependent sorting mechanism [15, 27]. Even though many studies have described significant contributions to ILV formation pathways, exosome biogenesis is still not exhaustively studied. Therefore, since current knowledge of exosome biogenesis is not fully specific to exosome secretion, and also not shown in all cell types [26], further studies on exosome biogenesis is still needed.

Once late endosomes become fully mature MVBs, they are transported to the cell periphery and fuse with the plasma membrane to release ILVs as exosomes [1, 32, 33]. The mechanisms of MVB mobilization, docking and fusion involve a large network of proteins, including the actin cytoskeleton, microtubules and associated molecular motors such as kinesins and myosins, molecular switches (small GTPases), tethering factors and SNARE proteins [7, 32, 34-38]. Proteins and protein complexes organise the tethers and work together with Rab proteins to direct the vesicle targeting [34]. The activated Rab proteins (Rab GTPases) such as Rab7, Rab11, Rab27 and related Ral-1 regulate vesicle formation, trafficking and fusion. They control movement through interaction of the vesicles with cytoskeletal components, tethering/docking of these vesicles to the cell periphery [32, 37, 39-41].

MVB trafficking requires actin and microtubule cytoskeletons, motor proteins to transport and tether MVBs to the plasma membrane [33]. After docking of MVBs to the plasma membrane, soluble N-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs) regulate the fusion of the MVB lipid bilayer with the plasma membrane to release ILVs [36]. SNAREs are the core fusion engine in membrane fusion and are recycled after each fusion event [35]. SNARE proteins are classified into four subfamilies based on their SNARE motifs; Qa-, Qb-, Qc-(t-) and R- (v-) SNAREs, which are highly conserved and diverged early in eukaryotic evolution [42]. They are assembled in a *trans* configuration and formed as helical core complexes, mediated by the SNARE motifs. The assembly starts at the N termini of the SNARE motif followed by a zipper-like fashion towards the C-terminal membrane anchors. The function of SNARE complexes is to provide the mechanical force exerted on the membrane to proceed the fusion of two lipid bilayers and then distort membranes to form a fusion pore releasing ILVs of MVBs into the extracellular environment as exosomes [35].

Microvesicles

Similar to exosomes, many types of machinery are involved in microvesicle biogenesis. Unlike exosome biogenesis which has been intensively studied, microvesicle biogenesis has

only recently started to emerge as a focus of study [43]. Microvesicles, also classified as ectosomes, are directly generated from the plasma membrane [44]. Microvesicles are generated by the formation of outward buds in specific sites of the membrane and then released into the extracellular space by fission [45]. Several molecular rearrangements are involved including changes in lipid and protein composition and even Ca^{2+} level at the specific sites of the membrane to elicit in membrane budding [46, 47]. Ca^{2+} level changes alter the lipid composition of the plasma membrane and the externalization of phosphatidylserine also plays a role in microvesicle formation[48].

Microvesicles have also been shown to be enriched in cholesterol and are raised from cholesterol-rich lipid rafts [49]. Furthermore, the depletion of cholesterol significantly reduces microvesicle shedding. Other factors such as molecular rearrangements in the plasma membrane, cell shape maintenance proteins, cytoskeletal elements and their regulators are also involved in microvesicle biogenesis [50]. The regulators of actin dynamics, RhoA (a member of the small GTPases family) and its downstream associated protein ROCK and LIM kinases are essential for microvesicle biogenesis [51]. A calcium dependent enzyme, calpain which regulates cytoskeletal proteins is involved in microvesicle shedding [52]. Inhibition of calpain could supress PAK1/1 activation to decrease polymerization of actin, formation of filopodia, and furthermore interfere the generation of microvesicles. ARF6 also plays a key role in microvesicle formation and shedding [53]. ARF6-GTP-dependent activation of phospholipase D recruits the extracellular signal-regulated kinase (ERK) to the plasma membrane, and then ERK phosphorylates and activates myosin light-chain kinase (MLCK) which is an important regulator of actin polymerization and myosin activity. This process is essential for microvesicle release and inhibition of ARF6 could block microvesicle shedding. Both exosomes and microvesicles play important roles in physiological and pathological cellular processes.

EV function

Endosomal exosomes were considered as the main mediators that affect recipient cells. However, it is difficult to efficiently separate exosomes from other subtypes of EVs by current isolation methods, so it is difficult to definitively assign a function to a particular type of vesicle. Furthermore, not only do the formation and secretion of ILVs employ multiple mechanisms, resulting in heterogeneous exosomes, other EVs also overlap in their biophysical properties [54]. Moreover, there is currently no consensus on markers to distinguish exosomes from other EVs.

The techniques used to isolate small EVs results in a heterogeneous mix of sizes, origin and molecular composition, with an unknown portion of them being exosomes [55]. Therefore, they may contain a mixture of endosomal and non-endosomal small EVs [56] and even some non-vesicular molecules such as various dense lipoproteins [57]. Nevertheless, many studies have discovered a significant function of EVs to target cells and demonstrated their potential in many pathophysiological fields such as cancer, immune responses, various diseases and regenerative therapeutics [6, 58]. Even though there are many studies that describe the function of exosomes, most of these studies may contain a mixture of EVs with different subtypes due to their preparation method, so the observed function, assigned to exosomes, may be elicited by multiple EV types [26].

EVs carry proteins, lipids and nucleic acids and can be released by most cells and taken up by recipient cells to trigger various phenotypic effects [59]. The lipid bilayer of EVs can protect their contents, transit through the extracellular fluid, and internalise into recipient cells. Different recipient cell types take up heterogeneous EVs through different pathways which are highly specialised and specific processes [60]. EVs bind to appropriate receptors on target cells through receptor-ligand interaction and enter these cells through three major EV uptake pathways: signalling, fusion and endocytosis [43, 60].

Many studies have shown the diverse biological functions of EVs. EVs released by B lymphocytes present MHC-peptide complexes to specific T cells which suggested EVs played a role in adaptive immune responses [61, 62]. Proteins and mRNAs of EVs can be transferred into target cells and mRNAs can be translated into corresponding proteins [63]. For example, selective mRNAs and miRNAs were found in mast cell EVs and involved in the immune response [13].

Genetic communication between cells might also occur via the trafficking of EVs through the systemic circulation, similar to how hormones impact their recipient cells. EVs derived from stem cells play a pivotal role in tissue regeneration [64, 65]. EVs not only play important roles in many aspects of biology such as intercellular vesicle traffic, immunity, neurobiology and microbiology, but also have important roles in disease pathogenesis such as tumour progression, neurodegenerative propagation and HIV and prion spread [6, 66]. For example, Tumor cells can release EVs into microenvironments to elicit tumor progression via numerous mechanisms such as promoting angiogenesis, suppressing immune responses and tumor cell migration in metastases [66, 67]. More recently, Mesenchymal stem cells have been shown to be prolific producers of EVs and have been investigated for their potential therapeutic applications.

Mesenchymal stem cells and EVs

Mesenchymal stem cells (MSCs) are multipotent stem cells derived from mesenchyme, which develops from the mesoderm [68]. MSCs are capable of self-renewal and differentiation into skeletal and connective tissues such as bone, fat, cartilage and muscle [69]. The main roles of resident MSCs in adults are self-repair and to maintain cellular tissue homeostasis. Due to their plastic adherence properties when cultured *in vitro*, MSCs can be easily isolated from various organs and tissues such as bone marrow, adipose tissue, muscle tissue, skin, teeth, periosteum, trabecular bone, synovium, skeletal tissues, brain, spleen, liver, kidney, thymus, pancreas and blood vessels [69, 70]. MSCs are considered to be ideal candidates for tissue regeneration and tissue engineering, and interest in their biological roles and clinical potential has dramatically increased over the last three decades [71].

There are over two thousand clinical trials registered on clinicaltrials.gov investigating therapeutic applications of MSCs in many diseases, such as Bronchopulmonary Dysplasia, Multiple sclerosis, Autoimmune Diseases, Alzheimer's disease, liver diseases, osteoarthritis, kidney disease, myocardial infarction and Graft Versus Host disease (ClinicalTrials.gov). Initially the therapeutic applications of MSCs were investigated to replace injured cells, based on their differentiation potential. However, less than 1% of the transplanted MSCs could reach the target tissue, such as the infarcted myocardium in treatment of myocardial infarction [72]. Nonetheless, MSCs restored heart function more rapidly compared to the slow and inefficient differentiation process of cardiomyocytes [73]. MSCs have also been shown to be effective in treating degenerative diseases such as osteoarthritis for both animals and humans [74, 75].

Furthermore, it has been demonstrated that MSCs can be effective in the modulation of immune responses, anti-inflammatory affect, tissue repair and regeneration in many therapeutic applications *in vitro* and *in vivo*. Therefore, MSCs are proposed to exert their beneficial effects by paracrine secretion rather than from their differentiation [76] [77], for which most MSC clinical trials were rationalized. However, to date, none of the identified soluble secreted mediators alone are able to sufficiently mediate the MSC therapeutic effects [78]. Subsequently many studies have shown that the paracrine effects of MSCs were mediated in part by the secretion of EVs [64, 79]. Thus extracellular vesicles derived from MSCs might be a safer cell-free alternative to cell therapy [80]. More recently the research focus on the mechanism of therapeutic action of MSCs, which was previously attributed to their differentiation and paracrine efficacy and has now focused on the role of EVs. MSC-derived EVs play an important role in the regulation of normal physiological, tissue regenerative and the pathological propagation processes, and MSCs are considered to be prolific producers of EVs when compared to other cell types [81].

MSC-derived EVs have been shown to contain at least 730 different proteins [82]. These proteins reflected both features of MSCs and EVs. For example, 53 proteins of MSC-derived EVs were related to self-renewal genes associated with MSCs, and 25 proteins were differentiation genes of MSCs. In their study, Kim et (2014) showed that MSC-derived EV proteins not only included surface markers of MSCs, but also MSC-specific proteins involved in signalling pathways to facilitate self-renewal and differentiation. MSC-derived EVs also contain proteins associated with EV biogenesis, trafficking, docking and fusion. Furthermore, EV proteins such as the surface receptor PDGFRB, EGFR, and PLAUR, signalling molecules of RAS-MAPK, RHO and CDC42 pathways, cell adhesion molecules and additional MSC antigens are associated with promotion and modulation of MSC therapeutic potential. These proteins may play a role in the efficacy of MSC-derived EVs in tissue repair and tissue regeneration. Even though EV miRNAs were estimated to be less than one copy per EV [83], some EVs might be enriched with certain miRNAs. 171 miRNAs were identified in MSC-derived EVs [84]. The most abundant 23 miRNAs could target 5481 genes to regulate many specific pathways and biological processes, such as miR-130a-3p and miR-199a, which induce cellular proliferation, promote angiogenesis and inhibit apoptosis. Furthermore, the proteome of purified MSC exosomes as profiled by mass spectrometry and antibody arrays, contain 938 unique gene products found in exosome database website http://exocarta.org that encompass a wide range of biochemical and cellular processes including cellular communication, structure and mechanics, inflammation, exosome biogenesis, tissue repair and regeneration and metabolism [85].

Therapeutic applications of mesenchymal stem cell-derived extracellular vesicles

To date, the therapeutic potential of MSC-derived EVs have been studied in both animal models and various clinical applications for many disease areas, such as cardiovascular disease, acute kidney injury, liver disease, lung disease, cutaneous wound healing, cancer suppression [73, 86-88]. EVs also have also been tested as potential diagnostic tools, anti-tumor therapeutics, drug delivery vehicles and vaccines [86, 89]. Here, we focus on the

therapeutic potential of MSC-derived EVs in a number of applications in regenerative medicine.

One of the first reports of MSC-derived EVs was of those derived from human bone marrow MSCs. These EVs had a beneficial impact on tubular epithelial cells through delivering mRNA cargo to activate regenerative programmes and resulted in recovery from acute kidney injury *in vitro* and *in vivo* [90]. Furthermore, intravenous administration of human MSC-derived EVs had the same efficacy as MSCs themselves on the treatment of acute kidney injury by inhibiting apoptosis and stimulating tubular cell proliferation in a rat model [87]. They also protected the kidney from the development of chronic injury, which highlights the potential of MSC-derived EVs for regenerative medicine.

Recent studies include the use of MSC-derived EVs for the treatment of a number of neuropathological diseases, such as multiples sclerosis [91] and Alzheimers disease [92]. In a mouse model of multiple sclerosis the mice were treated with saline, placenta MSCs, low dose ($1.0X10^7$) or high dose ($1.0X10^{10}$) human placenta MSC-derived EVs. [91]. Both MSCs and MSC-derived EVs showed regenerative effects and prevented oligodendroglia degradation and demyelination, resulting in motor function improvement. Importantly animals treated with high-dose MSC-derived EVs or MSCs showed similar clinical outcomes, demonstrating that MSC-derived EVs possess the same therapeutic potential as MSCs. Another preclinical study showed that MSC-derived EVs could be a therapeutic strategy for the treatment of currently incurable Alzheimer's disease [92]. After 28 days of injection of 10 μg EVs and 1×10^6 MSCs separately into two groups of mice with induced Alzheimer's disease, both groups had similar beneficial effects in improvement of neurogenesis and cognitive function.

MSC-derived EVs are capable of reducing infarct size of myocardial injury through modulating the injured tissue environment, inducing angiogenesis, promoting proliferation, and preventing apoptosis [64]. The therapeutic effects of MSC-derived EVs on myocardial infarction has been demonstrated in a mouse model [93]. MSC-derived EVs could reduce infarct size to preserve cardiac function for an extended period through rapid activation of multiple cardioprotective pathways.

The function of MSC EVs in cartilage repair has been studied by investigation of the effects of human MSC-derived EVs on chondrocyte survival *in vitro* [94]. The chondrocytes could quickly endocytose the labelled MSC-derived EVs and rapidly phosphorylate AKT and ERK in chondrocytes within 1 hour to elicit the cellular proliferation of chondrocytes. MSC-derived EVs enhanced regeneration of the damaged cartilage through inducing proliferation, migration and matrix synthesis of chondrocytes, attenuating apoptosis and modulating immune reactivity. Furthermore, intra-articular injection of 100 μg/100 μl of embryonic MSC-derived EVs could efficiently repair osteochondral defects in a rat model [95]. The results from the MSC-derived EVs treatment group showed hyaline cartilage regeneration by the end of 12 weeks. In contrast, the defects of controls treated with PBS were filled with fibrous and non-cartilaginous tissue. Additionally, there were no adverse inflammatory responses in this experiment. In a preclinical study the efficacy of MSC-derived EVs secreted from synovial membrane were compared to induced MSC-derived EVs in the treatment of mouse osteoarthritis (OA) [96]. Intra-articular injection of only 8 μl of EVs (1.0×10^{10}/ml), from either source, into collagenase-induced OA mice attenuated OA. MSC-derived EVs showed a more significant effect than synovial membrane MSC-derived EVs. Furthermore, EVs from adipose

tissue-derived MSCs could repair damaged cartilage through increasing the proliferation and migration of chondrocytes in a rat model of OA [97]. These numerous studies demonstrate the possibility of treating chronic conditions with MSC-derive-EVs to address current unmet medical needs.

Alternate therapeutic delivery methods of MSC-derived EVs

As researchers have begun to unlock the therapeutic potential of MSC-derived EVs in the field of regenerative medicine, alternate delivery methods are being explored. These include the encapsulation of EVs in hydrogels, or incorporation into biodegradable scaffolds such as polylactide (PLA) and polyethyleneimine (PEI). These methodologies represent ways of cell-free delivery methods with the benefits of MSCs, which can be sustained over long periods of time.

Hydrogels are a 3D network of polymers with hydrophilic properties that can swell in an aqueous solution and absorb biologic fluids and therefore have the potential to act as delivery vectors in tissue engineering. A biodegradable hydrogel was used to encapsulate ES cell differentiated MSC-derived EVs in a rat hepatic regeneration model [98]. The EVs were encapsulated in PEG hydrogels, which acted as a sustained-release EV depot to treat liver disease in rats [98]. The MSC-derived EV-laden hydrogels could gradually release EVs and result in accumulation in the liver for one month, compared to 24 hours clearance after conventional bolus injection. This study not only demonstrated the anti-apoptosis, anti-fibrosis and regenerative properties of MSC-derived EVs, but also demonstrated a sustained systemic delivery method which could be employed for treatment of a variety of diseases.

Alternatively, EVs can be incorporated into solid 3D scaffolds when modelling structures such as bone. In a rat model of calvaria bone tissue damage, MSC-derived EVs were delivered on 3D PLA and PEI scaffolds to determine their ability to repair bone lesions [99]. Human MSCs, MSC-derived EVs and 3D PLA or PEI-engineered EVs were evaluated in a number of combinations for their capability for bone defect regeneration *in vitro* and *in vivo*. It was found that there was more host tissue in-growth in the implant of 3D-PLA + MSC EVs, 3D-PLA + EVs + MSCs samples than 3D printed PLA scaffolds only and 3D-PLA + MSCs samples. Abundant ECM, formation of nodules and visible blood vessels in 3D-PLA + MSC EVs, 3D-PLA + EVs + MSCs, 3D-PLA + PEI-EVs, 3D-PLA + PEI-EVs + MSCs samples. This finding demonstrates that MSC-derived EVs could contribute to osteogenic regeneration, improve the mineralization process and develop an extensive vascular network. Furthermore, the calvarial bone defect was completely repaired in 3D-PLA + EVs + MSCs, 3D-PLA + PEI-EVs, and 3D-PLA + PEI-EVs + MSCs samples when evaluated for up to 16 weeks, which demonstrates the potential of engineered MSC-derived in tissue engineering of bone defects. In another study on cartilage regeneration, MSC-derived EVs were evaluated using 3D printed ECM and Gelatin-Methacaryloyl (GelMA) hydrogels in a rabbit OA model [100]. The 3D printed ECM/GelMA/EV scaffold had the best therapeutic effect in cartilage regeneration when compared to 3D printed GelMA and 3D printed ECM/GelMA scaffold. The defect region with the 3D printed radially oriented ECM/GelMA/EVs had facilitated cartilage regeneration and repaired tissue with a mixture of fibrocartilage and hyaline-like cartilage. These studies suggest a promising application of MSC-derived EVs in 3D printing for tissue engineering of bone and cartilage.

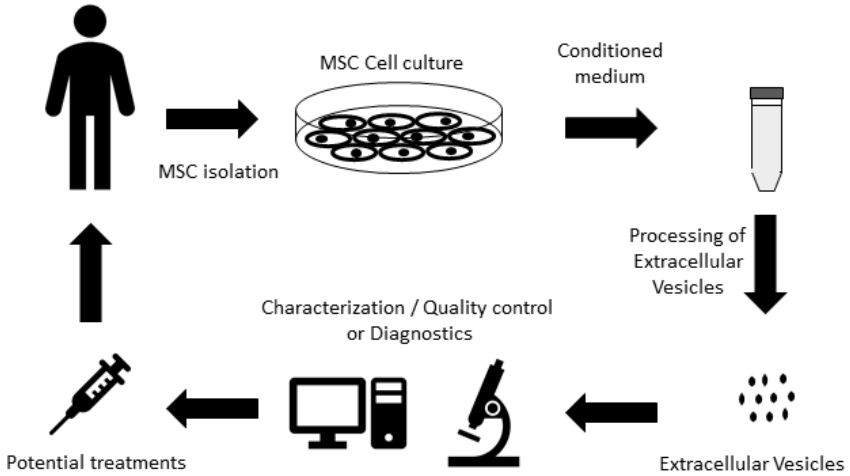

Figure 2: Workflow of MSC-derived EVs for therapeutic and diagnostic applications. MSCs can be isolated from patients from a variety of tissues. MSCs are cultured *in vitro* and the conditioned culture medium is collected and subjected to extracellular vesicle isolation and/or purification. The isolated MSC-derived EVs can be used for diagnostic purposes or undergo quality control before being used in autologous and/or allogeneic therapeutics.

Clinical trials using MSC-derived EVs

Overall, MSC-derived EVs have been evaluated for their therapeutic potential for the treatment of various diseases both *in vitro* and in animal models. Based on these results findings a number of clinical trials have begun to evaluate the therapeutic potential of MSC-derived EVs for the treatment of particular diseases and the procedure similar as in Figure 2. Using the key search words of ‘exosomes’ and ‘extracellular vesicles’ on the clinical trials website (https://clinicaltrials.gov/) reveals 172 and 51 registered clinical trials, respectively. Although some of these studies include MSC-derived EVs, very few clinical studies have been published. MSC-derived EVs have improved therapy-refractory graft-versus-host disease (GvHD) in patients [81]. These MSC-derived EVs were isolated from allogeneic MSC cultured medium and delivered to steroid-refractory GvHD patients in escalating doses. The clinical GvHD symptoms significantly declined shortly after the start of MSC-derived EVs treatment. The GvHD patients were stable and had no side effects. Another clinical trial displayed efficacy outcomes using EVs derived from umbilical cord MSCs to treat chronic kidney disease [101]. These results demonstrated that MSC-derived EVs could safely improve the inflammatory immune reaction and overall kidney function in chronic kidney disease patients through MSC EV administration in two doses, the first intravenous and second intra-arterial.

Based on the preclinical and clinical studies, human MSC-derived EVs are considered as promising products in regenerative medicine and tissue engineering. Many studies have compared the beneficial effects of MSCs and MSC-derived EVs and showed that they had similar therapeutic outcomes. This indicates that MSC-derived EVs possess the same therapeutic potential as MSCs. The use of MSC-derived EVs might serve as an alternative, cell-free therapy over MSC transplantation for tissue regeneration [82] and have “off-the – shelf” therapeutic potential. Furthermore, clinical applications of MSC-derived EVs are advantageous over MSC cell-based therapy, as they have lower immunogenicity, capacity to cross biological barriers and less safety concerns, such as the possibility of MSC

differentiation or tumour generation [89, 102, 103]. The preclinical results using MSC EVs in tissue engineering have given exciting promise to their use as powerful tools as therapies to tackle a wide a range of unmet disease burden.

Despite the progress in the field, EV isolation method may yield different EV subtypes as they co-exist but may differ in their functional properties [104]. The heterogeneity of MSCs which include tri-, bi, and uni-potent populations [105] need to be addressed as they may impact on therapeutic outcomes of trials using EVs derived from different MSC populations. It should also be noted that some of the clinical studies have been terminated without publication. Furthermore, some experiments have demonstrated better results when using MSCs and MSC-derived EVs together, compared to the cells or EVs alone [91, 99]. Other considerations, include the dose requirement, as some studies required higher doses of EVs or multi-dose injections to achieve significant therapeutic outcome [91, 101]. Another short-coming is the half-life of EVs. Cellular therapies using MSCs are able to continuously release the beneficial paracrine factors (including EVs), while EVs have a relatively short half-life and therefore might be unable to retain sufficient levels present at the defect region [104]. However, this drawback might be offset by using alternate delivery methods such as bioengineered scaffolds, such as PEI, encapsulation with PEG hydrogels or gelMA to maintain the sustainedly release of the MSC-derived EVs [98-100]. These bioengineering techniques for EV delivery might open up new avenues for therapeutic application.

Along with rapid development of the EV field, MSC-derived EVs have gained significant attention for their use in regenerative medicine. MSC-derived EVs bearing proteins, lipids and RNAs could impact the target cells to exert their therapeutic effects. The cellular fate of EVs is still not well understood [26] and many questions of MSC-derived EV biodistribution are unanswered. Furthermore, the therapeutic mechanism of MSC-derived EVs still remains elusive [107]. Many MSC-derived EV studies *in vitro* and *in vivo* have verified that they are capable of enhancing tissue repair and mediating regeneration in various diseases and enhancing therapeutic outcomes. MSC-derived EVs have the theoretical advantages of being a safer regenerative tool when compared to cell-based therapies. However, we are in the early stage of using MSC-derived EVs in regenerative medicine. Standardised techniques for culture conditions and large-scale culturing, effective isolation, optimal dosing and safe storage need to be methodically determined before large scale clinical applications. We believe that MSC-derived EVs hold great promise in cell-free therapy, with the potential to be applied in a wide range of diseases.

Conflict of interest and funding

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Publication 3

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