Regenerative effects of mesenchymal stem cell-derived exosomes

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Cover illustration: Left: MSC adherent on exosome-immobilised titanium surface, image by Furqan Ali Shah. Right: MSC internalised PKH67-labelled exosomes (green), image by Xiaoqin Wang.

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To my beloved family

不忘初心，方得始终

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ABSTRACT

Mesenchymal stem cells (MSCs) play pivotal roles for bone regeneration by virtue of their osteogenic differentiation ability and immunomodulatory capacity. Recently, secretion of exosomes/extracellular vesicles (EVs) has been suggested as a new mechanism of MSC-based therapy. MSC-derived EVs/exosomes have shown promising effects in tissue regeneration and immunomodulation, which are attributed to their regulatory effects in various processes. The overall objective of this thesis was to explore the cell-to-cell communication and cell-to-material surface interaction mediated by MSC-derived EVs/exosomes. The emphasis was placed on their functions in the regeneration capacity of MSCs and the determination of the microRNA and protein contents of these EVs/exosomes in order to obtain an insight into the underlying mechanisms of the EV-/exosome-mediated biological effects.

The results demonstrated that exosomes secreted from MSCs in the mid and late stage of osteogenic differentiation induced osteogenic lineage commitment, but only exosomes from the late differentiation induced the mineralisation of the extracellular matrix. MSC-derived exosomes were internalised by a subpopulation of homotypic cells. The differentially expressed microRNAs were osteogenesis related and predicted to enrich pathways involved in the regulation of osteogenic differentiation and general mechanisms by which exosomes exert their functions. *In vitro* ageing increased the secretion of EVs and in some contexts altered the protein profiles of EVs. The top abundant proteins in high passage (HP, “aged”) and low passage (LP, “young”) EVs shared similar but not identical functional features with an overlap of the enriched pathways related to endocytosis and regulation of cell proliferation and survival. The differentially expressed proteins in HP EVs were predicted to enrich GO biological process related to transport and secretion. Both HP and LP EVs promoted MSC proliferation in autocrine and paracrine manners and in a dose-dependent fashion. In contrast to MSC-

derived exosomes in suspension, exosomes which were immobilised on titanium (Ti) surfaces accelerated and increased MSC adhesion, influenced the early morphology and promoted the growth of MSCs on titanium. Proteomic analysis of the exosomal protein revealed identified proteins with predicted GO molecular function related to adhesion, structure and morphology, and growth factor and growth factor receptor activity.

In conclusion, MSC-derived EVs/exosomes possess regenerative effects, in terms of the stimulation of the proliferation and osteogenic differentiation of MSCs, and influence the behaviour of MSCs on titanium surfaces. The expression of exosomal cargoes is altered during osteogenic differentiation and *in vitro* ageing and their predicted functions partially correspond to the observed effects. It is suggested that the MSC-derived EV-/exosome-mediated effects on the regeneration capacity of MSCs are at least partially attributed to the transfer of functional exosomal cargoes.

Keywords: aging, cell adhesion, cell-material interaction, exosomes, extracellular vesicles, mesenchymal stem cells, osteogenic differentiation, proliferation, regeneration, titanium

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Sammanfattning på svenska

Mesenkymala stamceller (MSC) är en typ av adulta stamceller som är viktiga för benregeneration. MSC kan både differentiera till benceller samt påverka andra celler immunologiskt och på så vis påverka benregeneration. Exosomer är en typ av extracellulära vesiklar (EV) som frisätts från celler till den extracellulära miljön och som kan fungera som budbärare mellan celler. De kan interagera med celler i närmiljön eller transporteras i blodet till mer distala celler. EV/exosomer frisätts från de flesta, om inte alla, celler och deras innehåll och funktion korrelerar med vilken celltyp och under vilket stimuli/miljö de frisätts. MSC exosomer har i olika studier bland annat visats påverka olika regenerationsprocesser som benläkning, hjärtregeneration och njurregeneration.

Syftet med den här avhandlingen var att utforska EV/exosomer från MSC med fokus på deras funktion vid regeneration samt deras protein- och mikroRNA-innehåll. Vidare avsåg vi att studera deras roll vid cell-cell kommunikation samt cell-material interaktion. Vi har undersökt detta närmare i tre olika studier. I den första studien studerade vi exosomer/EV som frisatts från MSC när dessa differentieras till osteoblaster. Vi undersökte exosomer/EV närmare vid tre olika stadier; expansion, tidig och sen differentiering. Studien visade att exosomer/EV frisätts under hela differenteringsprocessen. EV/exosomer från differentierade celler kan påverka andra MSC att börja differentiera till osteoblaster men endast när exosomer/EV kommer från sent differentierade celler kan de påverka andra celler att mineralisera den extracellulära matrisen. Vi kunde också identifiera osteogenrelaterade mikroRNA i EV/exosomer som åtminstone delvis kan förklara effekten inducerad av EV/exosomer.

I den andra studien undersökte vi hur MSC frisätter EV/exosomer när de åldras och deras funktion på både ”unga” och ”gamla” MSC. För att undersöka detta använde vi oss av en *in vitro* modell där MSC odlades från låga passager (LP, ”unga” celler) och höga passager (HP, ”gamla” celler). Vi kunde visa att ”gamla”, HP MSC frisätter mer EV/exosomer jämfört med ”unga”, LP MSC. Båda typerna av vesiklar kunde öka proliferation och överlevnad av både ”unga” och gamla” celler. Vidare så kunde vi detektera nästan 2000 olika proteiner i de olika typerna av EV, varav många hade med cellproliferation och överlevnad att göra.

I den tredje studien fäste vi EV/exosomer till titanytor och studerade *in vitro* hur exosomer fästa till ytor påverkar MSC med avseende på celladhesion, proliferation och differentiering. Vidare jämförde vi det med EV/exosomer som tillsatts direkt till cellodlingsmediet eller med ytor utan några EV/exosomer. För detta använde vi oss av två grupper av exosomer/EV; isolerade från MSC som prolifererar samt från MSC som differentierar. Vi kunde visa att titanytor med ytbundna exosomer stimulerade adhesionen av MSC, påverkade cellernas morfologi och ökade cellernas proliferation. Med hjälp av proteomik kunde proteiner detekteras i exosomerna som är av betydelse för celladhesion, morfologi och tillväxt.

Sammanfattningsvis har denna avhandling visat att MSC bildar och frisätter EV/exosomer med regenerativa effekter vad avser stimulering av proliferation och osteogen differentiering av MSC samt påverkan av MSC på titanytor. Exosomernas innehåll förändras under osteogen differentiering och åldrande *in vitro* och deras förutsagda funktioner motsvarar delvis de observerade cellulära effekterna.

LIST OF PAPERS

This thesis is based on the following studies, referred to in the text by their Roman numerals.

I. Wang X, Omar O, Vazirisani F, Thomsen P, Ekström K.

Mesenchymal stem cell-derived exosomes have altered microRNA profiles and induce osteogenic differentiation depending on the stage of differentiation.

PLoS One. 2018; 13(2): e0193059.

II. Wang X, Philip J, Vazirisani F, Tsirigoti C, Thomsen P, Ekström K.

The impact of *in vitro* aging on the release of extracellular vesicles from human mesenchymal stem cells.

In manuscript.

III. Wang X, Shah FA, Vazirisani F, Johansson A, Palmquist A, Omar O, Ekström K, Thomsen P.

Exosomes influence the behavior of human mesenchymal stem cells on titanium surfaces.

Submitted for publication.

List of papers not included in the thesis

I. Ekström K, Omar O, Granéli C, Wang X, Vazirisani F, Thomsen P.

Monocyte exosomes stimulate the osteogenic gene expression of mesenchymal stem cells.

PLoS One. 2013;8(9): e75227.

II. Nawaz M, Camussi G, Valadi H, Nazarenko I, Ekström K, Wang X, Principe S, Shah N, Ashraf NM, Fatima F, Neder L, Kislinger T.

The emerging role of extracellular vesicles as biomarkers for urogenital cancers.

Nat Rev Urol. 2014;11(12): 688 - 701. Review.

Contents

Abbreviations

Aβ	β-amyloid
AD	Alzheimer disease
AFM	atomic force microscopy
Ago2	argonaute-2
AIH	autoimmune hepatitis
AKI	acute kidney injury
ALIX	apoptosis-linked gene 2-interacting protein X
ALP	alkaline phosphatase
ALT	alanine transaminase
AR	androgen receptor
ARF6	ADP ribosylation factor 6
AST	aspartate aminotransferase
AT-MSC	adipose tissue-derived MSCs
ATP	adenosine triphosphate
β-TCP	tricalcium phosphate
BCA	bicinchoninic acid
(b)FGF	(basic) fibroblast growth factor
BGM	basal growth media
BM-MSC	bone marrow derived MSC
BMP-2	bone morphogenetic protein-2
c-JNK	c-Jun N-terminal kinases
CCI4	carbon tetrachloride
CCK-8	Cell counting kit-8
CDC42	cell division cycle 42
CM	conditioned media
CP-MSC	chorionic plate-derived MSC
cryo-EM	cryo-electron microscopy
CTGF	connective tissue growth factor
CXCR4	C-X-C chemokine receptor type 4
DAPI	4′,6-diamidino-2-phenylindole
DC	dendritic cell
DLL4	delta-like 4
DLS	dynamic light scattering
DMEM-LG	Dulbecco’s modified Eagle’s medium with low glucose

DRP1	dynamin-related protein 1
ECM	extracellular matrix
EFNA3	Ephrin A3
EGFR	epidermal growth factor receptor
EMT	epithelia-to-mesenchymal transition
ER	endoplasmic reticulum
ESCRT	endosomal sorting complex required for transport
EV	extracellular vesicle
FBS	fetal bovine serum
FZD	Frizzled
GA	glioma-associated
GF	growth factor
GFR	growth factor receptor
GO	gene ontology
Grp 94	glucose-regulated protein 94
GSC	glioma stem-like cell
GSH	glutathione
GSK	glycogen synthase kinase
GVHD	graft-versus-host disease
(h)FN	(human) fibronectin
HGF	hepatocyte growth factor
Hh	hedgehog
HIF-1α	hypoxia inducible factor-1α
HP	high passage
hPBMC	human peripheral blood mononuclear cell
HRS/HGS	hepatocyte growth factor-regulated tyrosine kinase substrate
HSC	hematopoietic stem cells
HSC70 (Hsp70)	heat shock protein 70
HUVEC	human umbilical vein endothelial cell
ICAM	intracellular adhesion molecule
IFNγ	interferon gamma
IGF/IGFR	insulin-like growth factor/insulin-like growth factor receptor
IL/ILR	interleukin/interleukin receptor
ILV	intraluminal vesicles
IPA	Ingenuity pathway analysis

IR	ischaemia reperfusion
IRAK1	interleukin 1 receptor associated kinase 1
LDH	lactate dehydrogenase
LP	low passage
LRP	low-density lipoprotein receptor-related protein
LUAD	lung adenocarcinoma
M-PER	mammalian protein extraction reagent
MAPK	mitogen-activated protein kinase
MCAo	middle cerebral artery occlusion
MCP-1	monocyte chemoattractant protein-1
MDA	malondialdehyde
MFG-E8	milk fat globule epidermal growth factor 8
MHC	major histocompatibility complex
MI	myocardial infarction
MPO	myeloperoxidase
MSC	mesenchymal stem cell
mTOR	mammalian target of rapamycin
MVB	multivesicular body
Myo1e	myosin 1E
NCOR1	nuclear receptor co-repressor 1
NEP	neprilysin
NF-κB	nuclear factor-κB
nLC-MS	nano-liquid chromatography coupled to an Orbitrap mass spectrometer
NLRP3	NACHT, LRR and PYD domains containing protein 3
nSMase	neutral sphingomyelinase
NTA	nanoparticle tracking analysis
OCPC	ortho-cresolphthalein complexone
ODM	osteogenic differentiation media
OPG	osteoprotegerin
PBS	phosphate buffer saline
PD(T)	population doubling (time)
PDGF	platelet-derived growth factor
PGE2	prostaglandin E2
PI3K/Akt	phosphatidylinositol 3-kinase/protein kinase B
PLD2	phospholipase D2
PM	plasma membrane

pre-microRNA	precursor microRNA
PS	phosphatidylserine
PTEN	phosphatase and tensin homolog
qPCR	quantitative polymerase chain reaction
Rab (RAB)	Ras-related proteins in brain
RhoA	ras homolog gene family member A
RNPs	ribonucleoproteins
RT	room temperature
RUNX2	runt-related transcription factor 2
SASP	senescence-associated secretory phenotype
SDF-1α	stromal cell-derived factor 1α
SEC	size exclusion chromatography
SEM	scanning electron microscopy; standard error of mean
SM	sphingomyelin
SOD	superoxide dismutase
STAB2	stabilin 2
STAM	signal transducing adaptor molecule
STAT3	signal transducer and activator of transcription 3
Stau	staufen
TCPS	tissue culture treated polystyrene
TEM	transmission electron microscopy
TGFβ	transforming growth factor beta
Ti	titanium
TLR	toll-like receptor
TMT	tandem mass tag
TNF-α	tumour necrosis factor-α
tRNA	transfer RNA
TRPS	tunable resistive pulse sensing
Tsg101	tumor susceptibility gene 101 protein
UC	umbilical cord
VEGF(R)	vascular endothelial growth factor (receptor)
VPS4	vacuolar protein sorting-associated protein 4
VSMC	vascular smooth-muscle cell

1 Introduction

1.1 Extracellular vesicles/exosomes

Extracellular vesicles (EVs) are particles that are naturally released from the cell and delimited by a lipid bilayer, containing cytosol from the secreting cells, but not containing a functional nucleus, indicating the lack of ability to replicate [1]. The secretion of EVs appears to be a conserved process throughout evolution, as both eukaryotic and prokaryotic cells have been reported to release vesicles into the extracellular space. However, in the present thesis, the term "EVs" only refers to vesicles secreted by eukaryotic cells. An early evidence of the presence of EVs was reported in 1969, in which membrane-enclosed vesicles, named as "matrix vesicles", were found to locate in the matrix of cartilage and to be associated with calcification [2]. For decades, EVs were found in different biological fluids and secreted by various of mammalian cells. Nevertheless, these vesicles were initially assumed to be secreted by the outward budding of the plasma membrane (PM) of cells. In 1983, another more complicated EV secretion pathway was demonstrated by Harding *et al.* [3] and Pan and Johnstone [4], in which vesicles were formed in the intracellularly endosome pathway, particularly in multivesicular bodies (MVBs), and secreted by the fusion of MVBs with the PM, resulting in the release of intraluminal vesicles (ILV) into the extracellular space. Thereafter, the term "exosome" was proposed by Johnstone *et al.* in 1987 to describe the released vesicles of endosomal origin [5]. However, for a long time, exosomes were assumed to function as a "waste bin" for cells to dispose of unwanted components. A great deal of attention was then paid to another breakthrough in 2007, showing that exosomes, containing mRNA and microRNA, mediated cell-to-cell communication via the transfer of genetic material [6]. As of today, EVs/exosomes research is growing exponentially and a huge number of studies have been published.

1.1.1 Classification and nomenclature

EVs are heterogeneous because of their diverse origin, nature and features. Over the years, EVs have been classified in different ways and assigned various names based on size, biogenesis, cell origin or function. Currently, a commonly accepted way to classify EVs is based on their biogenesis, in which EVs are categorised into three broad classes: exosomes, microvesicles/

ectosomes and apoptotic bodies [7]. Apoptotic bodies are vesicles formed during apoptosis when the cell cytoskeleton breaks and the PM bulges outwards. Apoptotic bodies contain parts of a dying cell and consist of vesicles with the most heterogeneous diameter size ranging from 200 nm to 5 μm [7, 8]. Microvesicles/ectosomes are thought to be formed by the outward budding of the PM of viable cells, in a size range of 100 nm to 800 nm [9]. Exosomes are of endosomal origin and are thought to have the smallest size range (30 nm- 150 nm) among all three groups of EVs. However, different classifications have been proposed as a result of the knowledge that has been accumulated on the diversity of EVs. This is illustrated by the overlap of size and density among different subpopulations of EVs and the much more diverse morphology of EVs than previously observed [10, 11], as well as the challenge encountered in isolating a pure subpopulation of EVs based on the currently available techniques.

Along with the development of the EV research field, more and more evidence shows the overlaps between different subgroups of EVs. A generic means of classification and nomenclature has therefore recently been suggested [12]. EVs can be classified based on their physical characteristics, such as size, density and morphology, or based on their biochemical composition, for example, positive for a specific molecule, or referring to the secretion conditions or cell origin. The following are two examples using the recently proposed generic means of classification and nomenclature [13]. When applying size as the criterion for classification, the use of the generic terms small EVs and middle/large EVs for vesicles of < 200 nm and > 200 nm respectively is suggested. Another alternative way is to classify EVs based on their density, in which vesicles are grouped into low-, middle- and high-density EVs, with each density range defined.

The development of EV research has highlighted some limitations of the current classification and nomenclature and has revealed the need for a consistent classification and nomenclature for exchanging information within the field and communicating with other research fields. Debates and discussions on these issues, i.e., nomenclature, are on-going and efforts to provide new and better technical solutions are emerging. Although different operational terms for EVs have been suggested, “exosome” is still one of the most commonly used terms in the current literature. However, due to the difficulty involved in obtaining solid evidence of the endosomal origin of studied vesicles in reality, the term “exosome” is often used in a less strict

manner compared with the initial definition. Indeed, some researchers classified the subpopulation of microvesicles, which is indistinguishable from exosomes, i.e., sharing a similar size and density, enriching classical markers such as CD63 and CD81, and the formation involved the tumour susceptibility gene 101 protein (TSG101) and the vacuolar protein sorting-associated protein 4 (VPS4), components of the endosomal sorting complex required for transport (ESCRT) machinery, as exosomes [14, 15]. In the present thesis, the term "EVs/exosomes" is used to refer to an exosome-enriched vesicle preparation, isolated using high-speed ultracentrifugation or a commercial chromatography isolation kit, Exospin, and characterised by applying complementary techniques.

1.1.2 Biogenesis of EVs/exosomes

The heterogeneity of EVs reflects the complexity of EVs biogenesis and the existence of various mechanisms and pathways regulating the formation and release of EVs. As previously mentioned, the theory of the direct outward budding of the PM of cells as the main mode for EVs secretion was replaced by the finding that the endosomal pathway was demonstrated for the formation of MVB, in which ILVs were formed by the inward budding of the endosomal membrane and subsequently exocytosed into the extracellular environment as exosomes [2, 4, 5, 16]. For a long time, EVs budded directly from the PM and formed via the endosomal pathway were considered to be regulated by distinguishable machineries. However, growing evidence shows that the same machineries can play similar roles either at the PM for direct budding or in the intracellular endosomal compartments for the biogenesis of exosomes [15, 17-21]. These observations indicate that similar yet not identical machineries exist to regulate the biogenesis of different subpopulations of EVs/exosomes.

Although the biogenesis of EVs and involved mechanisms are as yet incompletely understood, current knowledge has implicated ESCRT-dependent and ESCRT-independent mechanisms (Figure 1). Upon endocytosis, early endosomes, with tubular extensions, form intracellularly. Following maturation, ILVs are assembled by the inward budding of late endosomal membrane to form MVB. The most described mechanism in driving the formation of ILVs and MVB is ESCRT-dependent machinery. The human ESCRT consists of 33 proteins which are assembled into four complexes, ESCRT-0, ESCRT-I, ESCRT-II and ESCRT-III, with associated proteins VPS4 complex and Bro1 domain proteins including apoptosis-linked

gene 2-interacting protein X (ALIX) [22]. The four complexes are numbered according to the order in which they act in the pathway and play distinct roles. ESCRT-0, consisting of hepatocyte growth factor-regulated tyrosine kinase substrate (HRS, also known as HGS) and signal transducing adaptor molecule (STAM), together with clathrin, recognise and sequester ubiquitinated transmembrane proteins in the endosomal membrane. Following the recruitment of TSG101 of the ESCRT-I complex by HRS, the ESCRT-II complex is recruited via ESCRT-I and together initiate the local budding of the endosomal membrane with sorted cargo. ESCRT-III participates in protein deubiquitination and subsequently drives vesicle scission [1, 9, 22]. Thereafter, MVBs, loading with ILVs, are formed and undergo either fusion with lysosomes, resulting in the discharge and digestion of their ILVs in the lumen of lysosomes, or fusion with the PM, leading to the release of exosomes into the extracellular space.

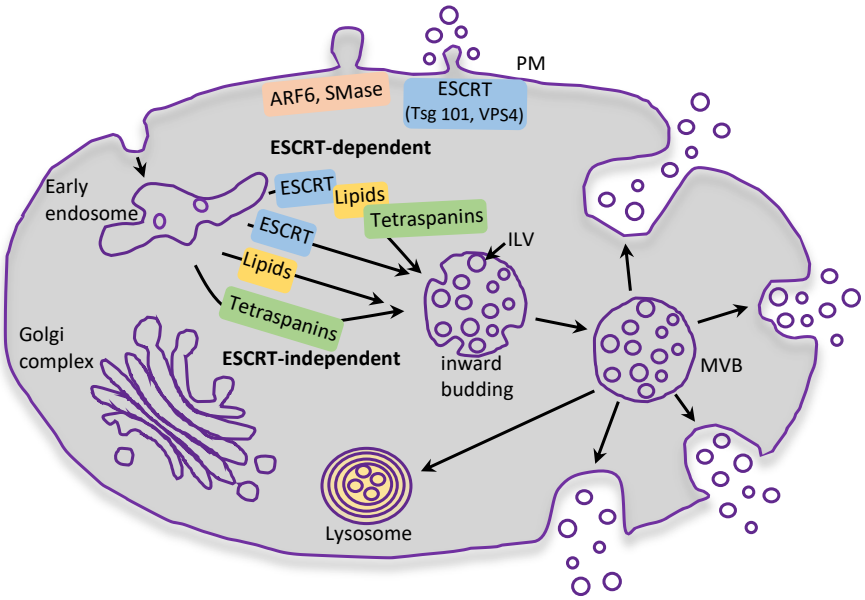


Figure 1. Biogenesis of EVs/exosomes. The biogenesis of exosomes and PM-derived EVs is regulated by both ESCRT-dependent and ESCRT-independent mechanisms. (Figure inspired from [1])

An increasing number of studies have provided a comprehensive overview on the role of individual ESCRT proteins in exosome biogenesis and secretion. RNA interference in HeLa cells to silence ESCRT-0 genes, HGS and STAM1,

or ESCRT-I gene TSG101 decreases exosome secretion [18]. Partially in line with this result, silencing of TSG101in MCF-7 breast tumour cells showed to decrease exosomes secretion [23]. However, contradictory results have been reported when silencing other ESCRT genes in different cell types. Baietti *et al.* showed that the depletion of ALIX or VPS4B impaired exosome secretion in MCF-7 cells [23], whereas Colombo *et al.* reported that the exosome secretion was increased by the depletion of ALIX or VPS4B in HeLa cells [18]. Together, these findings indicate the complexity and heterogeneity of mechanisms regulating EV/exosome secretion, which may also be partially contributed by the differences of parental cell types. Moreover, the study demonstrated that the ESCRT machinery not only regulates the amount of exosomes released but is also involved in the exosomal cargo loading [18, 23]. This is supported by the observation of reduced amounts of CD63 and major histocompatibility complex (MHC) class II in exosomes secreted by TSG101 or STAM1 knockdown HeLa cells [18], as well as reduced CD63 in exosomes secreted by TSG101 or ALIX knockdown MCF7 cells [23]. In addition to the regulation of exosome formation through the endocytotic pathway, ESCRT machinery, particularly the TSG101 and VPS4 components, was also involved in the formation of EVs directly budding from the PM. The interaction of TSG101 with a specific (protein) sequence, the PSAP sequence present in the gag protein of retroviruses [15] or arrestin domain-containing protein 1 [24], induced the budding of EVs at the PM.

In addition, ESCRT-independent machinery mediated by lipids, tetraspanins and small GTPase has been implicated in the regulation of EV/exosome biogenesis. The lipid metabolism enzyme, neutral sphingomyelinase (nSMase), has been shown to hydrolyse sphingomyelin (SM) into ceramide, which was required for the transfer of exosome-associated domains into the lumen of endosomes [25]. The inhibitor of nSMase, GW4869, reduced the secretion of proteolipid protein-bearing exosome [25] and the exosomal protein, flotillin 1 [26], as well as exosomal microRNA [27]. Similarly, phosphatidic acid, hydrolysed by phospholipase D2 (PLD2), was also shown to play a role in inward budding and increase of exosome secretion [19, 28]. Various members of tetraspanins, CD63, CD9 and CD82, have also been shown to regulate exosomes formation and secretion [26, 29]. The overexpression of CD9 or CD82 induces the secretion of β-catenin-bearing exosomes [26]. Some evidence also suggests the roles of small GTPase Ras-related proteins in brain (Rab) proteins, RAB11, RAB35, RAB7 and RAB27A/B, in endosome maturation and exosome secretion (reviewed by Stenmark) [30]. In details,

RAB11 and RAB35 were mainly associated with recycling and early endosomes respectively, whereas RAB7 and RAB27 were associated with late endosomal and secretory compartments. In comparison to exosome biogenesis, PM-derived EVs were also shown to be regulated by SMase [17] and small GTPase ADP ribosylation factor 6 (ARF6) [20]. The overexpression of ARF6 depolymerised the actin cytoskeleton and permitted the efficient release of PM-derived EVs. Interestingly, ARF6, together with its effector, PLD2, affected the budding of ILVs into MVBs, suggesting that ARF6 is also involved in the regulation of exosomes formation [19].

1.1.3 Molecular composition of EVs/exosomes

The molecular composition of EVs/exosomes has been studied using different approaches. Comprehensive data have been collected in database such as EVpedia (http://evpedia.info) and Vesiclepedia (http://microvesicles.org/). The contents of EVs/exosomes often reflect their parental cell sources and are influenced by their secretion conditions. In general, EVs/exosomes contain lipids, proteins and nucleic acids (Figure 2).

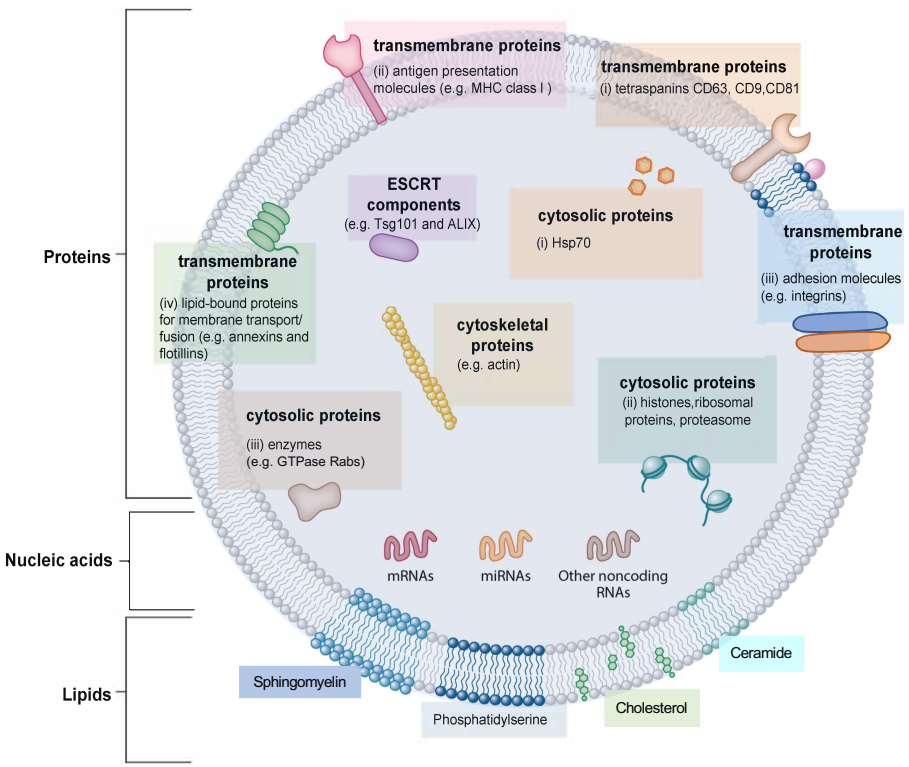


Figure 2. Molecular composition of EVs/exosomes. EVs/exosomes enclosed by lipid bilayer contain lipids, proteins and nucleic acids. The main specifics/families of lipids, proteins and nucleic acids that have been detected in EVs/exosomes are presented in the figure. (Figure adapted from [1])

The lipid composition of EVs has not been studied as much as the protein contents. Nevertheless, it has been reported that, in comparison with the total cell membrane, EVs/exosomes are enriched with several lipid species, including cholesterol, SM, phosphatidylserine (PS) and saturated fatty acids [18, 31]. Moreover, a recent study exploring the lipidomes of exosomes derived from three different cell types, Huh7 hepatocellular carcinoma cells, U87 glioblastoma cells and mesenchymal stem cells (MSCs), revealed a similarity in the lipidomes between Huh7 and MSC exosomes, but not U87 exosome [32]. A comparison of the lipidomes of two subpopulations of EVs showed the enrichment of different lipid species [32]. These observations hint at a mechanism that sorts selected lipid species into vesicles, which may be dependent on both cell origin and vesicle types. Such hypothesis is also supported by significantly different levels of specific lipids in urinary exosomes from prostate cancer patients and healthy donors [33]. On the basis of the features of the enriched lipids, it was proposed that the lipid composition contributes to the stability and structural rigidity of vesicles [31]. On the other hand, some studies have demonstrated that the lipids and lipid metabolism play important roles in the regulation of the biogenesis of both endosomal-origin exosomes and PM-derived EVs [17, 19, 25, 28, 34].

The protein contents of EVs have been studied using the antibody-based detection of specific proteins initially [3], while the high-throughput proteomic technique has enabled the large-scale identification of the global proteome of EVs. Early proteomic studies showed that both cell type-dependent and -independent proteins were detected in exosomes. The cell type-independent proteins often came from specific subcellular compartments, including the PM, cytosol and endosomes, and rarely from the nucleus, mitochondria, endoplasmic reticulum (ER) and Golgi apparatus [35, 36]. These proteins were therefore defined as common markers for exosomes, whereas proteins from compartments such as the ER often served as negative markers for exosomes. The typical enriched exosomal proteins, irrespective of cell origin, include transmembrane proteins such as (i) tetraspanins CD63, CD9, CD81 and CD82, (ii) integrins, (iii) proteins binding to the lipid raft such as flotillin and annexins; cytoskeleton proteins such as actin; the protein components of ESCRT machinery, i.e. TSG101 and ALIX; cytosolic proteins such as heat

shock 70-kDa protein (HSC70) and proteasome; and GTPase Rabs (reviewed in [1, 37]). However, as the cargo-sorting mechanism for different subpopulations of EVs is not yet fully understood, it is not clear whether these enriched exosomal proteins, often serving as markers, are specifically detected in exosomes or whether they are also present in another subpopulation of EVs, i.e. PM-derived EVs. Theoretically, proteins located in the cell membrane or cytosol may also have the chance to be sorted into PM-derived EVs. Indeed, a recent study showed that classical markers for exosomes, such as MHC, flotillin and HSC70, were similarly present in all the studied EVs, indicating the non-specificity of these proteins among subtypes of EVs [38]. Moreover, through a comprehensive comparison of the proteome of EVs recovered by different centrifugation speeds, density gradients and antitetraspanin-coated beads, the authors proposed syntein-1, TSG101 and tetraspanins CD63, CD81 and CD9 as a new panel of specific markers for small EVs, including exosomes [38]. In addition to the cell type-independent proteins, efforts have also been made to compare the protein contents of EVs secreted by different cell types [32, 38] or under various conditions, i.e. pathological or healthy conditions [39, 40], to unravel the specific proteins enriched in EVs that are reflective of cell origin. Recently, a new technique, the multiplex proximity extension assay, has been reported to be able to identify the cellular origin of exosomes recovered from different cell lines and body fluids [41]. Taken as a whole, the development of knowledge and techniques will enable us to acquire a better understanding of the EV biology and application of EVs, i.e. as biomarkers of disease.

Since the first evidence showing that exosomes contain RNA, particularly microRNA and mRNA [6], the RNA contents of EVs have been intensively studied. In addition to microRNA, several other species of non-coding RNAs have been identified in EVs/exosomes, including vault-RNA, Y-RNA and specific transfer RNA (tRNA) [42]. However, it was confirmed that ribosomal RNA was almost absent in EVs/exosomes [43]. The detected RNAs were shown to be resistant to RNase digestion, indicating their intravesical location [6]. Among these detected RNAs, microRNA has been attracted the most attention. A growing body of studies have suggested that microRNA carried via EVs/exosomes as one of the most important populations of extracellular circulating microRNAs, which is involved in the regulation of both physiological and pathological processes [44, 45]. Furthermore, altered microRNA profiles have been found in EVs/exosomes secreted from various differentiation stages of dendritic cells [46] and MSCs [47]. Another study

found that sumoylated hnRNPA2B1 mediated the targeting of microRNA to be sorted in exosomes [48]. These observations suggest the existence of specific sorting mechanisms for RNA cargo in EVs/exosomes, which requires further comprehensive investigation. Interestingly, a few studies have found the presence of DNA, both genomic and mitochondrial DNA, in EVs/exosomes [49-51]. However, a great deal of information is still lacking, i.e. (i) whether DNA is presented in bona fide exosomes or its presence is due to the co-isolation of other subtypes of EVs such as apoptotic bodies that are well-known to contain DNA; (ii) whether DNA is present inside EVs or on the surface of EVs and (iii) whether DNA is specifically sorted inside EVs, like other EVs constituents.

1.1.4 Isolation of EVs/exosomes

EVs/exosomes can be isolated using various techniques, including differential ultracentrifugation, filtration, size exclusion chromatography (SEC), precipitation and immunoaffinity capture (reviewed in [52, 53]). There is no one-size-fits-all approach. The selection of an isolation method therefore depends on the properties of the starting material (i.e. cell culture media or biological fluid, volume of the material), the aims of the study and the interested downstream analyses.

Differential ultracentrifugation, with or without a further purification step by extra washing or density gradient depending on the requirement of sample purity, is the most commonly used isolation method [54]. On the basis that vesicles with different sizes and density are sedimented by different centrifugation speeds, a low centrifugation speed (i.e. 300xg, 500xg or 2,000xg) is first applied to remove dead cells and cell debris. Following a medium-speed centrifugation (i.e. 10,000xg-20,000xg) to eliminate large vesicles, small vesicles are finally pelleted using high-speed centrifugation (i.e. 100,000xg or 120,000xg) [55]. Traditionally, various subpopulations of vesicles are isolated by different centrifugation steps. Large vesicles like apoptotic bodies are pelleted at 2,000xg or 3,000xg, middle size vesicles, such as PM-derived microvesicles, are isolated at 10,000xg-20,000xg, whereas exosomes are pelleted at 120,000xg [43]. However, it is acknowledged that such separation may not be sufficient, due to the overlapping of vesicle size among different subpopulations of EVs, as well as the existence of vesicles of the same size but different densities [7, 56]. Moreover, non-vesicle structures such as protein aggregates may be co-isolated. One solution partially to solve

these problems is to apply a density gradient. The total EV samples can be sedimented in iodixanol gradients or floated in sucrose gradients and EVs sedimented at specific densities are collected for further study [38]. Similarly, the principle for isolation by filtration or SEC is based on the differences in vesicle size. SEC has been shown efficiently to eliminate soluble proteins, thereby reducing the non-vesicle contamination [57, 58]. Such non-pelleting procedure also avoids the possible damage or aggregate of vesicles, thereby preserving the integrity of vesicles. Commercially, water-excluding polymers such as polyethylene glycol (PEG) have been used for the isolation of EVs [59]. The crude samples are first incubated with precipitation solution and, following a low-speed centrifugation, the precipitate containing vesicles is collected. Isolation by precipitation is simple, with high recovery, but it often results in samples with poor purity. In comparison to the above-described methods, isolation based on immunoaffinity allows to capture vesicles with specific protein markers, such as CD63, CD81 and CD9 [38]. Nevertheless, all the existing methods generally have their pitfalls, necessitating the optimisation of current techniques and the development of new techniques. Indeed, a variety of innovative techniques, such as microfluidics device-based isolation (reviewed in [52]), ion-exchange chromatography [60], acoustics purification [61], asymmetrical flow field-flow fractionation (AF4) [62], novel immunoisolation [63] and isolation based on lipid affinity [64], has been applied on EV/exosome isolation. Although some of the methods may be not widely applied yet, the strategy to develop and combine different techniques will offer an opportunity for improving the isolation of EVs/exosomes.

1.1.5 Characterisation of EVs/exosomes

The characterisation of EVs/exosomes is encouraged to be performed by using multiple complementary techniques to determine different properties of EVs/exosomes, such as morphology, size, density and specific protein compositions. To visualise the morphology of vesicles, various microscopy techniques can be applied, including transmission electron microscopy (TEM), cryo-electron microscopy (cryo-EM), scanning electron microscopy (SEM) and atomic force microscopy (AFM) (reviewed in [53]). In addition to providing high-resolution morphology, TEM and SEM can be combined with immunogold labelling to detect specific surface proteins such as CD63 [47], while SEM is able to provide three-dimensional surface topology information [65, 66]. In comparison to TEM and SEM, which require extensive sample preparation before visualisation, cryo-EM enables the chemical fixation of the

samples to be avoided and thereby provides the opportunity to retain the natural state of vesicles. Through application of cryo-EM, a recent study unravelled a vast morphological diversity of an EV subpopulation sharing the same density [10]. Similarly, AFM enables direct visualisation without sample processing and both the surface topology and the local stiffness and adhesion properties of vesicles can be determined [66]. Despite the morphological information, high-resolution microscopy-based methods can be used to determine the size of vesicles, but in a less statistical manner. Dynamic light scattering (DLS), nanoparticle tracking analysis (NTA) and tunable resistive pulse sensing (TRPS) are methods that enable the quantification of vesicle size when in suspension. DLS measures bulk scattered light from vesicles undergoing continuous Brownian motion and determines the size of vesicles based on the scattered intensity [67]. NTA tracks individual vesicle scattering over time to collect information on particle velocity and diffusivity for the calculation of the vesicle size distribution [47, 68]. TRPS detects transient changes in the ionic current, generated by the transport of the vesicles through a size-tunable nanopore, as a result of which the size of vesicles is indicated [68]. Moreover, both NTA and TRPS are able to quantify the concentration of particles for calculation of the total particle number. It has been suggested that the ratio of different quantification methods (i.e. protein amount: particle number) is able partially to indicate the purity of vesicle samples [69]. The density of vesicles is often determined by sucrose or iodixanol gradient centrifugation.

Selected proteins are used as EVs/exosomes markers, which can be detected by antibody-based methods such as flow cytometry and Western blot. It is recommended to select proteins covering different categories of markers, including (i) transmembrane and/or endosomal proteins indicating the lipid-bilayer structure of EVs, such as tetraspanin CD63; (ii) cytosolic proteins, which are able to bind to membrane or to cytosolic sequences of transmembrane proteins, indicating the enclosure of intracellular materials, such as TSG101 and flotillin; (iii) proteins specific for other intracellular compartments, i.e. ER-specific protein glucose-regulated protein 94 (Grp94) [70], which are not enriched in exosomes, serving as negative markers to indicate the specificity of EV subtypes. To detect these specific proteins, Western blot is one of the most commonly used methods. Alternatively, flow cytometry can be applied to determine the surface phenotypic features of EVs in a high throughput manner [71]. Due to the limited sensitivity and resolution of conventional flow cytometry, EVs need to be bound with micrometre-sized

latex beads conjugated with antibodies such as CD63, CD81 and CD9 before detection. The pitfall of an approach like this is the lack of phenotypic signature of a single vesicle, as one bead may bind to multiple vesicles. Recently, advanced flow cytometry with increased resolution has been developed to enable single vesicle detection [72]. In addition to protein compositions, the determination of lipid content and refractive index is suggested for characterisation of EVs [73].

1.1.6 EV-/exosome-mediated cell-to-cell communication

Upon release, EVs/exosomes travel in the extracellular space and are able to target the adjacent cells, as well as distant cells. As the first evidence showing that exosomal mRNA can be transferred and translated in the recipient cells, the secretion of EVs/exosomes was suggested as a new route to mediate cell-to-cell communication [6]. To date, a growing body of studies has intensively investigated the intercellular communication mediated by EVs/exosomes and the subsequent consequence on the regulation of recipient cells. However, the specificity of targeting EVs/exosomes to particular recipient cells and the underlying mechanisms remain largely unknown. A previous study showed that PM-derived EVs from platelets transferred tissue factor to monocytes but not to neutrophils, indicating the preferred interaction with monocytes [74]. Whereas another two studies showed that PM-derived EVs from platelets and neutrophils resulted in different effects on the same recipient cells, macrophages [75, 76]. These observations indicate that the selection of targeting cells may depend on the cell origin of EVs, the subtypes of EVs, as well as the features of recipient cells, which together contribute to the complexity of routes for EVs to interact with cells.

A variety of routes have been suggested for EVs to interact with cells, including receptor/ligand binding, membrane fusion, internalisation via (i) clathrin-, or caveolin-mediated endocytosis, (ii) phagocytosis, and (iii) micropinocytosis (reviewed in [77]). Receptor/ligand binding may trigger the direct activation of specific signalling pathways in the recipient cells, resulting in rapid responses on regulation of recipient cells. Such hypothesis is partially supported by the discovery that exosomes carry tumour necrosis factor receptor 1 (TNFR1), which is capable of binding to tumour necrosis factor-α (TNF-α) [78]. Moreover, another study showed that the blocking of exosome internalisation by cytochalasin D did not inhibit the exosome-mediated secretion of interleukin-1β (IL-1β), whereas the blocking of surface

interaction mediated by exosomal fibronectin abrogated IL-1β production, together indicating that internalisation was not essential for these biological effects mediated by exosomes [79]. However, such activation mode may depend on the functional features of the receptor and/or ligand, as well as the quantity of receptor or ligand harbouring on the surface of EVs/exosomes.

In addition to direct activation, the other consequence of receptor/ligand binding is to dock EVs/exosomes on the PM of recipient cells and facilitate the subsequent membrane fusion or internalisation. Several transmembrane proteins such as tetraspanins and integrins have been shown to play important roles in the process of cellular uptake of exosomes. A previous study demonstrated that dendritic cells (DC) internalised exosomes and, moreover, the targeting of exosomes to DCs was mediated via milk fat globule epidermal growth factor 8 (MFG-E8), CD11a, CD54, PS and the tetraspanins CD9 and CD81 on the exosome, and αv/β3 integrin, and CD11a and CD54 on the DCs [80]. Fusion with the PM of recipient cells results in the direct release of the intravesical contents. The observation of membrane fusion was achieved by using a self-quenched fluorescent lipid probe R18 [46, 81] and the fusion efficiency was enhanced by low pH condition [81]. The endocytosis-mediated uptake also requires the fusion of EV/exosome membrane with the membrane of intracellular compartments, such as endocytic compartments, to enable the intraluminal compositions of EVs/exosomes to gain access to the cytosol of recipient cells. Although the detailed intracellular fate of EVs contents is not yet fully understood, various studies have shown biological effects induced by the exosomal contents, which indirectly confirm the intracellular release of EVs/exosomes contents. For example, exosomal mRNA was translated in the recipient cells [6] and exosomal microRNA regulated the target gene expression in the recipient cells [46, 82]. Furthermore, a recent study showed that both epidermal growth factor receptor (EGFR) and androgen receptor (AR) can be transferred via EVs to the nucleus of recipient cells and the transported exogenous EGFR and AR were active and able to stimulate the nuclear pathways, respectively [83].

Due to the complexity of the *in vivo* environment and the fact that almost all cell types secrete EVs/exosomes which are detected in blood and other various biological fluids, it is difficult to determine the fates of EVs/exosomes *in vivo*. Nevertheless, it is crucial to evaluate the biological effects of EVs/exosomes *in vivo* for their further application. Although EVs/exosomes have been injected into animals, due to technical limitations, the direct and accurate

tracking of the cellular internalisation and organ distribution of EVs/exosomes *in vivo* has been difficult to achieve. Some efforts have been made to tackle this challenge. For example, melanoma cells transfected with plasmid-expressing fusion protein consisting of luciferase and MFG-E8 were used to produce exosomes with luciferase activity [84]. These exosomes were injected intravenously and detected in the blood circulation four hours later, after which they were distributed to the liver and lungs. Another study utilised the Cre-LoxP system to induce a colour switch in recipient cells that expressed reporters when they internalised EVs released from cells that expressed Cre recombinase and, as a result, the release and internalisation of EVs *in vivo* were visualised [85].

1.2 Mesenchymal stem cells (MSCs)

1.2.1 Historical background of MSCs

The discovery of MSCs began in the 1960s when Friedenstein showed for the first time that the transplantation of bone marrow fragments or bone marrow cell suspension formed new bone and new bone formation required a certain density of bone marrow cells [86]. These results indicated an osteogenic potential in bone marrow, which may be driven by a subpopulation of bone marrow cells. It was subsequently determined that the bone marrow cell suspension included two main populations of cells: hematopoietic stem cells (HSCs) and non-hematopoietic stromal cells [87]. The non-hematopoietic stromal cells were initially thought only to provide physical and functional support to hematopoiesis [88], but *in vitro* culture showed the density-independent differences in comparison to HSCs [87]. These stromal cells exhibited capacities to adhere to plastic and initiate clonal growth in a density-insensitive fashion (a colony-forming unit-fibroblastic feature), and thus distinguished from HSCs. These features of stromal cells therefore supported the hypothesis that the bone formation *in vivo* upon transplantation was due to the differentiation of stromal cells, indicating the osteogenic potential of bone marrow stromal cells [89]. Afterwards, the multipotency of bone marrow stromal cells to differentiate to various mesenchymal lineages, such as osteogenic, chondrogenic, adipogenic and myogenic lineages, has been documented (reviewed in [90]). The term “mesenchymal stem cells” (MSCs) was proposed for this heterogeneous population of stromal cells with self-renewal and multipotent capacities [91]. Although there has been a large-scale

debate in the literature regarding the terminology and definition of "mesenchymal stem cells", the description of MSCs is currently often used to refer to cells characterised *in vitro* with defined features, rather than a strict definition based on the stem cell biology [92]. To date, the functions and therapeutic application of MSCs have been extensively studied, as reflected by hundreds of on-going clinical trials using MSCs for diverse diseases and a vast body of published studies [92].

1.2.2 Characterisation and sources of MSCs

Although MSCs were initially found in bone marrow stroma, many other tissues, such as adipose tissue, skin, lung, synovial membrane, dental pulp, scalp tissue, muscle, periosteum, corneal limbus, uterine cervix, fetal/neonatal tissue, and biological fluids, for example, nasal olfactory mucosa, breast milk, peripheral blood, endometrial and menstrual blood and umbilical cord blood, have been found to be sources of MSCs (reviewed in [93]). Among these, bone marrow derived MSC (BM-MSC) is the most studied, but the lack of source tissue and painful harvest have limited its application. One of the alternative sources of MSCs, adipose tissue, has become a popular replacement for its abundance and convenience of harvest. In general, the adipose tissue-derived MSCs (AT-MSCs) are isolated from adipose tissues by enzymatic digestion and differential centrifugation to remove adipocytes [94]. The isolated cells are further purified by a combination of washing steps and culture expansion with similar media for BM-MSC to obtain adherent and fibroblast-like cells. AT-MSCs are similar to BM-MSCs in terms of their morphology, self-renewal property and surface phenotypic features, but they appear to possess some differences in the multiple lineage differentiation capacity [95-97]. It was suggested that this difference may partially reflect the difference in the niches of these cells residing in the respective tissue origin, and the different protocols for *in vitro* isolation and expansion [94-96].

Due to the diversity of sources and heterogeneity of isolated MSCs, the need to standardise the isolation and characterisation of MSCs has emerged. The Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy (ISCT) has proposed the minimal criteria for the characterisation of MSCs. The identifying characteristics include (i) the capacity of plastic adherence when maintained in standard culture conditions, (ii) the expression of specific surface markers CD105, CD73 and CD90, (iii) the lack of expression of hematopoietic lineage markers CD45, CD34, CD14

or CD11b, CD79a or CD19and HLA class II and (iv) the capacity to differentiate into osteoblasts, chondroblasts and adipocytes *in vitro* [98]. In addition to the minimal criteria suggested for the characterisation of MSC, the International Federation for Adipose Therapeutics and Science (IFATS) and the ISCT have together suggested including CD13 as an alternative or supplement to CD105 for characterisation of AT-MSCs [94].

1.2.3 Osteogenic differentiation of MSCs

One of the initially discovered key features of MSCs was the capacity for bone formation upon transplantation *in vivo* [86, 87], indicating their capacity to differentiate into osteogenic lineage cells. Later, MSCs were successfully isolated and expanded *in vitro*, and the culture condition for MSCs osteogenic differentiation, by supplementation with a cocktail of chemical stimuli, was optimised via evaluation of osteoblastic morphology, the expression of typical osteogenic differentiation markers, such as alkaline phosphatase (ALP), and the mineralisation of extracellular matrix (ECM) [99]. To date, the osteogenic differentiation process of MSCs and the mechanisms regulating this process have been extensively studied. Moreover, MSCs have emerged as an attractive candidate for tissue engineering, partly because they are capable of differentiating into osteogenic progenitors and subsequently regenerating bone. Various approaches have been explored to enhance the osteogenic differentiation of MSCs for their application on bone regeneration.

Several molecules and signalling pathways have been identified to regulate the osteogenic differentiation of MSCs in a stage-specific manner. In the early stage of differentiation, runt-related transcription factor 2 (RUNX2) functions as a master transcription factor to determine the fate of MSCs to differentiate towards the osteogenic lineage. After osteogenic commitment, the expression of another key transcription factors, Osterix or β-catenin, activates the production of downstream osteoblastic markers, such as ALP and osteocalcin, to induce the osteoblastic terminal differentiation for forming matured osteoblasts that can further differentiate to osteocytes. Signalling pathways, the bone morphogenetic protein (BMP) pathway, the canonical Wnt/β-catenin pathway and the mitogen-activated protein kinase (MAPK) pathway, are the main pathways that have been shown to regulate these transcription factors and in turn influence the osteogenic differentiation process (reviewed in [100]). In brief, extracellular BMPs binding to transmembrane receptors, type I and type II BMP receptors (BMPR), trigger the recruitment and phosphorylation of the

cytoplasmic proteins Smads (particularly Smad1, 5 and 8), which in turn associate with Smad 4. The Smad complex is then translocated to the nucleus and activates the expression of target genes including RUNX2 for the induction of osteogenic differentiation [101]. Alternatively, the binding of BMPs with BMPRs may instead activate the MAPK pathway. The activation of the MAPK pathway is able to upregulate the expression of both RUNX2 and Distal-Less Homeobox 5 (Dlx5), which are able independently to induce the transcription of Osterix to mediate osteoblast maturation [102, 103]. The MAPK pathway can also be triggered by growth factors, such as the fibroblast growth factor (FGF) [104]. In addition, the Wnt signalling pathway plays pivotal roles in the regulation of osteogenic differentiation. Upon Wnt protein binding to its receptor, Frizzled (FZD), and co-receptors, low-density lipoprotein receptor-related protein 5 and 6 (Lrp5/6), cytoplasmic proteins dishevelled (Dsh) and axin are recruited to the membrane leading to the inhibition of β-catenin degradation. β-catenin thus accumulates in the cytoplasm and subsequently translocates to the nucleus and interacts with transcription inducer factors, CBP/p300 and lymphoid enhancer-binding factor/T-cell-specific transcription factor (LEF/TCF), consequently resulting in the expression of target genes for the stimulation of osteogenic differentiation [104]. Moreover, these pathways were found to crosstalk to one another and build a complex regulation network for osteogenic differentiation [103, 105, 106].

Besides the transcription factors and signalling pathways, microRNAs have been shown to be part of this regulatory network. Several microRNAs have been found to be involved in determining the fate of MSCs and regulating osteoblastogenesis (reviewed in [107-109]). A previous study showed that a selected panel of microRNAs, miR-96, miR-124 and miR-199a, were differentially expressed during lineage commitment and these lineage-specific microRNAs regulated the gene expression of lineage-related transcription factors, such as SOX9, and components of signalling pathways related to cell differentiation [110]. Furthermore, a series of studies have illustrated some specific microRNAs controlling osteogenic differentiation and the mechanical concepts were explored. For example, miR-218 was found to promote osteogenic differentiation in a feed-forward manner (pro-osteogenic). In detail, miR-218 stimulated the Wnt pathway by down-regulating Wnt signalling inhibitors, including Dickkopf2 (DKK2). In turn, the activated Wnt signalling upregulated the expression of miR-218 and, together, this positive feed-forward loop resulted in enhanced osteogenic differentiation [111]. On the

other hand, microRNA can also function as a negative regulator of osteogenic differentiation (anti-osteogenic). A microRNA cluster, miR-23a~27a~24-2, was found to repress the translation of RUNX2 and its downstream target, stabilin 2 (STAB2), leading to the suppression of osteogenic differentiation, whereas RUNX2 inhibited the transcription of the microRNA cluster to re-activate STAB2 to promote osteogenic differentiation [112]. Another more comprehensive study revealed a panel of 11 microRNAs targeting RUNX2 and the majority of them were upregulated during osteogenic differentiation and attenuated RUNX2 protein accumulation [113]. However, the temporal expression of RUNX2 and these specific microRNAs during osteogenic differentiation indicated that the function of these microRNAs, anti-osteogenic or pro-osteogenic, appeared to be stage dependent. RUNX2 is a master switch to stimulate osteogenic lineage commitment but it is inhibitory at the late stages of osteoblast maturation and osteocyte formation [114]. Thus, the attenuation of RUNX2 by these miRNAs in the late stage of differentiation indeed promoted the terminal differentiation [113]. Moreover, the multiple-targeting property of microRNA allows its regulation on different key factors for osteogenic differentiation; for example, miR-218 was shown to function on both RUNX2 and inhibitors of the Wnt signalling pathway [111, 113]. Taken together, microRNAs are able positively or negatively to regulate osteogenic differentiation, which may be dependent on the stage of differentiation. Although microRNA functions as a negative regulator for the translation of its target mRNAs, the promotion of differentiation can be achieved directly by down-regulating inhibitors of pro-osteogenic factors/pathways or indirectly by relieving its inhibition on activators of osteogenic differentiation ("double-negative" mode) [107].

The network consisting of transcription factors, microRNAs and signalling pathways increases the complexity of the regulation on osteogenic differentiation of MSCs, which is not yet fully clear. However, decades of studies have deepened our understanding of this regulatory network, which provides various options to modify MSCs for their therapeutic application. For instance, MSCs were transfected for the overexpression of pro-osteogenic microRNAs (i.e. miR-21) or the inhibition of anti-osteogenic microRNAs (i.e. miR-221) to enhance osteogenic differentiation for bone regeneration [115, 116]. The combination of MSCs, scaffold and microRNAs or other bioactive molecules, such as BMP2, has been suggested to be promising regenerative tools in orthopaedics [117].

1.2.4 Immunoregulatory effects of MSCs

In addition to their regenerative property, MSCs possess broad immunoregulatory abilities to influence both innate and adaptive immune responses [118]. MSCs exert their immunoregulatory function through interaction with various immune cells, such as neutrophils [119, 120], monocytes/macrophages [121, 122] and T cells [123]. Neutrophils are the most abundant cell type in the innate immune system, circulating in the bloodstream or resting in the bone marrow. They are able to migrate and accumulate within minutes at the site of inflammation that is caused by injury or infection. MSCs have been shown to inhibit the apoptosis of both resting and IL-8-activated neutrophils and thus probably promote the preservation of the storage pool of neutrophils in the bone marrow niche [120]. The anti-apoptotic effect of MSCs was dramatically enhanced when the toll-like receptor-3 (TLR3) of MSCs was activated. In addition to prolonging the survival of neutrophils, TLR3-activated MSCs also amplified the function of neutrophils via promoting the immunophenotypic change of neutrophils [119]. Monocytes/macrophages are another key player in the inflammatory response. Upon stimulation by inflammatory signals, monocytes differentiate into macrophages, which can be further polarised into at least two different phenotypes: pro-inflammatory phenotype M1 and anti-inflammatory phenotype M2 [118]. MSCs were found to promote monocyte/macrophage migration and recruitment to the inflamed tissues and, moreover, to regulate the phenotypic polarisation of macrophages [121]. This finding was supported by evidence from both *in vitro* co-culture experiments and *in vivo* models. For example, in a sepsis model, the administration of MSCs decreased lethality via the increased production of IL10 that attenuated inflammation and in turn prevented further tissue damage [122]. The observed protective effect of MSCs was eliminated by the depletion of macrophages or the administration of IL10-neutralising antibody, together supporting the hypothesis that the MSCs induced the polarisation of macrophages toward the M2 phenotype *in vivo* [122]. In addition, MSCs were also shown to inhibit the proliferation of T cells and favour the differentiation of regulatory T cells (Treg) that function as suppressors of T cell-mediated immune responses [123].

The immunoregulatory property of MSCs has emerged their function as a sensor and switcher of inflammation, which in turn contributes to tissue regeneration. It has been proposed that MSCs regulated inflammation in a phase-dependent manner [121]. Tissue injury initiates an acute inflammatory

response, which peaks 24h after the injury and is completed by the first week. A complex cascade of pro-inflammatory cytokines is released in a temporal and spatial manner during this period. It has been suggested that MSCs possessed a pro-inflammatory ability to trigger the recruitment and accumulation of inflammatory cells at the injury site, thus helping to boost inflammation in the early phase, when there was an insufficient level of pro-inflammatory cytokines [124]. However, in the late stage of inflammation, MSCs appeared to adopt an immune-suppressive phenotype to dampen inflammation and promote tissue regeneration. This hypothesis was partially supported by the finding that LPS or TNF-α activated MSCs released prostaglandin E2 (PGE2) to trigger the reprogramming of macrophages toward the M2 phenotype and the further secretion of IL10 and this effect was eliminated if the MSCs lacked receptors such as TLR4 [122]. MSCs express several TLRs, which can be activated in a stimulus-dependent way. A previous study showed that MSCs secreted different patterns of cytokines upon the activation of specific TLRs. It was therefore proposed that MSCs were polarised toward two different phenotypes by downstream TLR signalling: pro-inflammatory MSC1 and immunosuppressive MSC2 [125]. Taken together, the current literature supports the belief that MSCs may possess various phenotypes that are able to switch in response to the surrounding microenvironment and thus result in the regulation of immune responses.

1.2.5 Ageing-related changes of MSCs

Ageing is a complex natural process with a progressive loss of physiological integrity contributed by cellular and molecular hallmarks such as genomic instability, telomere attrition, cellular senescence, stem cell exhaustion and altered intercellular communication [126]. Among these, stem cell, including MSC, ageing/exhaustion is one of the critical hallmarks characterised by the loss of efficient cellular function and response to the environment [127]. Previous studies have shown that both *in vitro* ageing (passage number in culture) and *in vivo* ageing (donor age) resulted in changes of MSCs [128, 129]. The ageing-related changes of MSCs included a decline of proliferation capacity, an increase in cellular senescence and apoptosis, the loss of multiple lineage differentiation potential, a shift in differentiation potential from osteogenic to adipogenic lineage, the loss of *in vivo* bone formation and impaired immunomodulatory function (reviewed in [130]). The declined proliferation and increased cellular senescence and apoptosis may result in a reduced number of stem cells in the stem cell pool, i.e. bone marrow [127].

Moreover, a previous study showed that ageing increased bone marrow fat [131], which may be partially due to the loss of osteogenic differentiation capacity, and favoured the differentiation of aged MSCs to the adipogenic lineage [132]. Indeed, it has been demonstrated that bone marrow adipose tissue was inversely associated with measurements of bone density and strength [133]. These observations may partially explain the development of osteoporosis and the delayed healing of bone fracture/defects in the aged population [130].

In addition to the impaired regeneration capacity, ageing also led to the loss of the immunoregulatory functions of MSCs. Previous studies showed that aged MSCs lacked the anti-inflammatory protective effect in young endotoxemic mice, which was partially due to the age-dependent decrease of immunoregulatory cytokine expression [134, 135]. Consistently, another study revealed that AT-MSCs from old donors failed to alleviate symptoms in an experimental autoimmune encephalomyelitis mouse model and, moreover, a possible mechanism that aged AT-MSCs secreted less hepatocyte growth factor (HGF) in comparison with cells from young donor *in vitro* was demonstrated [136]. Together, these observations indicated a change in the secretome of MSCs in conjugation with ageing.

Furthermore, the mechanisms underlying these functional changes of MSCs have been investigated in some studies. Transcription factors, such as PPARγ [137], p38 MAPK [135], Wnt/β-Catenin [138], and Akt/mTOR [139], and the signalling pathways they regulate have been shown to be involved in the regulation of age-related changes in MSCs. In addition, studies also demonstrated that several miRNAs, miR-10a [140], miR-141-3p [141], miR-543 and miR-590-3p [142], were able to regulate MSC ageing.

1.2.6 Secretome of MSCs

Early research interests in MSCs focused primarily on their multipotential lineage differentiation capacity. Hence, an attempt was made to achieve the therapeutic application of MSCs by transplantation of MSCs with the hope to differentiate them to specific cell types to repair/regenerate injured tissues. However, regardless of the administration routes and animal models, the low engraftment percentage of transplanted MSCs, the short window in which MSCs exerted their effects and the fact that MSC-conditioned medium (CM) or secreted factors alone stimulated beneficial effects similar to those of MSCs shed light on the importance of MSC secretome-mediated paracrine effects

[143, 144]. The secretome is the set of factors/molecules, including soluble factors and EVs, secreted to the extracellular space [93]. In this section, interest will focus on the soluble factors secreted by MSCs, while the secretion of EVs/exosomes by MSCs will be introduced in Section 1.3.

MSCs have been shown to secrete a broad spectrum of soluble factors including growth factors and cytokines, implicated as being involved in both regeneration and immunoregulation [145, 146]. Instead of the direct differentiation of MSCs to a specific cell type at the site of injury, MSCs secreted a series of trophic factors, such as growth factors (GFs), which were suggested to promote a regenerative microenvironment in various tissue injury models, such as lung, liver and kidney injuries [147, 148]. The trophic factors, such as FGFs, insulin-like growth factors (IGFs), HGF and platelet-derived growth factors (PDGF), were detected in MCS CM and possessed an anti-apoptotic effect and promoted proliferation leading to improved cell survival at the site of injury [145, 149]. Moreover, most of these GFs, as well as stromal derived factor-1 (SDF-1) [149], another MSC secreted factor, have a chemotactic effect on inducing the migration of progenitor cells to the site of injury. Tissue injury is often accompanied by damage of blood vessels, while MSC-secreted vascular endothelial growth factors (VEGFs) and angiopoietins (ANGs) were able to induce angiogenesis [145]. Although the therapeutic effects of transplanted MSCs may not result directly from the differentiation of MSCs, MSCs indeed secreted factors that were capable of stimulating differentiation. For example, MSCs secreted BMP2, which is stimulus of osteogenic differentiation [100].

In addition, MSCs were found to secrete a variety of cytokines that mediate immunoregulatory effects. MSCs exert their immunoregulatory functions by direct cell-to-cell contact [150], and via the secretion of chemokines, such as monocyte chemoattractant protein-1 (MCP-1, also known as CCL2), which are able to stimulate the migration of immune cells, and pro- or anti-inflammatory cytokines, such as IL-6, TNF-α, interferon γ (IFNγ), IL10, PGE2 and indoleamine 2,3-dioxygenase (IDO), which are able to regulate the proliferation and phenotype polarisation of immune cells, including neutrophils, macrophages and T cells [121, 145, 146]. The secretion of immunoregulatory factors enables MSCs to partially control the inflammatory response during tissue repair. Tissue repair upon injury is a process with overlap of inflammation and tissue regeneration [151]. The MSC secretome consists of cytokines, which were suggested to be able to regulate

inflammation in a phase-dependent manner [121], as well as GFs and other trophic factors, which generated a microenvironment favouring tissue regeneration [147]. Together, the multiple functions of MSC secretomes emerge as an attractive therapeutic approach. Furthermore, the secretome of MSCs can be modified by preconditioning of MSCs using various strategies [152]. For example, the pre-activation of MSCs by TNF-α or LPS increased the secretion of VEGF, FGF2, HGF and IGF-1 [153], as well as immunomodulatory cytokines such as PGE2 [122].

1.3 MSC-derived EVs/exosomes

In addition to secreted soluble factors, a large body of studies has shown that MSCs secrete EVs/exosomes carrying bioactive molecules including lipids, proteins and various species of RNA. It is suggested that the MSC-derived EVs/exosomes are a crucial part of the MSC secretome and play pivotal roles by mediating paracrine effects. Growing evidence has suggested that MSC-derived EVs/exosomes possess immunomodulatory effects to regulate inflammation, contribute to the regeneration of various tissues, and participate in the progress of tumorigenesis. Moreover, it has been proposed that the tentative mechanism of the observed biological effects of MSC-derived EVs/exosomes is the transfer of functional cargoes to recipient cells. Consequently, the biological activities of the recipient cells are regulated.

1.3.1 Cargoes of MSC-derived EVs/exosomes

Irrespective of cell types, EVs/exosomes share some common enriched lipids and proteins, as described in Section 1.1.3. Nevertheless, accumulating evidence reveals that the cargoes of EVs/exosomes reflect their parental cell origin and affect the functions of EVs/exosomes. In this section, the cargoes of MSC-derived EVs/exosomes, including lipids, proteins and RNA, will be summarised on the basis of the current literature.

Lipids

Lipids are the essential components of the membrane structure of EVs/exosomes and have been shown to play important roles in the biogenesis of EVs/exosomes. However, fewer studies have focused on investigating the lipid contents in comparison with other contents in EVs/exosomes. A previous study compared the lipidomes of exosomes and microvesicles secreted from

three different cell types, including MSCs and two cancer cell lines, U87 and Huh7 [32]. The results indicated both cell type- and vesicle type-dependent lipid contents. MSC exosomes shared similar enriched lipid species with Huh7 exosomes but not with U87 exosomes, including glycolipids, fully saturated free fatty acids, cardiolipins, lyso-derivatives of PS, phosphatidylglycerols and phosphatidylinositols. Moreover, the MSC exosomes and microvesicles were shown to enrich different lipid species. MSC exosomes enriched glycolipids and free fatty acids, whereas MSC microvesicles enriched ceramides and sphingomyelins. In agreement with the observation of the diversity of lipid contents in MSC EVs, another study isolated three subpopulations of MSC EVs/exosomes based on their affinities for three membrane lipid-binding ligands, cholera toxin B chain (a GM1 ganglioside-specific ligand), annexin V (a ligand for PS) and Shiga toxin B subunit (a ligand for globotriaosylceramide) [154]. It was determined that these three subpopulations of MSC EVs/exosomes were distinguished by their membrane lipid composition, proteome and RNA cargo, and were likely to have different biogenesis and functions. Interestingly, a recent study showed that the supplementation of tailored lipids in the culture of MSCs reduced the release of exosomes, but the exosomes recovered from a lipid-supplemented culture exhibited a stronger stimulation of cell migration [155]. Evidence provided by these studies together indicates the impact of lipid contents on the biogenesis and functions of MSC-derived EVs/exosomes, which require further investigation.

Proteins

In comparison to the lipid contents, the protein contents of MSC-derived EVs/exosomes have attracted more attention since the therapeutic effects of MSC-derived EVs/exosomes were reported. To date, almost 2,000 proteins have been detected in MSC-derived EVs. The first proteomic study of MSC EVs revealed four groups of protein candidates potentially associated with the therapeutic effects of MSC EVs: i) surface receptors including PDGF receptor B (PDGFRB), EGFR and plasminogen activator urokinase receptor (PLAUR); ii) signalling molecules, for example Ras related protein (RRAS)/NRAS, MAPK1 and cell division cycle 42 (CDC42); iii) cell adhesion molecules such as fibronectin1 (FN1) and integrins; and iv) MSC-associated antigens and common exosomal markers, such as CD9, CD63, CD81, CD44, CD73 and CD105 [156]. A further functional prediction revealed that these signalling molecules were involved in pathways including RAS-MAPK, RHO and

CDC42 pathways. The authors proposed a possible action mode for the candidate molecules, namely that the GFRs and FN/integrins activated their downstream pathways RAS-MAPK, RHO and CDC42, resulting in the actin cytoskeleton reorganisation needed for cell adhesion and migration [156]. It was suggested that the broad spectrum of biological activities, cell adhesion, migration, proliferation and differentiation, which were regulated by these candidate molecules, contributed to the MSC EV-mediated regenerative effects. This hypothesis was partially supported by another study showing that MSC exosomes carrying functional Wnt4 protein, which were able to promote the nuclear translocation of β-catenin, resulted in the enhanced proliferation and migration of skin cells [157]. Recently, it was also reported that MSC EVs enriched VEGF protein, which activated VEGFR in endothelial cells to promote angiogenesis [158]. In line with these findings, Lai *et al.* explored the proteome of MSC exosomes by using both mass spectrum and antibody array, and identified proteins including 1) GFs/GFRs, such as members of FGF, IGF, HGF, PDGF, VEGF and transforming growth factor-β (TGFβ); 2) cell adhesion molecules, including a variety of collagens and integrins, FN1, intracellular adhesion molecules (ICAMs) and MFGE8; 3) signalling molecules, for instance, MAPKs, small GTPase Rabs, Wnt5a and LRPs; 4) ILs/ILRs, such as IL10, IL8 and IL1RL2 and 5) cell structure proteins, including actin and tubulin [159]. Moreover, several enzymes were shown to be present in the proteome of MSC exosomes. These enzymes included glyceraldehyde-3-phosphate dehydrogenase (GAPDH), phosphoglycerate kinase (PGK), phosphoglucomutase (PGM), enolase (ENO), pyruvate kinase m2 isoform (PKm2), ecto-59-nucleotidase (also known as CD73) and 20S proteasome [159-161]. The first five enzymes are responsible for catalysing five connected reactions of glycolysis and it was therefore suggested that they play important roles in generating glycolytic adenosine triphosphate (ATP) [160]. While CD73 is an enzyme capable of dephosphorylating extracellular adenosine monophosphate (AMP) into adenosine, which has been shown to activate adenosine receptor and subsequently phosphorylated phosphatidylinositol 3-kinase/protein kinase B (PI3K/Akt) pathway. CD73 is also involved in immunoregulation [161]. The MSC exosomes carry functional 20S proteasome, which could potentially reduce the misfolded proteins [159]. The discovery of another enzyme, neprilysin (NEP, also known as CD10), in MSC exosomes and its function of degrading intracellular and extracellular β-amyloid (Aβ) in the neuroblastoma cell line further supported the enzymatic activity of proteins carried by MSC exosomes [162]. Taken as a whole, the

current evidence indicates that the protein contents play critical roles in MSC EV/exosome-mediated functions.

mRNA

Besides the protein contents of MSC-derived EVs/exosomes, the RNA cargoes have been extensively investigated. The RNA cargoes of MSC EVs have been determined by quantitative reverse transcription polymerase chain reaction (qPCR), microarray, or RNA sequencing in a variety of studies. Various species of RNA, including mRNA, tRNA, microRNA, among others, have been detected in MSC EVs/exosomes. The mRNA contents of MSC EVs has been profiled by RNA sequencing, showing that MSC EVs preferentially enriched mRNAs for transcription factors and GFs [163, 164]. Several studies supported this finding, showing that MSC EVs contained intravesicular HGF mRNA and keratinocyte GF (KGF) mRNA, which could be delivered to tubular cells and lung cells respectively and translated into functional proteins [165, 166]. Moreover, the MSC EV-treated tubular cells secreted more HGF, resulting in the strong stimulation of cell growth and differentiation [165]. In agreement with this study, Tomasoni *et al.* demonstrated that MSC exosomes carried IGF1R mRNA, but not IGF1 mRNA, and IGFR mRNA and proteins were detected in IGF1R-lacking cells after being exposed to MSC exosomes [167]. Together, these studies indicated that MSC EVs/exosomes carried functional mRNAs, specifically various GF/GFR mRNA, which can be transferred to and translated in recipient cells.

microRNA

In addition to the protein-coding RNA, non-coding RNAs were also detected in MSC EVs/exosomes. Interestingly, a previous study found that tRNA was the most abundant RNA species in exosomes derived from both BM-MSC and AT-MSC [168]. However, the sorting mechanism and function of these enriched tRNAs in MSC exosomes have not as yet been investigated. In contrast, a large body of studies have shed light on the microRNA contents of MSC EVs/exosomes. Two early studies back-to-back reported that only a specific fraction of microRNAs was packaged into MSC EVs/exosomes when compared with the total microRNAs identified in their parental cells [169, 170]. Chen *et al.* found that both precursor (pre-) and mature microRNAs were presented in MSC-secreted EVs and the EV-associated pre-microRNAs were capable of becoming mature microRNAs under treatment by RNase III *in vitro*

[169]. However, the main components of the RNA-induced silencing complex (RISC) required for microRNA-mediated regulation, endoribonuclease Dicer and argonaute-2 (Ago2), were not detected in the MSC EVs and the authors therefore questioned whether the EV-associated microRNAs were functional [169]. In contrast, another study showed the presence of a variety of selective ribonucleoproteins (RNPs), including staufen 1 and 2 (Stau 1 and Stau 2), AU-rich element binding protein (HuR), T cell internal antigen-1 (TIA) and Ago2, in exosomes derived from both MSCs and human liver stem cells [170]. These RNPs are involved in the traffic and stabilisation of RNA. For example, Stau 1 and 2 are implicated in the transport and stability of mRNA, while Ago2 is involved in microRNA transport and processing [170]. This result indicated that functional mRNA and microRNA were transferred via MSC EVs/exosomes. Two other recent studies also supported this hypothesis by showing that the MSC exosome-mediated therapeutic effects were diminished after the knockdown of Ago2 or Drosha, the other crucial enzyme regulating microRNA maturation, indirectly indicating that the MSC exosomal microRNAs were functional [171, 172]. Furthermore, a growing body of studies has validated that various specific microRNAs were transferred via MSC EVs/exosomes and were responsible for the biological effects induced by MSC EVs/exosomes. Among these, a selected list of representative studies is presented in Table 1. To summarise, MSC exosomal microRNAs have been implicated in different biological effects mediated by MSC EVs/exosomes, such as anti-apoptosis, increased proliferation, the regulation of angiogenesis, anti-fibrosis and immunomodulation/anti-inflammation.

Table 1. List of MSC exosomal microRNA cargoes that mediate biological effects in various target organs/cells via specific mechanisms.

Species/ MSC origin	Animal model /target cells	Responsible microRNA	Possible mechanisms for the biological effects	Ref.
Rat /BM-MSC	Rat stroke model induced by MCAo/ primary neuron and astrocytes	miR-133b	Downregulate RhoA expression to stimulate neurite outgrowth of neural cells	[82, 173]
Rat /BM-MSC	Diabetic streptozotocin rat model housed in enriched environment	miR-146a	Reduce the expression of IRAK1, NF-κB and TNF-α to exert anti-inflammatory effects on damaged astrocytes	[174]
CP-MSC	CCl_4-treated rat/ human hepatic stellate cell LX2	miR-125b	Suppress the activation of Hh signalling to reduce fibrosis in the liver	[175]

Mouse /BM-MSC	Liver antigen S100 induced AIH mice model/injured AML12 hepatocyte induced by LPS and ATP	miR-223	Downregulate NLRP3 and Caspase-1 and reduce the expression of TNF-α, IL-1β and IL-17	[176]
Mouse /BM-MSC	Myocardial infarction mouse model/ HUVEC	miR-210	Repress EFNA3 to promote the proliferation, migration and tube formation capacity of HUVEC and improve angiogenesis in post-MI heart	[177]
Rat /BM-MSC	Rat model with balloon-induced vascular injury/ VSMC	miR-125b	Repress Myo1e to inhibit the proliferation and migration of VSMC and neointimal hyperplasia	[178]
Human /WJ-MSC	Rat model with acute renal ischaemia reperfusion injury/ injured renal tubular epithelial cells	miR-30	Reduce the expression of DRP1 to inhibit mitochondrial fission and cell apoptosis	[179]
Mouse /MSC line C3H10T1/2	HUVEC	miR-30b	Downregulate angiogenic inhibitor DLL4 to promote tube-like structure formation	[180]
Human /BM-MSC	Acute GVHD mice model/CD3-/CD28-stimulated hPBMC	Predicted to be miR-125a-3p	May be responsible for the inhibition of T-cell proliferation and differentiation	[181]
Human /BM-MSC	Human breast carcinoma-derived cell lines (MDA-MB-231, MCF-7 and T47D)	miR-100	Downregulate mTOR/HIF-1α axis to inhibit VEGF expression	[182]
Mouse /BM-MSC	Mouse breast cancer induced by injection of 4T1 breast cancer cells/breast cancer cell line 4T1	miR-16	Inhibit VEGF to suppress angiogenesis	[183]
Human /UC-MSC	Mouse lung adenocarcinoma induced by injection of H1299 LUAD cells/ LUAD cell line H1299 and PC-9	miR-410	Reduce PTEN expression to increase proliferation and decrease apoptosis in LUAD cells	[184]
Human /GA-MSC	Mouse glioma induced by injection of GSC /GSC	miR-1587	Downregulate tumour suppressor NCOR1 to increase the proliferation and clonogenicity of GSC	[185]

Ref, reference; MCAo, middle cerebral artery occlusion; RhoA, ras homolog gene family member A; IRAK1, interleukin 1 receptor associated kinase 1; NF-κB, nuclear factor-κB; CP-MSC, chorionic plate-derived MSC; CCl_4, carbon tetrachloride; Hh, hedgehog; AIH, autoimmune hepatitis; NLRP3, NACHT, LRR and PYD domains containing protein 3; HUVEC, human umbilical vein endothelial cell; EFNA3, Ephrin A3; MI, myocardial infarction; VSMC, vascular smooth-muscle cell; Myo1e, myosin 1E; WJ, Wharton Jelly; DRP1, dynamin-related protein 1; DLL4, delta-like 4; GVHD, graft-versus-host disease; hPBMC, human peripheral blood mononuclear cell; mTOR, mammalian target of rapamycin; HIF-1α, hypoxia inducible factor; UC, umbilical cord; LUAD, lung adenocarcinoma; PTEN, phosphatase and tensin homolog; GA, glioma-associated; GSC, glioma stem-like cell; NCOR1, nuclear receptor co-repressor 1

1.3.2 Functions of MSC-derived EVs/exosomes

It has been proposed that MSC-derived EVs/exosomes mediate their biological functions in a content-dependent fashion [162], protein based (i.e. enzymatic activity or signalling transfer) or RNA based (i.e. microRNA mediated regulation), which is partially summarised in the above Section 1.3.1. This section will focus on MSC-derived EV/exosome-mediated functional outcomes in various injury and/or disease models and will shed light on our current understanding of how MSC-derived EVs/exosomes mediate cell-to-cell interaction and the implicated molecular and cellular mechanisms.

Functions on tissue regeneration

Earlier, the therapeutic effects of MSC exosomes were reported in a myocardial ischemia/reperfusion (MI/R) injury model, which showed that the administration of MSC exosomes reduced infarct size by 45% compared with control and that the cardiac function and geometry were enhanced after MI/R injury [186, 187]. In a glycerol induced acute kidney injury (AKI) model, or in a renal injury model induced by ischaemia reperfusion (IR), MSC exosomes ameliorated IR-caused kidney dysfunction and induced the morphological and functional recovery of AKI [172, 179, 188]. In a CCI4-induced fibrotic liver model, MSC exosomes were shown to alleviate hepatic inflammation and collagen deposition, recover serum aspartate aminotransferase (AST) activity and reduce the surface fibrous capsules [175, 189]. Hypoxia-induced pulmonary hypertension was shown to be inhibited by the administration of MSC exosomes via ameliorating hypoxia-induced lung inflammation and lung vascular remodelling [190]. In an MCAo-induced experimental stroke model, MSC exosomes promoted neural plasticity and neurite remodelling in the ischaemic boundary zone, leading to improved functional recovery. Moreover, in the neurodegenerative disorders, for example, an Alzheimer disease (AD) model, MSC exosomes were able to rescue cognition and memory impairment,

based on the results of the Morris water maze test, and downregulate the neuroinflammation [191]. In a rat skin burn model, MSC exosomes accelerated re-epithelialisation and angiogenesis to promote wound healing [157, 192]. The implantation of MSC exosomes with tricalcium phosphate (β-TCP) scaffolds in a critical-sized bone defect model enhanced angiogenesis and osteogenesis, indicating the improvement of bone regeneration [193, 194]. In line with this latter observation, the direct injection of MSC exosomes in bone fracture was also shown to facilitate the acceleration of fracture healing [195].

Functions on immunomodulation

In addition to the therapeutic effects on various tissue injury models, MSC exosomes also showed promising effects in autoimmune disease models because of their immunomodulatory potential. In a liver antigen S100-induced autoimmune hepatitis model, MSC exosomes were shown to alleviate hepatic inflammation and the serum level of AST and alanine transaminase (ALT), and improve liver structure [176]. In another autoimmune disease model, 2,4,6-trinitrobenzene sulfonic acid (TNBS)-induced colitis, the injection of MSC exosomes attenuated the severity of colitis exhibited by a decrease of the disease activity index (DAI) and histological colonic damage and the downregulation of inflammation and oxidative stress [196]. It is noteworthy that the first case of successful treatment with MSC exosomes in a patient with steroid-resistant GVHD has been reported [197], which is a hallmark achievement and may move MSC EVs/exosomes toward clinical application. A recent study further confirmed the therapeutic effects of MSC exosomes in a mice GVHD model by showing the prolonged survival of mice with GVHD and the reduced pathological damage of multiple GVHD-target organs [181].

Functions on tumorigenesis

While MSC exosomes show trophic effects on the regeneration/healing of various tissues and the attenuation of the symptoms in different autoimmune disease models, MSC exosomes also play important roles in tumorigenesis (reviewed in [198, 199]). MSC exosomes have been shown to promote tumour growth in gastric carcinoma [200], lung adenocarcinoma [184], glioma [185] and breast cancer [201]. However, in some studies, MSC exosomes negatively regulated tumour growth via the suppression of angiogenesis [183]. The contradictory effects of MSC exosomes on tumorigenesis appear to be due to the exposure of MSCs in different surrounding microenvironments, which in

turn affect the properties of exosomes secreted by MSCs. Further comprehensive studies are required to demonstrate the roles of MSC EVs/exosomes in tumorigenesis.

MSC-derived EV-/exosome-mediated cell-to-cell interaction

MSC-derived EVs/exosomes have been shown to mediate interaction with many different cell types, including homotypic MSCs and heterotypic cells: i) tissue-specific cell types, i.e. endothelial cells (EC), fibroblasts, renal tubular epithelial cells, hepatic stellate cells, neurons, astrocytes and the neuroblastoma N2a cell line and osteocyte-like cell line MLO-Y4 and ii) immune cells, such as macrophages, T cells and B cells.

Previous studies have revealed that MSC exosomes increase HUVEC proliferation, migration and tube formation capacity via the transfer of MSC exosomal microRNA, miR-30b and miR-210, to target DLL4 and EFNA3 respectively, which consequently promoted angiogenesis [177, 180]. Beside the exosomal microRNA-mediated effects, MSC exosomes also transferred Wnt4 to HUVEC to activate β-catenin to exert proangiogenic effects [192]. Several studies have demonstrated that MSC exosomes induce apoptosis resistance and the proliferation of tubular epithelial cells via the internalisation and transfer of exosomal RNA contents including mRNAs and microRNAs. Bruno *et al.* revealed a subset of MSC exosomal mRNAs, which were involved in the regulation of transcription, proliferation and immunoregulation and were probably able to translate in recipient cells upon exosome internalisation [188]. Collino *et al.* suggested that MSC exosomal microRNA-dependent effects induced a pro-regenerative gene signature in the injured kidney [172]. Furthermore, a recent study showed that MSC exosomes-enriched miR-30, transferred to tubular epithelial cells, led to the inhibition of mitochondrial fission and cell apoptosis [179]. MSC exosomes were also shown to interact with hepatic cell lines, such as HL7702, AML12 and LX2, decrease the expression of collagen type I and III, TGF-β1 and the phosphorylation of Smad2 and reverse the TGF-β1-induced epithelia-to-mesenchymal transition (EMT) [189]. Moreover, two microRNAs were shown to be responsible for the protective effects of MSC exosomes on injured hepatic cells. Exosomal miR-125 reduced liver fibrosis via the inactivation of Hh signalling, while miR-223 downregulated inflammatory cytokines NLRP3 and caspase-1 to reduce inflammation [175, 176]. In the central nervous system, MSC exosomes mediated communication with both neurons and astrocytes via the

transfer of exosomal microRNA, miR-133b, to reduce the expression of its targets, connective tissue growth factor (CTGF) and RhoA, thereby resulting in the stimulation of neurite outgrowth [82, 173]. In addition, MSC exosomes carrying enzymatically active NEP, which can be transferred to N2a cells, resulting in reduced secretion and the intracellular formation of Aβ [162]. Dermal fibroblasts play important roles in skin wound healing. MSC exosomes were shown to carry Wnt4, which could be transferred to dermal fibroblasts and further promoted the nuclear translocalisation and activation of β-catenin to enhance cell proliferation and migration [157]. A previous study also showed that MSC exosomes to interact with homotypic cells to promote the proliferation, migration and osteogenic differentiation of MSCs via the regulation of the PI3K/Akt pathway [194]. Furthermore, exosomes derived from various stages of osteogenic differentiation induced the osteogenic differentiation of MSCs in a stage-dependent manner and it is also worth noting that only exosomes from the late stages of differentiation induced the mineralisation of MSCs [47]. This induction of osteogenic differentiation via MSC exosomes was suggested to be mediated via the transfer of osteogenesis-related exosomal microRNAs [47]. A recent study revealed that MSC exosomes ameliorated the apoptosis of osteocytes induced by hypoxia/serum deprivation and alleviated increased osteoclastogenesis [202].

In addition to the interaction with homotypic cells and a variety of tissue-specific cell types, MSC exosomes have been shown to mediate communication with immune cells. A previous study revealed that MSC exosomes activated TLR4, leading to the upregulation of the myeloid differentiation primary response 88 (MyD88)-dependent nuclear translocation of NFκB in the monocyte THP1 cell line [203]. Moreover, MSC exosomes stimulated a high level of IL10 and a low expression of IL1β and IL12P40, indicating an induction of the anti-inflammatory M2 phenotype [203]. In agreement with this observation, another study showed that MSC exosomes triggered macrophage polarisation towards the M2 phenotype, indicated by an increased expression of the M2 surface signature, CD36, CD51 and CD206 [204]. This result was also confirmed *in vivo*. MSC exosomes promoted the infiltration of M2 macrophages to the site of injury after the initial inflammatory response, which was characterised by an increase in the expression of M2 markers, Arginase1 and Chitinase 3-like 3, and a reduction in the M1 marker, nitric oxide synthase 2 [204]. MSC exosomes therefore influenced the balance of M1/M2 at the injury site, resulting in a reduced

IL6/IL10 ratio. In addition, MSC exosomes were able to inhibit B-cell proliferation and differentiation in a dose-dependent manner [205]. Similarly, the proliferation and differentiation of T cells were also downregulated by MSC exosomes. Moreover, MSC exosomes induced the apoptosis of activated T cells and increased the ratio of $T_{reg}/T_{effector}$ cells and IL10 secretion [206].

To summarise (Table 2), a large body of evidence indicates several, diverse functions of MSC EVs/exosomes, which are attributed to the MSC EV/exosome-mediated cell interaction. The transfer of exosomal cargoes results in the regulation of various physiological processes, including apoptosis, cell proliferation, migration, osteogenic differentiation, angiogenesis and immune responses/inflammation.

Table 2. The functions of MSC-derived EVs/exosomes in various injury and disease models

Functional category	Injured tissue /disease model	Functional outcome	Possible cellular and molecular mechanisms	Ref.
Tissue regeneration	Myocardial infarction/ myocardial IR injury	Reduced infarct size; enhanced cardiac function and geometry	Exosomal enzyme-mediated glycolysis to increase the generation of ATP for restore of bioenergetics; reduced oxidative stress; activated pro-survival signalling Akt/GSK3; inhibited apoptosis pathway c-JNK; enhanced angiogenesis.	[160, 177, 186, 187]
	AKI/renal IR injury	Ameliorated IR injury-induced kidney dysfunction; induced morphological and functional recovery of AKI	Induced resistance to apoptosis/necrosis; inhibited mitochondrial fission; stimulated proliferation	[172, 179, 188, 207]
	CCl_4-induced fibrotic liver	Alleviated hepatic inflammation and collagen deposition, reduced the surface fibrous capsules and made the textures soft; reduced serum level of AST	Decreased collagen type I and III, TGF-β1 and the phosphorylation of Smad2; inhibited EMT; downregulated inflammatory cytokines NLRP3 and caspase-1 via the transfer of exosomal miR-223; inactivated Hh signalling via the transfer of exosomal miR-125b	[175, 189] [176]

	Hypoxia-induced pulmonary hypertension	Ameliorated hypoxia-induced lung inflammation and lung vascular remodelling	Prevented pulmonary influx of macrophages; inhibited STAT3 activation to regulate hypoxia-related microRNAs, miR-17 superfamily and miR-204	[190]
	MACo-induced stroke model	Promoted neural plasticity and neurite remodelling; improved post-stroke functional recovery	transfer of exosomal miR-133b to reduce the expression of inhibitors of axonal growth, CTGF and RhoA; stimulated neurite outgrowth	[82, 173]
	Neurodegenerative disorders/AD	Ameliorated cognitive decline; rescued synaptic dysfunction; reduced plaque deposition and Aβ levels in the brain; downregulated neuro-inflammation	Transfer of exosomal enzyme NEP to degrade Aβ; mediated the upregulation of miR-21 to inactivate STAT3 and NFκB, leading to the downregulation of pro-inflammatory cytokines TNF-α and IL-1β, and upregulation of anti-inflammatory cytokines IL-4 and IL-10.	[162, 191]
	Cutaneous wound/skin burn injury model	Accelerated re-epithelialisation and angiogenesis to promote wound healing	Activated AKT pathway to reduce heat-stress-induced apoptosis; delivery of exosomal Wnt4 to activate β-catenin, resulting in the proliferation and migration of dermal fibroblasts and EC and the tube formation of EC	[157, 192]
	Bone fracture/defect	Promoted bone regeneration and angiogenesis	Promoted the proliferation and migration of MSCs; induced or promoted the osteogenic differentiation and mineralisation of MSCs; transfer of osteogenesis-related exosomal microRNA; regulated the PI3K/Akt pathway; downregulated apoptosis and osteoclastogenesis	[47, 193-195, 202]
Immuno-modulation	Autoimmune hepatitis	Improved liver structure; alleviated hepatic inflammation; reduced serum level of ALT and AST	Decreased expression of pro-inflammatory cytokines TNF-α, IL-1β and IL-17; exosomal miR-223 mediated downregulation of NLRP3 and caspase-1;	[176]

	TNBS-induced colitis	Attenuated severity of colitis indicated by decrease of DAI and histological colonic damage; decreased inflammation in the colon; enhanced colon antioxidant defence	Inhibited NFκBp65 signalling pathways to downregulate pro-inflammatory cytokines TNF-α and IL-1β and increase anti-inflammatory cytokines IL-10; suppressed oxidative perturbations by decreasing the activity of MPO and MDA and increasing SOD and GSH; inhibited apoptosis by reducing the cleavage of caspase-3, caspase-8 and caspase-9	[196]
	GVHD	Reduction of diarrhoea volume and enhanced cutaneous wound healing in cutaneous and mucosal GVHD patient; prolonged survival of mice with acute GVHD; ameliorated the systemic symptoms of GVHD in mice; reduced damage of multiple GVHD target organs, including the large and small bowels, liver and skin in mice	Suppressed the proliferation of CD4+ and CD8+ T cells; inhibited the functional differentiation of T cells to an effector phenotype; preserved circulating naïve Treg cells; MSC exosomal miR-125a-3p was predicted to be partially responsible for the effects	[181, 197]
Tumorigenesis	Gastric carcinoma	Promoted tumour growth and angiogenesis	Activated ERK1/2 and p38 MAPK pathway to promote VEGF and CXCR4 expression in tumour cells	[200]
	Glioma	Increased tumour burden and decreased survival	Transfer of exosomal miR-1587 downregulated NCOR1 resulting in the promotion of GSC growth	[185]
	LUAD	Promoted tumour growth	Transfer of exosomal miR-410 downregulated PTEN expression, leading to the increased proliferation and decreased apoptosis of LUAD cells	[184]
	Breast cancer	Suppressed angiogenesis	Transfer of exosomal miR-16 to downregulate VEGF in tumour cells	[183]

GSK, glycogen synthase kinase; c-JNK, c-Jun N-terminal kinases; STAT3, signal transducer and activator of transcription 3; MPO, myeloperoxidase; MDA, malondialdehyde; SOD, superoxide dismutase; GSH, glutathione; CXCR4, C-X-C chemokine receptor type 4

1.4 Bone formation around implants

Bone-anchored implants, in particular, titanium implants are widely used in clinics. When titanium implants, such as dental or orthopaedic implants are introduced into bone, the bone may interact with the implant surface, resulting in a direct structural and functional connection between bone and implant. This process, termed as osseointegration, was first discovered by P-I Brånemark in the late 1960s [208]. Bone formation around the implant is a critical step towards achieving successful osseointegration and the subsequent stability and sustainability of the implant. The process of bone formation around an implant was previously regarded as identical to that occurring after bone injury or fracture, but several biological events, such as the absence of an intermediate chondrogenic callus around implants, actually differ. The tissue response at the implant surface after implantation includes three sequential phases: the inflammatory phase, the bone regeneration phase and the bone remodelling phase (reviewed in [209, 210]). The implant properties, such as surface composition, topography and roughness, and the microenvironment, consisting of the ECM, recruited cells and their secretome, at the bone-injury site, are important. On the one hand, various studies have shown that the surface properties influence protein absorption, platelet adhesion, inflammation, osteogenic differentiation and cytokine secretion, indicating the regulation of cellular behaviour by implant surface properties [209]. On the other hand, the behaviour of the recruited cells and their secretome in the surrounding microenvironment of the implant consequently influenced the bone formation around the implant and the subsequent interaction of bone and implant surface [210]. A comprehensive understanding of this regulatory loop will provide fundamental knowledge for the development of novel implants, especially for application in compromised tissue conditions, such as osteoporosis and diabetes.

Upon implantation, the titanium implant surface comes in contact with various biological components, such as proteins and platelets released from blood due to the rupture of blood vessels. Various cell types, including inflammatory cells (i.e. macrophages), bone-forming cells (i.e. MSCs and/or osteoblasts), and bone remodelling cells (i.e. osteoclasts), sequentially recruited to or differentiated at the implantation site, together with their secretome, contribute to a complex microenvironment surrounding the implant. The new bone formation can occur at the existing bone surface where woven bone is produced by the recruited osteoblastic cells and gradually advances towards

the implant surface (distance osteogenesis) [211, 212]. Alternatively, based on the observation of direct contact between woven bone and the titanium implant surface during osseointegration, it is also suggested that new bone can be formed directly on the implant surface by the migration and differentiation of MSCs and osteoblastic lineage cells at the implant surface and the subsequent mineralisation of their produced ECM (contact osteogenesis) [213]. Hence, the cell-material interaction and cell-to-cell communication play pivotal roles in bone healing and regeneration around the titanium implant.

1.4.1 Cell-material surface interactions

Cellular behaviour on the implant surface, including recruitment/migration and adhesion, proliferation and osteogenic differentiation, is regulated by the signalling cues presented on the cell surface (i.e. receptors and/or ligands), in the microenvironment surrounding the implant (i.e. soluble factors including cytokines, chemokines and GFs) and on the implant surface (i.e. surface features such as topography and absorbed proteins).

The implant surface properties can influence the cellular behaviour indirectly via adsorbed proteins during blood clot formation. The surgical trauma caused by implantation initiates the blood clot formation and results in the adsorption of proteins and platelets on the implant surface, which provides a signal cue for the recruitment of cells to the implant surface. For example, PDGF secreted by platelets is a potent chemoattractant for various cell types including MSCs [214]. The adsorbed protein profiles can be modified via changes of surface properties, which in turn influence the cellular behaviour, such as cell adhesion, on the surface. In addition, implant surface properties may impact on the secretion of the recruited cells, which may modify cellular behaviour in an autocrine and/or paracrine fashion. For instance, a variety of cytokines and chemokines, such as MCP-1, TNFα, IL-1β and IL6, are secreted following the infiltration of inflammatory cells to the site of injury. A previous study showed the co-existence of monocytes/macrophages and MSCs surrounding the implant, however, the predominant cell population and cytokine secretion appear to be influenced by the surface properties [215]. Cells adhering to a smooth machined titanium surface expressed a higher level of MCP-1, TNFα and IL-1β, indicating the favoured recruitment of monocytes/macrophages and secretion of proinflammatory cytokines on machined surfaces. In contrast, cells on rougher oxidised surfaces exhibited the predominant expression of CXCR4, suggesting the presence of more MSCs on oxidised surfaces in

comparison to machined surfaces. The pro-inflammatory cytokines TNFα and IL-1β have been shown to augment the migration of MSCs [216]. On the other hand, the recruited MSCs also express a broad range of cytokines and GFs, including SDF-1α, PGE2 and BMP2, playing crucial roles in regulating the behaviour of MSCs as well as inflammatory cells [214]. PGE2 is an important immunoregulatory factor and is able to polarise macrophages to anti-inflammatory phenotypes [122]. The binding of SDF-1α with its receptor CXCR4 on MSCs further triggers the increased recruitment and early osteogenic differentiation of MSCs [217].

Importantly, a number of studies have shown a profound impact of the implant surface properties, especially the surface topography, on different aspects of cellular behaviour [218]. Learning from the nature of the osteoclast resorption pit that was shown to be chemotactic for osteoprogenitors and to stimulate rapid osteogenic differentiation, the current biomimetic implants are inspired to be designed with the topographical features of an osteoclast resorption pit [219]. Surfaces with microscale to nanoscale roughness have also been extensively studied. Microrough surfaces have been shown to favour cell adhesion [220] and promote osteogenic differentiation [221]. The rapid osteogenic differentiation of MSCs was induced without chemical stimuli when cultured on nanostructured titanium surfaces, indicated by the increased expression of osteogenic markers including ALP, osteocalcin, osteopontin and osteoprotegerin (OPG) [221]. OPG functioned as a decoy receptor for the receptor activator of NFκB ligand (RANKL) to inhibit osteoclast maturation and result in decreased bone remodelling. In addition, osteoblasts cultured on a microrough surface regulated bone remodelling via the increased secretion of TGFβ1 [222]. TGFβ1 promotes the proliferation of MSCs and osteoblasts and stimulates ECM production. On the other hand, TGFβ1 regulates bone remodelling via effects on the production of OPG. The microrough surface thus promotes new bone formation via favouring osteogenesis over osteoclastic resorption. Hitherto, the biological response to advanced implant surface modifications has been primarily evaluated experimentally *in vitro* and *in vivo* and results from human conditions are scarce.

1.4.2 Titanium surface modification

The first generation of titanium implants machined with a smooth surface texture has been successfully used in the clinic for more than half a century

[209]. Over the years, several different strategies have been investigated to further improve the functional outcomes of titanium implants, especially for application in compromised tissue conditions where the clinical results could be optimised. In an attempt to enhance the bone healing and regeneration to promote osseointegration, efforts have been made to modify the surface chemistry, wettability, charge and topography using various approaches. The most commonly used approaches include grit blasting, acid etching, electrochemical anodisation, laser treatment and different types of coating, such as a calcium phosphate/hydroxyapatite (HA) coating [209]. These physiochemically modified surfaces have been shown to promote osteogenic differentiation and osseointegration to varying degrees. Based on the results from *in vitro* and *in vivo* studies, highly roughened surfaces, such as surfaces modified by grit blasting, tend to favour mechanical anchorage and primary fixation to bone, whereas surfaces with nanoscale topography focus more heavily on the intentional regulation of molecular and cellular events, in terms of protein adsorption, cell adhesion and other cellular behaviours, during the early stage of healing [223]. The provision of a biomimetic implant surface with biological cues to influence the cell and tissue behaviours is yet another interesting strategy based on the nature of the biological process. For example, learning from integrin-mediated cell adhesion by binding to different ligands, such as FN, type I collagen, osteopontin and bone sialoprotein, the specific amino acid sequence RGD shared among these ligands has been coated to generate a surface favoured cell adhesion [218]. Moreover, a variety of bioactive components, such as peptides [224, 225], proteins [226] and GFs [227], have been applied to functionalise the implant surface with a specific purpose. The vast majority of these latter attempts have not yet reached the *in vivo* and clinical stages.

2 Aim

The overall objective of this thesis was to explore the cell-to-cell communication and cell-to-material surface interaction mediated by MSC-derived EVs/exosomes and the functions of MSC-derived EVs/exosomes in the regeneration capacity of MSCs, in terms of the proliferation and osteogenic differentiation of MSCs. Moreover, the contents of these EVs/exosomes, with the emphasis on microRNA and proteins, were determined to obtain an insight into the underlying mechanisms of the EV/exosome-mediated biological effects.

2.1 Specific aims

The specific aims of the papers included in this thesis were as follows.

- To determine whether exosomes (i) are secreted by MSCs during osteogenic differentiation, (ii) are internalised by recipient MSCs and influence osteogenic differentiation in a stage-dependent manner and (iii) contain altered microRNA profiles related to osteogenic differentiation and exosome function (Paper I)
- To investigate whether (i) *in vitro* ageing influences the release of EVs by MSCs quantitatively, (ii) the protein contents of EVs from LP ("young") and HP ("aged") MSCs differ and (iii) EVs from LP and HP MSCs regulate the proliferation of MSCs in an autocrine and/or paracrine manner (Paper II)
- To explore (i) whether MSC-derived exosomes can be immobilised on Ti surfaces and influence the behaviour of MSCs, in terms of cell adhesion, morphology, growth, migration and osteogenic differentiation, on Ti surfaces, (ii) whether the behaviour of Ti-adherent MSCs is differentially affected by the parental sources of the exosomes (expanded *vs* differentiated MSCs) and/or the routes of exosome delivery (immobilisation on the Ti surface *vs* suspension in the culture) and (iii) whether the predicted functional features of exosomal protein cargoes are linked to the observed effects (Paper III).

3 Materials and Methods

3.1 Materials

3.1.1 MSCs

Human adipose-derived MSCs were isolated and characterised by American type culture collection (ATCC, Manassas, VA). hMSCs at passage 2 (P2, Lot number 59193163) were purchased and cultured under different conditions in Papers I-III.

3.1.2 Titanium discs

In Paper III, the titanium (Ti) discs 6 mm in diameter and 1 mm in thickness were machined from pure titanium (ELOS Medtech, Gørløse, Denmark). The discs were cleaned and sterilised by ultrasonication and in a series of baths containing heptane, acetone and ethanol, before use in all of the experiments.

3.2 MSC culture and conditioned media collection

3.2.1 Expansion and *in vitro* ageing of MSCs

hMSCs were seeded at 5,000 cells/cm^2 for expansion as follows. In Paper I, hMSCs were cultured in home-made exosome-free medium prepared according to Thery *et al.* [55]. Briefly, Dulbecco's modified Eagle's medium with low glucose (DMEM-LG) was supplemented with all nutrients, including 2mM L-glutamine, 1% penicillin-streptomycin and 20% fetal bovine serum (FBS), and centrifuged for 16h at 100,000x g, 4°C. After centrifugation, the supernatant was filtered to sterilise it. The exosome-free basal growth medium (BGM) was prepared by diluting one volume of the depleted medium with one volume of medium supplemented with all the nutrients except FBS. hMSCs, at P4-P6, were expanded in the exosome-free BGM supplemented with 10 ng/ml of human basic FGF (bFGF).

The *in vitro* ageing of MSCs in Paper II was performed by continuously subculturing cells up to P15-16. MSCs at P5-6 were defined as young, low-passage cells (LP MSC), while MSCs at P15-16 were defined as aged, high-

passage cells (HP MSC). In Paper III, hMSCs at P4-P6 were used for expansion culture. In Papers II and III, MSCs were first seeded in serum containing BGM. The culture media were replaced by serum-free MSC NutriStem XF medium two to three days after seeding.

3.2.2 Osteogenic differentiation of MSCs

To induce osteogenic differentiation (Paper I), hMSCs at P6 were seeded at 18,000 cells/cm^2 in 2 μg/cm^2 human fibronectin (hFN)-coated tissue culture flasks. After overnight incubation, cells were exposed in home-made osteogenic differentiation media (ODM, including exosome-free media supplemented with 100 nM dexamethasone, 45 μM ascorbic acid and 20 mM β-glycerophosphate) for 21 days (d). Fresh culture medium was added every three days. To evaluate the osteogenic differentiation capacity of LP and HP MSCs in Paper II, MSCs were seeded at 30,000 cells/cm^2 in BGM and following exposure in home-made ODM, as described above. In Paper III, MSCs at P5, when reaching about 80% confluence, were exposed to MSCgo™ Osteogenic XF for about 10 d.

3.2.3 Conditioned media collection

The conditioned medium (CM) was collected every three days during the expansion and osteogenic differentiation culture in Paper I. A total of eight groups of CM, P6, D3, D6, D9, D12, D15, D18 and D21 respectively were collected. CM from LP and HP MSCs (LP CM and HP CM) respectively were collected when the cells reached approximately 90% confluence (Paper II). In Paper III, CM from MSC expansion (CM1) was collected after three days of serum-free culture, while CM from MSC osteogenic differentiation culture (CM2) were collected in the middle and late stages of differentiation. All the CM were centrifuged at 500 x g for 10 min (Papers I and III), or at 300 x g for 5 min (Paper II), to eliminate cells before being stored at -80°C for the isolation of EVs/exosomes. In each study, at least three different batches of CM were collected.

3.2.4 Characterisation of MSCs

The phenotypic signature, morphology, proliferation and osteogenic differentiation capacity of LP MSCs and HP MSCs respectively were characterised in Paper II. The phenotypic signature was analysed using a BD Stemflow hMSC analysis kit based on the definition of MSCs. The co-expression of surface markers CD73, CD90 and CD105 and the absence of

CD11b, CD19, CD34, CD45 and HLA/DR were examined using a BD FACS Melody™. The data were further analysed by FlowJo™10. The morphology and confluence of LP and HP MSCs were observed by light microscopy at d4 and d6 of culture. The proliferation capacity of LP and HP MSCs was indicated by the population doubling time (PDT), calculated according to the formula PDT = [duration of culture * log (2)]/ [log (number of cells harvested) – log (number of cells seeded)]. The osteogenic differentiation of LP and HP MSCs was induced, as described in Section 3.2.2, and evaluated by the quantification of ALP activity after two weeks (described in Section 3.9.3) and ECM mineralisation after three weeks (described in Section 3.9.4).

3.3 EV/exosome isolation

3.3.1 Ultracentrifugation

Exosomes were isolated from the CM using a series of differential centrifugation (Papers I and III). In short, CM were centrifuged at 16,500 x g for 20 min, with (Paper I) or without (Paper III) filtration through a 0.22 μm filter, to deplete cell debris and large vesicles. Exosomes were then pelleted by ultracentrifugation at 120,000 x g for 70 min (Paper I) or for two hours (Paper III) in a T-647.5 rotor (Sorvall wx Ultra series). The exosome pellets were resuspended in phosphate buffer saline (PBS) and stored at -80°C until use. All the steps were performed at 4°C.

3.3.2 Exo-spin isolation

EVs from LP CM and HP CM (Paper II) were isolated using an Exo-spin™ exosome purification kit (Cell Guidance Systems), according to the manufacturer's instructions. Briefly, CM were centrifuged at 16,000 x g for 30 min to remove cell debris. Afterwards, the supernatant was mixed with ½ volume of Exo-spin™ buffer and incubated at 4°C for two hours following a second centrifuge step at 16,000 x g for one hour. The exosome-containing pellet was resuspended in 100 μl PBS and further purified by passing through Exo-spin™ mini columns.

For proteomic analysis (Papers II and III), EVs/exosomes were first pelleted by ultracentrifugation at 120,000 × g for two hours at 4°C in a T-647.5 rotor (Sorvall wx Ultra series) and resuspended in 1 ml PBS. The samples were further purified by the application of Exo-Spin™ midi columns (Cell Guidance Systems), according to the manufacturer's instructions. High-purity

EV/exosome samples (determined by nanoparticle tracking analysis and protein quantification) were collected from fractions 7-14 and concentrated using a 10 kDa Amicon Ultra-0.5 device (Merck Millipore). All EV/exosome samples were stored at -80°C until use.

3.4 EV/exosome characterization

3.4.1 Nanoparticle tracking analysis (NTA)

NTA was applied to determine the size distribution and particle concentration of EV/exosome samples using the NanoSight LM10/LM14 system (Malvern) (Papers I-III). Three representative groups of exosome samples (Exo_P6, Exo_D3 and Exo_D21) were selected for analysis (Paper I). All EV/exosome samples were diluted with PBS to an appropriate concentration range before analysis. A syringe pump was used for automatic injection before each capture. The setting of the instrument was kept constant for all experiments. Three 60-second videos were captured for each dilution and these videos were analysed using particle tracking (Nanosight software 3.2). A representative histogram of each sample was selected to show the size distribution of particles. The mean and mode size of samples were averaged within each group of EVs/exosomes across all video replicates. The results were presented as the average mean size ± standard error of the mean (SEM) (nm) and modal size ± SEM (nm) of the nanoparticles. The total particle number in the stock EV/exosome solution was back-calculated based on the dilution ratio for NTA (Papers II and III). At least three different batches of each group of EV/exosome samples were analysed.

3.4.2 Western blot

Western blot was performed to detect the common exosomal markers (Papers II and III). Equal amounts of proteins (20 μg) from each EV/exosome sample were loaded and separated on a 10% Mini-Protean Precast Gel and transferred to PVDF membranes. The membranes were blocked with 5% non-fat milk powder in 1x Tris-buffered saline -Tween (TBST) at room temperature (RT) for 1.5 hours to avoid non-specific protein binding. Afterwards, the membranes were incubated overnight at 4°C with the following primary antibodies: mouse monoclonal anti-Tsg101 (clone 4A10, 1:500, Abcam), rabbit monoclonal anti-flotillin1 (EPR6041, 1:1000, Abcam), mouse monoclonal anti-CD63, (sc-5275, 1:500, Santa Cruz Biotechnology), rat

monoclonal anti-heat shock protein 70 (anti-Hsp70, clone 1B5, 1:1000, Enzo Life Sciences, only in Paper III) and rat monoclonal anti-Grp94 (clone 9G10, 1:1000, Enzo Life Sciences). After incubation with primary antibodies, the membranes were rinsed three times with TBST and incubated for one hour at RT with appropriate secondary antibodies: goat anti-mouse (1:2000, Santa Cruz Biotechnology), goat anti-rat (1:10000, Enzo Life Science) and goat anti-rabbit (1:10000, Santa Cruz Biotechnology). Finally, the membranes were rinsed three more times before being developed using the Immun-Star™-WesternC™ Chemiluminescent detection kit (Bio-Rad), according to the manufacturer's instructions. The specific proteins were detected by applying the ChemiDoc XRS1 system with image Lab Software (Bio-Rad).

3.4.3 Transmission electron microscopy (TEM)

In Paper I, three representative groups of exosomes from the expansion (P6), early (D3) and late (D21) osteogenic differentiation of hMSCs respectively were selected for visualisation by TEM. Ten microlitres of exosomes were loaded onto formvar carbon-coated grids, fixed in 2% formaldehyde, washed and immunolabelled with an anti-CD63 (BD Biosciences) antibody, followed by 10 nm gold-labelled secondary antibody (Sigma Aldrich). The exosomes were post-fixed in 2.5% glutaraldehyde, washed, contrasted with 2% uranyl acetate and air-dried before being visualised under TEM (Tecnai F20).

3.5 Delivery of EVs/exosomes

3.5.1 Delivery via suspension

The EV/exosome samples were delivered in suspension in Papers I-III. In Paper I, the delivery of exosomes in suspension was based on the ratio of donor and recipient cells (approximately 10:1). Recipient MSCs at P6 were seeded at a density of 18,000 cells/cm^2 in hFN-coated tissue culture treated polystyrene (TCPS) plates. After overnight attachment, the cells were treated with different groups of exosomes including Exo_P6, Exo_D3, Exo_D6, Exo_D9, Exo_D12, Exo_D15, Exo_D18 and Exo_D21. Exosome suspension (5 µl/well) was supplied every day and medium was replaced every third day.

The delivery of EVs/exosomes in suspension was based on particle concentration in Papers II-III. Two different doses, 3x10^9 particles/ml and 3x10^{10} particles/ml, of both LP EVs and HP EVs suspension were applied for

the treatment of recipient MSCs, both LP and HP MSCs, seeding at 2,000 cells/well in BGM (Paper II). In Paper III, recipient MSCs at P5 were seeded at low density (3,000 cells/disc) on Ti discs or TCPS and at high density (15,000 cells/disc) on Ti discs. Exosome suspension, both Exo1 and Exo2, was supplied in culture media, BGM or ODM, at a concentration of $4x10^{10}$ particles/ml. Culture medium supplemented with EV/exosome suspension was refreshed every three days (Papers II and III).

3.5.2 Delivery via immobilisation on titanium surfaces

In Paper III, exosome samples, both Exo1 and Exo2, were diluted to a concentration of $4x10^{10}$ particles/ml with DMEM-LG basal media. One hundred µl of exosome solution was added to each Ti disc and incubated overnight at 4°C to immobilise on the surface of Ti discs. The remaining solution was removed prior to seeding recipient MSCs on Ti discs.

3.6 EV/exosome labelling and uptake

In Paper I, exosomes (Exo_P6, Exo_D3 and Exo_D21) were labelled with a PKH67 Green Fluorescent Cell Linker Kit for General Cell Membrane Labelling (Sigma-Aldrich) [228]. PKH67-labelled exosome samples were diluted in culture medium and added to hMSCs seeded on chamber slides with a density of 20,000 cells/cm^2. After 24 h of incubation, cells were washed with PBS, fixed with 2% formaldehyde for 15 min and washed again before mounting with Vectashield HardSet Mounting Medium with DAPI (Vector Laboratories).

3.7 Staining of MSCs

3.7.1 Alizarin red staining

After exosome treatment for 21 d, hMSCs were fixed and incubated with Alizarin Red solution (pH 4.0- 4.3) for five minutes to evaluate the mineralisation (Paper I). The extra dye was removed by rinsing with distilled water. Samples were observed using light microscopy (Nikon TE2000-U).

3.7.2 Actin red staining

In Paper III, MSCs cultured on Ti discs were stained for the observation of cell morphology and the quantification of adherent cell numbers. Briefly, 24 hours after seeding, MSCs were fixed with 4% formaldehyde for 10 min, washed twice with PBS and stained with ActinRed 555 ready probes reagent (Thermo Fisher Scientific), according to the manufacturer's instructions. After staining, the Ti discs were transferred to glass slides and mounted with DAPI-containing mounting media.

3.8 Microscopy and image analysis

3.8.1 Wide field fluorescence microscopy and confocal microscopy

Confocal microscopy was applied in Paper I to examine the internalisation of exosomes in recipient MSCs. Briefly, after exosome labelling and incubation with cells, recipient MSCs were observed under a confocal microscope (Nikon C2 Confocal) to determine whether PKH67-labelled exosomes were internalised and optical sections were taken for collecting Z stacks to confirm the intracellular localisation of the PKH67-labelled exosomes. Wide field fluorescence microscopy was applied to observe the morphology and quantify the number of adherent MSCs cultured on Ti discs in Paper III (Eclipse E600, Nikon).

3.8.2 Scanning electron microscopy (SEM)

In Paper III, SEM was used to characterise the Exo-Ti surfaces and analyse the morphology of adherent MSCs on Ti discs under different culture conditions and their interaction with exosomes. For sample preparation, Exo–Ti discs were subjected to fixation and post-fixation steps in 2% paraformaldehyde for 15 minutes and 2.5% glutaraldehyde for 10 minutes and contrasted by 2% uranyl acetate for 15 minutes. Ti discs with adherent MSCs were fixed in modified Karnovsky's fixative for two hours at 4°C, rinsed with 0.15 M sodium-cacodylate buffer and stained with 1% osmium tetroxide for two hours at 4°C, following steps to briefly dehydrate samples in a grade ethanol series (70, 80, 90, 95 and 100% ethanol). All samples for SEM were allowed to air-dry and were gold sputter-coated before being examined in an Ultra 55 FEG

SEM (Leo Electron Microscopy Ltd) with settings of 5 kV accelerating voltage, 5 mm working distance and 30 μm aperture size.

3.8.3 Image analysis

In Paper III, images for the quantification of cell adhesion were captured from five representative areas of each disc using a 10x magnification objective. DAPI-stained cell nuclei were counted in NIS-Elements D software (version 4.12) from a total of 45 images in each culture condition. Eighteen representative images from each culture condition were selected for the analysis of cell adhesion area in the software Image J (*imagej.nih.gov/ij*). The cell adhesion area was quantified using the plugin Macros. The results were expressed as average cell number per counting area and average percentage of surface coverage by adherent cells labelled with actin.

3.9 Colorimetric assays

3.9.1 microBCA assay

A Micro BCA Protein assay kit was used to quantify MSC cellular and exosomal proteins in Papers II and III. The principle of the assay is that Cu^{2+} is reduced by protein in an alkaline environment to form Cu^{+1}, which is thereafter detected by bicinchoninic acid (BCA). The water-soluble complex formed by Cu^{+1} and BCA exhibits colour for absorbance at 562 nm, which is linear with the protein concentration. Briefly, the protein samples were diluted properly and mixed with working reagents following a two-hour incubation at 37°C. A series of albumin (BSA) dilutions served as standard for the calculation of unknown protein concentration.

3.9.2 Cell adhesion and growth assay

A cell-counting kit-8 (CCK-8) was used to indirectly quantify cell growth (Papers II and III) and the number of adherent cells in the different culture conditions (Paper III). The principle is that the amount of the formazan dye generated by the activity of dehydrogenases in cells is directly proportional to the number of living cells. In short, 10 μl of CCK-8 solution was added to each well containing 100 μl of culture and incubated for two hours at 37°C following the reading of the absorbance at 450 nm.

3.9.3 Lactate dehydrogenase assay

Lactate dehydrogenase (LDH) is a soluble cytoplasmic enzyme found in almost all living cells, which catalyses the back-forward conversion of lactate to pyruvate. LDH is extracellularly released when the plasma membrane is damaged, and thus the leakage/concentration of LDH in the cell culture supernatant can be used to indicate the cell viability (Paper II). The cell-free culture supernatant was incubated with a reaction mixture to generate a red formazan when nicotinamide adenine dinucleotide (NADH), produced during LDH catalyses the conversion of lactate to pyruvate, reduces a tetrazolium salt. The level of formazan was then measured at 490 nm and considered to be directly proportional to the LDH activity. The assay was performed at the accredited laboratory at Sahlgrenska University Hospital, Gothenburg, Sweden.

3.9.4 Alkaline phosphatase activity

ALP activity was determined for the evaluation of osteogenic differentiation (Papers I and II). Briefly, the cells were rinsed with DMEM-LG and lysed with mammalian protein extraction reagent (M-PER). The ALP activity in the cell lysate was then quantified using p-nitrophenylphosphate as a substrate. The quantity of p-nitrophenol produced was measured at 409 nm, which was considered directly proportional to the ALP activity. The assay was performed at the accredited laboratory at Sahlgrenska University Hospital, Gothenburg, Sweden.

3.9.5 Extracellular matrix mineralisation

The deposition of calcium/phosphate mineral indicates the mineralisation of the ECM in bone tissues. Calcium and phosphate levels in ECM produced by cells were therefore quantified to determine the degree of ECM mineralisation and in turn indicate the progress of osteogenic differentiation (Papers I and II). The cells were rinsed with PBS, fixed in Histofix™ for 30 min and demineralised by incubation in 0.6 M HCl on an orbital shaker for 24 h at room temperature. The supernatant was collected to measure the calcium and phosphate levels using the ortho-cresolphthalein complexone (OCPC) method and the colorimetric assay of phospho-vanado-molybdic acid, respectively. Under alkaline conditions, calcium and OCPC formed a purple-coloured complex that was detected at about 570 nm and was directly proportional to the amount of calcium presented. Similarly, phosphate and the reagent formed a yellow-coloured complex under acidic conditions, which was detected at 340

nm and was directly proportional to the concentration of phosphate. The assays were performed at the accredited laboratory at Sahlgrenska University Hospital, Gothenburg, Sweden.

3.10 Gene expression analysis

3.10.1 RNA extraction

In Paper I, the total RNA of exosomes (Exo_P6, Exo_D3 and Exo_D21) and their parent cells was extracted using a miRCURY™ RNA Isolation Kit (Exiqon), according to the manufacturer's protocol. A miRCURY LNA™ Universal RT microRNA PCR RNA Spike-in kit (Exiqon) was used to control the quality of the RNA extraction and cDNA synthesis for the microRNA qPCR experiment. The RNA quality was assessed using an Agilent 2100 Bioanalyzer. In Paper III, the total RNA of MSCs was extracted using a RNeasy Micro kit (Qiagen). All the samples were DNase treated during RNA extraction to reduce genomic DNA contamination.

3.10.2 Quantitative polymerase chain reaction (qPCR)

qPCR was performed to analyse the expression of target genes of interest in adherent MSCs (Paper III). The reverse transcription of RNA was performed using a Grandscript cDNA synthesis kit. In order to select the most stable reference genes, a panel of five reference genes was screened in one third of the total samples and the expression profiles were evaluated using geNorm software. The primers of target genes were designed with Primer Blast (https://www.ncbi.nlm.nih.gov/tools/primer-blast/), validated and purchased commercially. The target gene panel included Ki67, stromal cell-derived factor 1α (SDF-1α), BMP-2, ALP and RUNX2. The qPCR analysis was performed in 10 µl reactions in duplicate for each sample on a CFX96 real-time system (Bio-Rad) using Grandmaster SYBR mix. An inter-plate calibrator was used to compensate for the variation between different runs. The expression of target genes was first normalised with the mean Cq value of the most stable reference genes. The relative expression was calculated using the delta-delta Cq method and assuming 90% PCR efficiency (k* $1.9^{\Delta\Delta Cq}$) [229].

3.10.3 MicroRNA profiling

MicroRNA profiles of exosomes and their respective parent cells were analysed in Paper I. In short, 10 µl RNA of each sample was reverse

transcribed in 50 µl reactions using the miRCURY LNA™ Universal RT microRNA PCR, polyadenylation and cDNA synthesis kit (Exiqon). cDNA was diluted 50 x and assayed in 10 ul PCR reactions, according to the protocol for miRCURY LNA™ Universal RT microRNA PCR. A commercially available microRNA panel containing 372 selected microRNAs, the miRCURY LNA™ Universal RT microRNA PCR Human panel I (Exiqon), was used for the analysis. The microRNA PCR was assayed using an ExiLENT SYBR® Green master mix and performed in a Light Cycler® 480 Real-Time PCR System (Roche) in 384 well plates. The amplification curves were analysed using Roche LC software, both for the determination of Cq and for melting curve analysis. The microRNA profiling was performed by Exiqon in Denmark.

3.11 Mass spectrometry of proteomic analysis

Relative quantitative mass spectrometry was performed to determine the protein content of EVs/exosomes (Papers II and III). A reference sample was produced by pooling an equal amount of protein from each sample after homogenisation to represent the mean of all samples. An equal amount of protein from each sample, including the reference sample, was trypsin digested into peptides and subjected to an isobaric mass tagging reagent. Each sample was labelled with a unique tandem mass tag (TMT) and then mixed before sample fractionation and clean-up. The samples were analysed by nano-liquid chromatography coupled to an Orbitrap mass spectrometer (nLC-MS). In short, the mass-to-charge (m/z) ratio of the peptides was determined, followed by fragmentation for peptide sequence information and relative quantification. The analysis was performed in the MultiNotch mode where the quantification occurred in MS3, reducing the interference of co-isolated peptides. MS raw data for each set were merged during the database search for protein identification and relative quantification. The ratios of the reporter ion intensities, carrying in each TMT, in MS/MS spectra were used for the quantitation of peptide abundance. Only peptides detected with a specific ion reporter and unique to the specific protein were considered for quantification. Relative quantification was determined using the Proteome Discoverer. The nLC-MS analysis of protein profiles of EVs/exosomes was performed in the Proteomic Core Facility, University of Gothenburg.

3.12 Bioinformatic analyses

3.12.1 MicroRNA target prediction and pathway analysis

In Paper I, the microRNA raw data were background filtered and normalised, based on the average of the assays detected in all the samples. The top 50 microRNAs with the highest standard deviation were used for the generation of a heat map showing microRNA profiles in both hMSCs and exosomes. Hierarchical clustering was performed in R using scripts from Bioconductor. The clustering was performed with Pearson's correlation and average linkage clustering. To compare the microRNA expression between different exosome groups, a pairwise t-test was performed. A cut-off p-value of < 0.05 was used to sort the differentially expressed microRNAs for the prediction of target genes and pathways. The target genes of candidate microRNAs were predicted based on two algorithms, DIANA-microT-CDS and DIANA-TarBase v 7.0 [230]. The microT threshold was set at a score of 0.8 when microT-CDS algorithms were utilised for target gene prediction. DIANA-mirPath v.3 was applied to perform the hierarchical clustering of microRNAs and all known KEGG pathways based on their interaction levels using predicted microRNA targets provided by the DIANA-microT-CDS algorithm and/or experimentally validated microRNA interactions derived from DIANA-TarBase v7.0. The option gene union in the software was selected to merge the results. The graphical output of the program provides an overview of the pathways modulated by selected microRNAs, facilitating the interpretation and presentation of the analysis results. The statistical significance value associated with the identified biological pathways was calculated automatically by mirPath software, in which Benjamini and Hochberg's false discovery rate was applied, with the significant threshold set at a p-value of < 0.05. The software is available at http://snf-515788.vm.okeanos.grnet.gr/.

3.12.2 Functional prediction of identified proteins

In Papers II and III, the MS raw intensity data were globally normalised before the calculation of relative abundance. The relative abundance of each identified protein was indicated by the relative ratio calculated on the basis of the reference sample. The top 200 abundant proteins were sorted based on the mean of the relative ratio (Paper II). The distribution profile of the relative ratio was checked using a density histogram to evaluate whether the data were

normally distributed. As the data were skewed, log2 transformation was performed to achieve normal distribution for an analysis by Student's t-test to reveal the proteins with significantly different expression levels (Papers II and III). A cut-off p-value of < 0.05 and a fold change of $\geqslant 2$ were set as criteria to sort significantly different proteins for the prediction of functional features. A heatmap was generated to cluster the identified proteins. Statistical analysis and heatmaps were generated in an R environment (University of Auckland). Gene ontology (GO) analyses of cellular component, molecular function and biological process were performed to predict the functional feature of the top 200 abundant proteins (Paper II) and all identified proteins (Paper III). Ingenuity pathway analysis (IPA) was performed to predict the pathways regulated by significantly different proteins (Papers II and III). GO terms were evaluated using the online database Gene Ontology Consortium tool (AmiGo; http://geneontology.org). Pathways analysis was conducted using the Ingenuity Pathway Analysis tool (Ingenuity; Qiagen).

3.13 Statistical analyses

In Paper I, the exosome treatment experiment was regarded as a randomised block experiment with groups as treatments and biological batches as blocks. The data were analysed using the Proc Mixed SAS procedure and comparisons between groups were made using the estimate statement (SAS Version 9.3). The corrections for mass significance were made using the Bonferroni method. In Paper II, an unpaired Student's t test was applied to compare differences between two groups and one-way ANOVA followed by the two-stage linear step-up procedure of Benjamini, Krieger and Yekutieli post-hoc testing was applied for comparisons of more than two groups. Two-way ANOVA followed by Dunnett's multiple comparison test was used for the analysis of CCK8 data. The statistical analyses in Paper II were performed using GraphPad prism Version 7.2. A non-parametric Kruskal-Wallis test following a Mann-Whitney test was applied to compare the difference in size and concentration of particles in exosome samples (Paper I) and all statistical comparisons in Paper III, using SPSS Version 10. In all studies, a p-value of < 0.05 was considered statistically significant.

4 Summary of results

4.1 Paper I

In this study, we showed the time-course secretion of exosomes by hMSCs during the entire process of osteogenic differentiation. We further investigated the biological effects of these exosomes, in terms of the induction of osteogenic differentiation, and the underlying mechanisms by exploring exosomal microRNA contents (Figure 3).

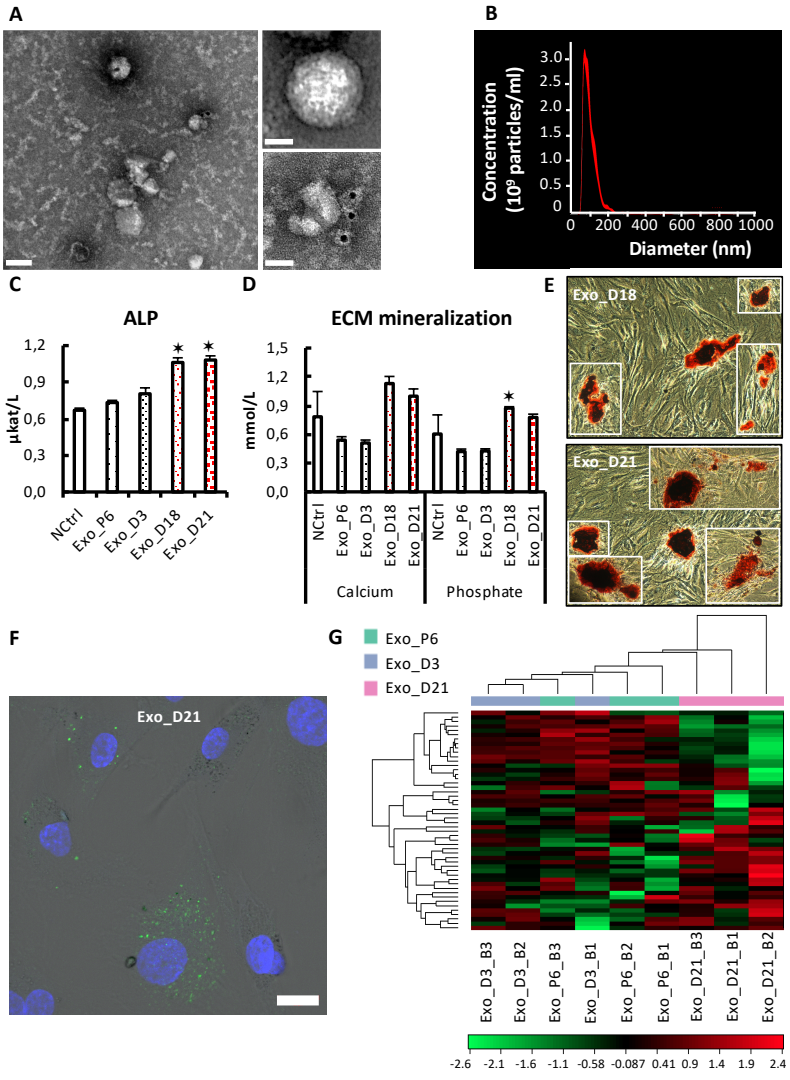


Figure 3. (A) TEM images of a representative group of exosomes, Exo_D21, show the size and shape of exosomes, and the selected exosomes with negative or positive labelling of CD63. Scale bar, 100 nm (low magnification) and 50 nm (high magnification). (B) An NTA histogram of a representative group of exosomes, Exo_D21, shows the average concentration and size distribution of all nine video captures. (C-E) An evaluation of osteogenic differentiation of hMSCs after exosome treatments via the determination of ALP activity (14d, C) and ECM mineralisation (21d, D and E). Selected control and/or treatment groups were presented. Significant difference based on Bonferroni-corrected p value < 0.05 was indicated by ✶. (F) The internalisation of a representative group of PKH67-stained exosomes, Exo_D21, in hMSCs. Scale bar, 20 μm. (G) Altered microRNA profiles of exosomes derived from expansion (Exo_P6), early (Exo_D3) and late (Exo_D21) stages of osteogenic differentiation of hMSCs.

The characterisation of hMSC-derived exosomes by TEM and NTA showed that different groups of exosomes shared a similar shape and size range. Irrespective of the source of parental cells, some exosomes were labelled with one or multiple anti-CD63 conjugated gold particles, while some were not labelled. The main population of the vesicles was distributed in a size range of 30-150 nm. Nevertheless, these exosomes induced the osteogenic differentiation of hMSCs in a stage-dependent manner. In detail, exosomes derived from hMSCs in various stages of osteogenic differentiation committed homotypic cells to differentiate towards an osteogenic lineage, but only exosomes from the late stages of osteogenic differentiation induced the mineralisation of ECM. Exosomes from expansion and both the early and late stages of osteogenic differentiation were internalised by a subpopulation of hMSCs. The internalised PKH67-labelled exosomes were localised in the cytoplasm of hMSCs. The internalisation of exosomes was irrespective of the parental source of exosomes, but it may be dependent on the recipient cells.

MicroRNA profiles of exosomes and their respective parent cells during expansion and the early and late stages of osteogenic differentiation were altered. However, comparisons of the top 50 abundant microRNAs in each sample revealed a high correlation of microRNA content between exosomes and their respective parent cells. Exosomal microRNAs were differentially expressed in the late stage of osteogenic differentiation compared with expansion in the early stage of differentiation. The set of differentially expressed exosomal microRNAs from the late stage of osteogenic differentiation were osteogenesis related. Target prediction demonstrated that these microRNAs enriched pathways involved in the regulation of osteogenic differentiation and general mechanisms relating to the way exosomes exert their functions, such as “Wnt signalling pathway” and “endocytosis”.

4.2 Paper II

In this study, we determined whether *in vitro* ageing affected the release and

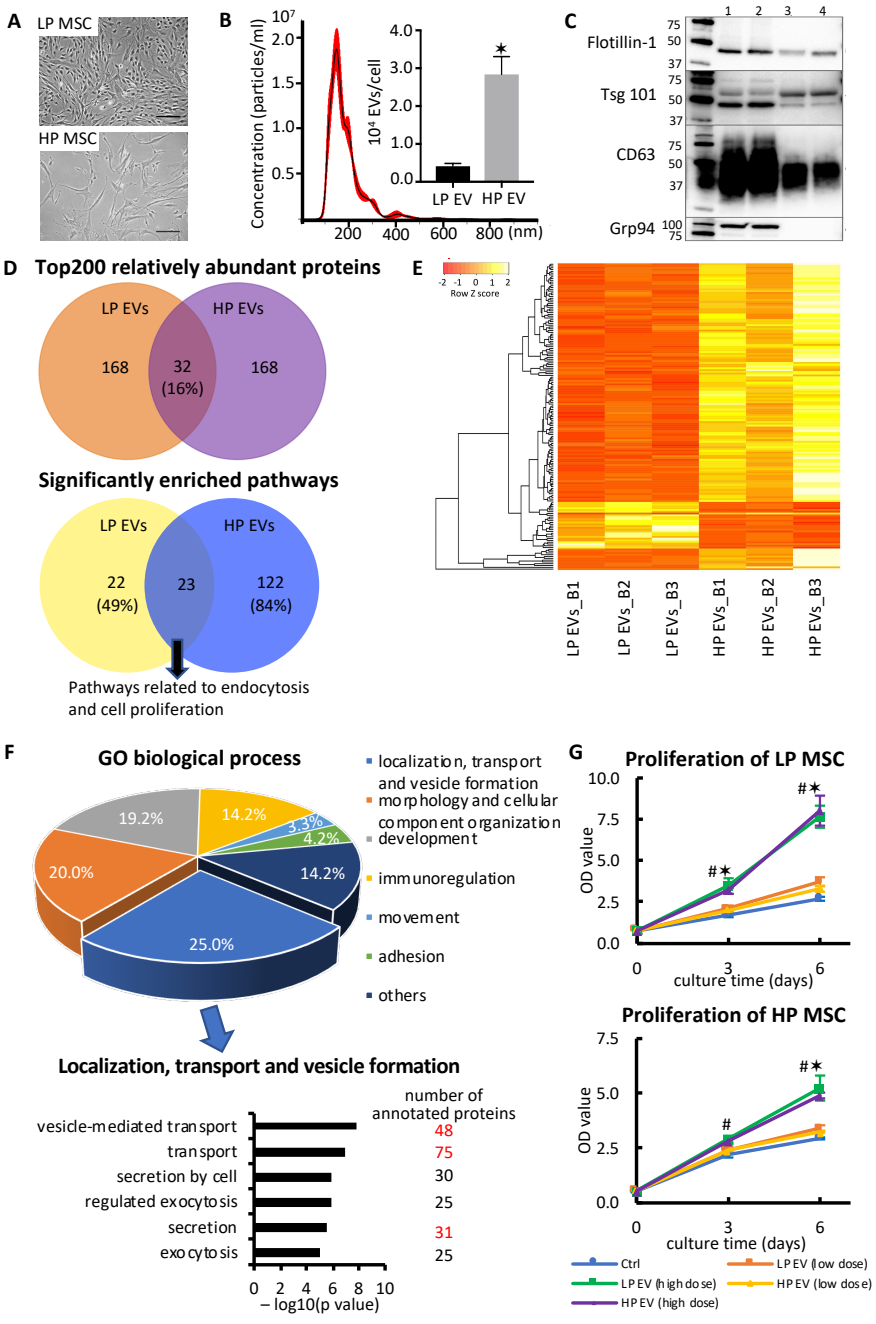


Figure 4. (A) Representative microscopic images show that in vitro ageing results in senescence-like morphology in HP MSCs. Scale bar, 500 μm. (B) An NTA histogram of a representative group of EVs, HP EV, shows the average size distribution of EVs. The inner column chart shows the number of EVs released per LP and HP MSCs. ✶ indicates a significant difference ($p < 0.05$) between LP EVs and HP EVs. (C) Representative Western blot images show the expression of EV-associated proteins, Flotillin-1, Tsg101 and CD63, and the absence of endoplasmic reticulum protein Grp94 in EVs. Lanes 1-4 are proteins from LP MSCs, HP MSCs, LP EVs and HP EVs, respectively. (D) The Venn diagram (top panel) shows that only 16% of the top 200 relatively abundant proteins are shared between LP EVs and HP EVs. Pathways related to endocytosis and cell proliferation are significantly enriched by both top 200 relatively abundant proteins in LP EVs and HP EVs. (E) A set of proteins has significantly different expression levels in LP EVs and HP EVs. (F) The pie chart shows the functional classification of the GO biological process enriched by significantly different proteins between LP and HP EVs. The bar chart shows the top six significantly enriched terms related to localisation, transport and vesicle formation. (G) Both LP EVs and HP EVs promote the proliferation of LP MSCs (top panel) and HP MSCs (bottom panel). The error bar indicates SEM, ✶ and # indicate a significant difference ($p < 0.05$) between controls and LP EVs (high dose), and between controls and HP EVs (high dose), respectively.

protein cargoes of EVs from MSCs, and the effects of EVs on MSC growth (Figure 4).

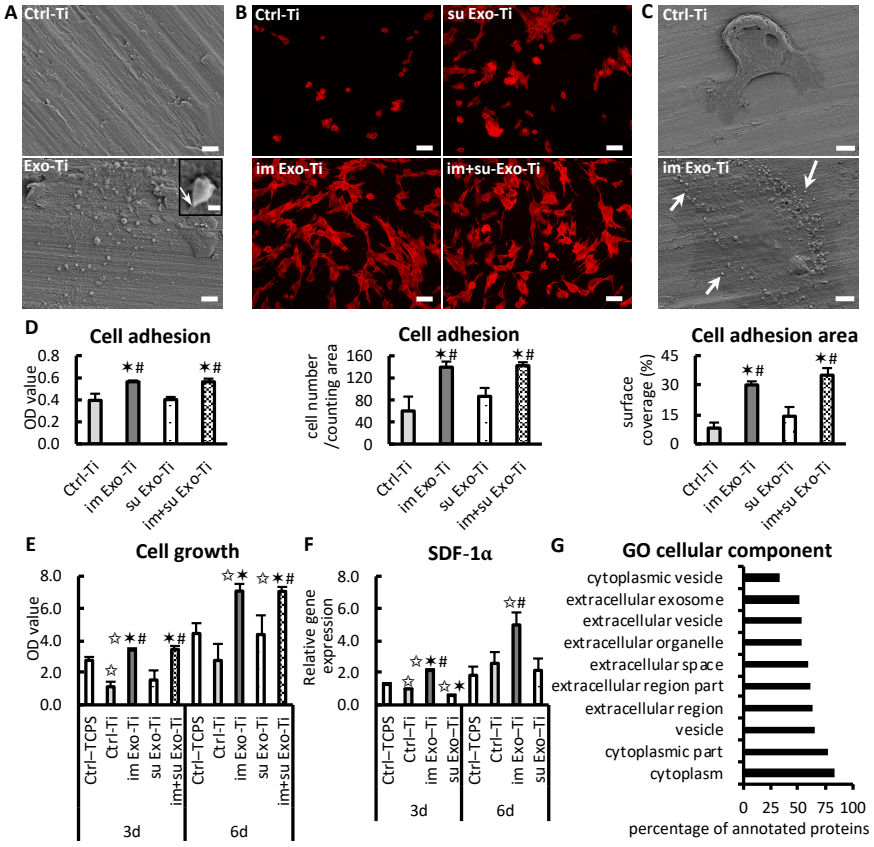
The characterisation of LP and HP MSCs showed that *in vitro* ageing retained the phenotypic signature of MSCs but resulted in morphological changes in HP MSCs, indicating cell senescence, and reduced regenerative capacity, in terms of proliferation and osteogenic differentiation capacities. EVs released from both LP and HP MSCs shared characteristics in terms of size and typical exosomal protein markers. However, HP MSCs possessed an ability to secrete more EVs than LP MSCs.

A proteomic analysis of the protein contents of both LP and HP EVs identified proteins that were EV associated. LP EVs and HP EVs shared a small portion of the top 200 relatively abundant proteins. The functional characteristics, in terms of GO cellular compartment, GO molecular function, GO biological process and Ingenuity canonical pathways, of the top abundant proteins in LP EVs and HP EVs were similar but not identical. Of particular note, some of the pathways significantly enriched by both the top abundant proteins in LP EVs and HP EVs were endocytosis related and involved in the regulation of cell survival and proliferation, such as the “STAT3 pathway”. A set of proteins with a significantly different expression between HP EVs and LP EVs were determined. These proteins were found to be mainly involved in biological

processes related to transport and secretion and were predicted to regulate pathways related to cellular morphology, growth/proliferation and development. The functional validation of LP and HP EVs showed that both EVs were capable of promoting MSC proliferation in a dose-dependent and recipient cell-associated manner. Moreover, both LP and HP EVs revealed enhanced cell viability of HP MSCs, indicated by reduced LDH activity.

4.3 Paper III

In this study, the exosomes, Exo1 and Exo2, were delivered via immobilisation on Ti surfaces or suspension in the culture. The biological effects of exosomes



	Top 3 terms (Fold enrichment)
adhesion	cell adhesion molecule binding (5.5)
	cadherin binding (5.8)
	cytoskeletal protein binding (2.2)
Structure and morphology	structural molecule activity (2.8)
	ECM structural constituent (4.7)
	structural constituent of cytoskeleton (4.0)
GF and GFR binding and activity	growth factor binding (4.1)
	PDGF binding (8.2)
	TGF beta binding (5.0)

Figure 5. In this summary figure, (A-F) treatment with Exo1 is selected as representative. (A) Representative SEM images of control (Ctrl-Ti) and exosome-immobilised (Exo-Ti) Ti surfaces show nanoscale vesicles with a heterogeneous size on the Exo-Ti surface. The inner plot shows the direct interaction of a single vesicle with the Ti surface. The white arrow indicates the attachment point of a vesicle on the Ti surface; scale bar, 1μm; scale bar of inner plot, 100 nm. (B) Representative fluorescence microscopic images show the distribution and morphology of MSCs adhering to different surfaces. The red colour indicates actin staining; scale bar, 100 μm. (C) Representative SEM images show the early morphology of MSCs adhering to Ti surfaces with or without immobilised exosomes. White arrows indicate the localisation and distribution of nanovesicles; scale bar, 5 μm. (D) The quantification of MSC adherence through the enzyme activity of live cells (left panel), the microscopic counting of cells (middle panel) and the image analysis of surface coverage of adherent cells (right panel). (E) The quantification of MSC growth on different surfaces on day 3 and day 6. (F) The gene expression of cell migration marker SDF1α. (D-F) The error bar indicates the standard error of the mean; ☆, ✶ and # indicate a significant difference compared with Ctrl-TCPS, Ctrl-Ti and su Exo-Ti, respectively ($p < 0.05$). (G) The top 10 significantly enriched GO cellular component terms of the global proteome of exosomes. (H) The functional classification of the significantly enriched GO molecular function terms. The top three significant terms related to adhesion, structure and morphology and GF and GFR binding and activity are listed in the table. Ctrl, control; im, immobilised; su, suspension; Exo, exosome; GF, growth factor; GFR, growth factor receptor

on the behaviour of MSCs on the Ti surfaces were further evaluated. The proteome of exosomes was explored to provide indications for the underlying mechanisms of observed effects (Figure 5).

Exosomes, from the culture of MSCs under expansion (Exo1) or osteogenic differentiation (Exo2), were isolated and characterised. Both exosomes exhibited a similar size distribution and presented typical exosomal protein markers. The overnight incubation of exosomes with Ti surfaces and examination by SEM showed that both exosomes were immobilised on Ti surfaces and interacted with Ti surfaces in a similar manner.

Exosomes were delivered in three ways to evaluate the biological effects on the behaviour of MSCs cultured on Ti surfaces: (i) immobilisation on Ti surfaces, (ii) suspension in the culture and (iii) a combination of (i) and (ii). Irrespective of the parental source of exosomes and whether there was a supplement of exosomes in suspension, the immobilisation of exosomes promoted the adhesion of MSCs to Ti surfaces.

Exosomes influenced the early morphology of adherent MSCs on Ti surfaces differently, depending on the routes of delivery. The delivery of exosomes via

immobilisation promoted the spreading of adherent MSCs, whereas the adherent MSCs on Ti surfaces in the culture with the delivery of exosomes only in suspension shared morphologies comparable to those of adherent MSCs on control Ti surfaces.

Exosomes increased the growth of MSCs on Ti surfaces. The results at cellular level demonstrated that the increase in MSC growth was mainly regulated by immobilised exosomes, irrespective of the parental sources of the exosomes. However, the gene expression of Ki67 on day 3 was upregulated by a suspension of Exo1 in the culture in comparison to the control or suspension of Exo2. The suspension of exosomes, Exo1 or Exo2, upregulated the gene expression of BMP2 on day 3 compared with the immobilisation of Exo1 or Exo2. Taken together, the results suggest some complexity of regulation on MSC growth by exosomes, which was dependent on the delivery routes and associated with the parental sources of exosomes.

Exosomes differentially regulated the expression of cell recruitment factor SDF-1α in MSCs cultured on Ti surfaces, which was dependent on the routes of delivery. Irrespective of the parental sources of exosomes, the delivery of exosomes via immobilisation significantly and continuously upregulated SDF-1α expression, while the delivery of exosomes in suspension downregulated SDF-1α expression.

Exosomes regulated the expression of osteogenic differentiation markers, RUNX2 and ALP, in MSCs under different culture conditions. Exosomes, irrespective of their delivery routes and parental sources, significantly enhanced the expression of RUNX2 on day 3 in MSCs cultured in ODM. Regardless of the culture conditions, BGM or ODM, the expression of ALP on day 3 was shown to be downregulated by immobilised exosomes. However, the suspension of Exo2 upregulated ALP expression on day 3 in MSCs cultured on BGM.

A proteomic analysis of protein cargoes of MSC exosomes showed that the identified proteins were mainly exosome associated. The prediction of the functional feature of the global proteome revealed that the molecular function of identified proteins was related to adhesion, structure and morphology, and GF activity. A quantitative comparison determined a set of proteins with a significantly differential expression level between Exo2 and Exo1. Pathway analysis predicted that this set of proteins would significantly enrich pathways

regulating cell morphology, adhesion, migration, growth and differentiation. Collectively, the MSC exosome proteome contains a robust profile of proteins with predicted regulation of MSCs behaviour.

5 Discussion

In the present thesis, EVs/exosomes were isolated by ultracentrifugation or chromatography-based Exo-spin isolation from MSCs cultured under three different conditions: self-renewal growth, osteogenic differentiation and ageing. The contents of EVs/exosomes, in terms of exosomal microRNA and proteins, were explored by applying qPCR-based microarray and mass spectrometry, respectively. Furthermore, the regenerative effects of EVs/exosomes, in terms of proliferation and osteogenic differentiation, and the effects of titanium surface-immobilised exosomes on the cellular behaviours of MSCs were evaluated by complementary methods. Interesting results have been obtained by design of *in vitro* experiments with specific aims, which are believed to partially contribute to our understanding of the functions of MSC-derived EVs/exosomes, and their potential future application as a regenerative tool. However, several methodological challenges also exist, including the purity of collected EV/exosome samples using the currently available techniques, the difficulty associated with the direct translation of the current obtained results to a physiological situation, as a single cell type was cultured to collect EV/exosome samples, and the lack of support from an *in vivo* study to confirm the observed *in vitro* effects. In this section, the main findings, as well as the limitations of the present thesis, will be critically discussed.

5.1 Methodological consideration

5.1.1 Cell types, sources and culture conditions

MSCs have been implicated as a promising candidate for regenerative medicine because of their multipotent differentiation capacities and immunomodulatory functions. Accumulating evidence indicates the functions of MSCs on their paracrine secretome, among which EVs/exosomes were found to be a novel and important member. MSC-derived EVs/exosomes have been therefore the focus of the present thesis. However, a large body of studies have suggested that the functions as well as the paracrine secretion of MSCs are not constitutive or fixed, but rather the result of crosstalk with the surrounding microenvironment that consists of various cell types and secreted factors. It is therefore suggested that MSCs have different phenotypes and are believed to be able to switch their phenotypes under physiological conditions. The phenotype changes may consequently lead to the modification of the MSC

secretome including EVs/exosomes. Thus, one of the limitations of the present thesis is the utilisation of a single cell type culture, MSC, for the collection of EV/exosome samples. The culture lacks interaction with other cell types, such as monocytes/macrophages, ECs and fibroblasts, which could have modified the changes of MSC-secreted EVs/exosomes, in terms of the amount and contents, and, as a result, it may not reflect the true or completed physiological effects. To test such hypothesis, a co-culture model can be applied. Indeed, the non-contact co-culture of MSCs with HUVECs has shown that the expression of exosomal pro-angiogenic microRNAs, such as miR-30b, mir-30c, miR-424 and let-7f, was reduced when compared with the co-culture of homotypic MSCs but increased in comparison with the co-culture of homotypic HUVECs [180]. This result indicates a possibility of changing exosomal contents in a co-culture system. However, no solid conclusion can be drawn, because both HUVECs and MSCs secrete exosomes and the reduction in exosomal pro-angiogenic microRNAs may be due to a contribution by HUVEC-secreted exosomes with less pro-angiogenic microRNAs, rather than an altered secretion of MSC exosomes. This observation also sheds light on one of the challenges involved in applying a co-culture system to investigate exosome secretion, as almost all cell types secrete relatively indistinguishable exosomes, due to their similarity with respect to physical features and the difficulty to separate by the current isolation techniques. One possible solution is to label heterotypic cells with different fluorescent lipid dyes, such as PKH67/PKH26 and DiI/DiO/DiD, however, it is not clear whether the pre-labelling of the parental cell membrane would affect the biogenesis of EVs/exosomes, as growing evidence indicates the potential roles of lipids and their activity in the biogenesis and functions of EVs/exosomes [19, 25, 28, 34, 155].

Nevertheless, instead of co-culturing MSCs with other cell types, the preconditioning of MSCs with a stimulus, such as cytokines or tissue extract, and the investigation of the subsequent secretion of MSC EVs/exosomes has indirectly confirmed the effects of components in the surrounding microenvironment. For example, the pretreatment of MSCs with pro-inflammatory cytokines, IFNγ combined with TNFα, did not affect the amount and size of secreted exosomes but changed the expression of exosomal microRNA involved in M2 macrophage polarisation and resulted in enhanced anti-inflammatory effects of prestimulated MSC exosomes compared with unstimulated MSC exosomes [231]. In line with this observation, another study showed dramatic changes in MSC exosomal protein contents when

MSCs were pretreated with IFNγ [207]. In addition, Xin *et al.* showed that the level of MSC exosomal miR-133b, which was responsible for the therapeutic effects of MSC exosomes in a rat stroke model, significantly increased after the exposure of MSCs to the cerebral tissue extract from rats suffering from stroke in comparison with that from normal rats [82]. This result suggests that the therapeutic effect of MSC-secreted EVs/exosomes in situ is probably stronger than that induced by injected EVs/exosomes from *in vitro* cultured MSCs. Taken as a whole, these observations indicate the necessity to take into consideration of the influence of other cell types and secreted factors when investigating the secretion and function of MSC-derived EVs/exosomes.

In the present thesis, we used MSCs derived from adipose tissues. MSCs can be isolated from various tissue sources, of which AT-MSC and BM-MSC are two of the most commonly used. The comparison of AT-MSC and BM-MSC, in the context of their morphology, surface phenotypic signature, self-renewal property and multipotent differentiation capacities, has been extensively studied [95-97]. Previous studies have shown that, although AT-MSC and BM-MSC shared similar morphology, self-renewal property and surface phenotypic features, AT-MSCs have a less potent osteogenic differentiation capacity in comparison with BM-MSCs in *in vitro* experiments [96, 97]. On the other hand, evidence has been presented to show that the *in vivo* transplantation of AT-MSCs is able to regenerate bone [232]. Moreover, another study revealed that the inferior osteogenic potential of AT-MSCs can be partially compensated for by the addition of platelet-rich plasma [95]. In addition, AT-MSCs are less affected by the donor age than BM-MSCs and the results obtained are therefore more reproducible. Together with the higher abundance and easier accessibility, AT-MSCs may be a better alternative to BM-MSCs based on a tissue engineering concept. Nevertheless, few studies have been performed to compare the secretome of AT-MSC and BM-MSC. Interestingly, Baglio *et al.* compared the exosomal RNA contents of both AT-MSCs and BM-MSCs and showed that AT-MSC exosomes and BM-MSC exosomes were similar in the RNA class distribution and composition [168]. Moreover, the majority of the top 20 abundant microRNAs (75%) were shared between AT-MSC exosomes and BM-MSC exosomes, whereas a comparison of the tRNA species in AT-MSC exosomes and BM-MSC exosomes revealed MSC tissue origin-related sorting [168]. Given the pivotal impact of MSC exosomal microRNAs on MSC exosome-mediated therapeutic effects, it can be hypothesised that AT-MSC exosomes may possess therapeutic effects, at least the exosomal microRNA content-dependent effects, similar to those of

BM-MSC exosomes. In addition, another study also showed that both AT-MSC exosomes and BM-MSC exosomes exhibited NEP activity, however, AT-MSC exosomes appeared to be more enriched with NEP than BM-MSC exosomes [162]. It was therefore suggested that AT-MSC exosomes were likely to have more pronounced therapeutic effects on AD. On the other hand, AT-MSC exosomes showed opposite effects on glioblastoma cell proliferation compared with BM-MSC exosomes [233]. Although exosomes from both sources were internalised in glioblastoma cells, AT-MSC exosomes promoted the proliferation of glioblastoma cells and did not show significant effects on the apoptosis of glioblastoma cells, whereas BM-MSC exosomes inhibited the proliferation and induced the apoptosis of glioblastoma cells [233]. Collectively, these results indicate the similarity yet not identical characteristics of AT-MSC exosomes and BM-MSC exosomes, which necessitates further more comprehensive studies.

Although the *in vitro* culture condition is less complex than the *in vivo* environment, an *in vitro* culture model is often useful to investigate and identify cellular and molecular mechanisms, diminishing the influence of multiple parameters and offering the flexibility to modify conditions based on specific experimental aims. In Paper II, an *in vitro* ageing model based on continuous passaging was developed to investigate the impact of ageing on MSC EV/exosome secretion. Previous studies have demonstrated the age-related changes of MSCs in both an *in vivo* ageing model (donor age) and an *in vitro* ageing model (passage number). Justesen *et al.* compared the colony formation, osteogenic and adipogenic differentiation capacities of human MSCs isolated from young donors, elderly healthy donors and elderly patients with osteoporosis [234]. The short-term (two weeks) culture of these different groups of MSCs showed no significant changes in the total number of colonies and no significant correlation between the gene expression level of osteogenic and adipogenic markers. Partially in line with these results, it was shown that, in the rat, the proportion of MSCs among the bone marrow cells was reduced as age increased and changes neither in phenotype nor in osteogenic and adipogenic differentiation capacities were detected [235]. Interestingly, another study established the long-term culture of MSCs isolated from both young and old donors and showed that MSCs from old donors exhibited a reduced maximal life span indicated by *in vitro* total PD and accelerated senescence evidenced by an increase in the number of senescence-associated β-galactosidase positive cells per PD in comparison with that of young donors [236]. Moreover, the early passage of MSCs from both young and old donors

had similar differentiation potentials indicated by the amounts of mineralised matrix *in vitro* and bone formation *in vivo*. Whereas, the late passage of MSCs from both young and old donors both showed decreased differentiation capacities, suggesting a culture age-dependent but donor age-independent changes [236]. Therefore, an *in vitro* ageing model appears to be more potent and practical than an *in vivo* ageing model for studies of the impact of ageing on the regenerative capacities of MSCs, in terms of proliferation and differentiation. Nevertheless, it is important to be aware that the *in vitro* ageing of MSCs may not exactly reflect their *in vivo* changes, as the complexity of the microenvironment *in vivo* indeed influences different functional aspects of MSCs [237]. It is therefore of critical importance to evaluate to what degree the *in vitro* results can be translated to physiological conditions *in vivo* in future research.

5.1.2 Purity of MSC-derived EVs/exosomes

The purity of EVs/exosomes is critical for the downstream studies; however, it is currently not possible to achieve absolute purified EVs/exosomes, due to the limitation of available techniques. One of the potential sources of contamination of EVs/exosomes recovered from the CM of a cell culture is the serum-derived EVs. Efforts were therefore made to eliminate contamination of serum EVs through the overnight centrifugation of media with 20% serum in Paper I, as previously recommended [55]. Nevertheless, such solution is only able to partially solve the problem, as serum is also enriched with proteins that cannot be efficiently eliminated and are therefore are often co-isolated with EVs/exosomes. On the other hand, prolonged centrifugation may be an option to remove more serum proteins, but the depleted serum may result in negative effects on cell growth due to the lack of nutrients. In an attempt to overcome this challenge, we adapted to serum-free culture in Papers II and III with the aims of obtaining relatively purified EV/exosome samples by collecting CM from a good quality cell culture and avoiding possible exogenous contamination.

Although it is not achievable to isolate absolute purified EV/exosome samples, the isolation method has a critical impact on the purity of samples. In the present thesis, two isolation methods have been applied: differential ultracentrifugation and a chromatography-based commercial isolation kit, Exo-spin. Differential ultracentrifugation is the most commonly used isolation method and it has been shown to result in relatively successful isolation in

terms of the recovery and specificity of EVs [238]. Chromatography-based isolation has been claimed to effectively eliminate protein complexes and the non-pelleting procedure benefits for the preservation of EV/exosome integrity [57]. According to the guideline of minimal information for studies of EVs 2018 (MISEV2018), these methods are recommended as isolation methods with intermediate recovery and specificity [13]. Moreover, we have applied multiple, complementary techniques to further confirm that the isolated samples are EVs/exosomes. Our characterisation data indicate that our selected isolation methods result in EV/exosome samples with reasonable purity for the downstream analysis in the present thesis. However, we should also be aware of the existence of protein contamination and the uncompleted separation of EV subtypes.

5.1.3 Normalisation of MSC-derived EVs/exosomes

The delivery of MSC-derived EVs/exosomes in the present thesis aimed to compare quantitatively the functions of different groups of EVs/exosomes. Currently, the normalisation of EVs/exosomes can be based on the characteristics of isolated EVs/exosomes, including particle counts, the total amount of a biomolecule type in the EVs, such as proteins, nucleic acids or lipids, and the content or activity of specific EV-associated molecules [13]. On the other hand, the normalisation can be based on the characteristics of the source material, such as the initial volume of biological fluid and the number of secreting cells [13]. In Paper I, we used a rough ratio of exosome donor and recipient cells for the normalisation of different groups of exosomes. The reason behind this was an attempt to avoid the bias encountered from the serum protein contamination. In Papers II and III, on the other hand, particle number-based normalisation was applied, as we observed that the number of EVs/exosomes recovered from MSCs cultured under consistent conditions appeared to be constitutive. Moreover, particle number-based normalisation may partially avoid the bias caused by potential protein contamination. Nevertheless, neither normalisation method applied in the present thesis reflects the physiological conditions. The functions of MSC EVs/exosomes that have been observed should therefore be mainly interpreted from an application point of view rather than directly translated into a physiological situation.

5.2 Contents of MSC-derived EVs/exosomes

The microRNA (Paper I) and protein contents (Papers II and III) of EVs/exosomes secreted from MSCs cultured under different conditions were explored in the present thesis to obtain an insight into the tentative mechanism for the MSC-derived EVs/exosome-mediated biological effects.

5.2.1 microRNAs

In Paper I, the microRNA contents of exosomes, Exo_P6, Exo_D3 and Exo_D21, representing exosomes secreted by self-renewal and early and late osteogenic differentiated MSCs respectively, were profiled using a selected microRNA panel. A qualitative comparison of the top 50 abundant microRNAs in exosomes and their respective parental cells revealed a high correlation, indicating that the microRNA signature of exosomes reflected their cellular origin. However, a further quantitative comparison of exosomal and cellular microRNA profiles revealed altered microRNA expression levels, indicating a possible sorting mechanism regulating the packaging of microRNAs during exosome biogenesis and secretion. This result is in line with the previous observation that EVs from MSCs and human liver stem cells respectively contained a pattern of microRNAs shared with their cell of origin, suggesting that microRNA patterns may be used as a signature to define the cellular origin of EVs [170]. Supporting this theory, several studies showed that exosomes, secreted from transgenic modified MSCs, mirrored their parental MSCs with the overexpression of a specific microRNA [239-245]. On the other hand, although the most abundant microRNAs were conserved in the cells and transported by EVs, the expression of a specific set of microRNAs was selectively enriched or absent in EVs in comparison to their parental cells, which indicated a selective mechanism of RNA packaging into EVs [168-170].

Moreover, a comparison of the microRNA profiles of exosomes secreted from MSCs undergoing self-renewal and various stages of osteogenic differentiation revealed altered expression pattern. MicroRNA profiles of exosomes from self-renewal and early osteogenic differentiated MSCs were clustered together, whereas exosomes from late osteogenic differentiated MSCs exhibited a more different expression pattern. A specific set of microRNAs was shown to be expressed differentially in exosomes from late osteogenic differentiated MSCs in comparison to that from self-renewal or early osteogenic differentiated MSCs. Consistently, a previous study also

showed differentially expressed exosomal microRNA contents during a shorter period, the first week of osteogenic differentiation [246]. In addition, it has been shown that the preconditioning of MSCs, such as stimulation with proinflammatory cytokines [231] and hypoxia [204, 247], modified MSC exosomal microRNA contents. These findings indicate that the exosomal microRNA contents are regulated by the exosome secretion conditions, i.e. under osteogenic induction.

Interestingly, the specific set of differentially expressed microRNAs in exosomes from late osteogenic differentiated MSCs were shown to be osteogenesis related. MiR-21 is one of the significantly increased microRNAs in exosomes from late osteogenic differentiated MSCs, which has been shown to target osteogenic inhibitor Spry1 and pluripotency marker Sox2, leading to osteogenic lineage commitment and differentiation [248, 249]. In addition, miR-21 regulates the PI3K-AKT-GSK3β pathway to stabilise β-catenin and activate RUNX2 transcription to promote the osteogenic differentiation of MSCs [250]. Evidence has been provided that the pro-osteogenic effects of miR-21 *in vivo* enhance bone formation in osteoporosis [251] and accelerate bone fracture healing [116]. Another top increased exosomal microRNA, miR-10b, has been shown to promote MSC migration [252], which may thereby contribute to MSCs homing to a bone injury site to promote bone regeneration. In contrast, the dramatically decreased exosomal microRNAs, such as miR-31, miR-221 and miR-144, have been found to regulate osteogenic differentiation negatively. MiR-31 downregulates RUNX2 downstream, Osterix [253] and special AT-rich sequence-binding protein 2 (SATB2) [254], to inhibit osteogenic differentiation. The inhibition of miR-31 in MSCs improves bone healing *in vivo* [255, 256]. Interestingly, miR-31 has been shown to be delivered via senescent EC-derived EVs to MSCs, leading to the downregulation of Wnt receptor FZD3 and thereby inhibiting the osteogenic differentiation of MSCs [257]. While reduced miR-221 was shown to upregulate ICAM1 to increase the migration and adhesion of MSCs, resulting in accelerated bone healing *in vivo* [258]. The anti-osteogenic effect of miR-144 is mediated by targeting Smad4 to inhibit osteogenic differentiation [259]. Taken together, a large body of studies have implicated the osteogenesis-related functions of the currently identified differentially expressed exosomal microRNAs. A functional prediction of these microRNAs revealed enriched pathways involved in the regulation of osteogenic differentiation, such as Wnt, MAPK, Hippo, mTOR and FoxO pathways, as well as pathways implicating the general function of exosomes, such as endocytosis, regulation of actin

cytoskeleton and protein digestion and adsorption. To summarise, the reduced expression of anti-osteogenic microRNAs and the increased expression of pro-osteogenic microRNAs in exosomes from late osteogenic differentiated MSCs implicated the functions of these exosomes on the induction of osteogenic differentiation and mineralisation.

5.2.2 Proteins

In Papers II and III, the global proteome of EVs/exosomes, LP EVs and HP EVs, representing EVs secreted from "young" and "aged" MSCs, and Exo1 and Exo2, representing exosomes secreted by self-renewal and osteogenic differentiated MSCs respectively, were profiled by relative quantitative MS. A total of 1,942 proteins were identified in the EV/exosome proteome, among which the majority have been reported to be EV associated, according to EVpedia (http://evpedia.info), one of the largest EV databases. Furthermore, GO cellular component analysis revealed that the significantly enriched terms with the highest fold enrichment were extracellular vesicles, extracellular exosomes and extracellular organelles. In line with the comparison with the EVpedia database, this finding again indicated that the identified proteins were mainly EV/exosome associated, as well as providing evidence that the isolation methods that were used recovered EVs/exosomes with a sufficiently high purity. Although the proteins identified among the proteome of each individual group of EVs/exosomes were overlapped, the majority of these proteins have differential expression levels, indicating a cellular origin-conserved but cellular status-specific sorting mechanism. Supporting this hypothesis, a previous study identified proteins in AT-MSCs and their secreted EVs and the comparison of cellular and EV proteomes revealed an overlap of about 90%, together with a small set of enriched proteins in the EV proteome [260]. Moreover, a recent study identified a total of 1,927 proteins in MSC exosomes across both culture conditions, serum starvation and serum starvation plus low oxygen conditions [261]. A differential expression analysis of exosomes from these two conditions revealed few significant differences in exosomes between these two conditions. The cellular origin-conserved packaging of proteins in EVs was also evident in other cell types. For example, the proteomes of EVs secreted by glioblastoma cells with or without the transgenic expression of EGFRvIII showed an 87% overlap of identified proteins [262]. However, a comparison of exosomal proteomes of MSCs and cancer cells revealed low correlation and a distinct pattern [32]. Taken together, the proteomic results from our studies and those of others support the

proposed cellular origin-conserved but cellular status-specific protein-sorting mechanism in EVs/exosomes.

The qualitative comparison of the relative abundance of identified proteins in LP EVs and HP EVs revealed that the majority of the top 200 relatively abundant proteins differed in LP EVs and HP EVs and only 32 proteins of the abundant proteins were shared between LP EVs and HP EVs (Paper II). This result indicated a specific sorting mechanism of the abundant protein profiles in LP EVs and HP EVs, which may be associated with the changes in their parental cells during the *in vitro* ageing. Furthermore, the functional characteristics of the top 200 relatively abundant proteins were predicted by GO analyses and IPA. The abundant proteins in LP EVs and HP EVs respectively significantly enriched various numbers and sets of GO functional terms and pathways. However, a portion of the enriched terms overlapped between LP EVs and HP EVs. It is particularly interesting that, among the enriched pathways, four pathways related to endocytosis, including "clathrin-mediated endocytosis signalling", "caveolar-mediated endocytosis signalling", "phagosome formation" and "phagosome maturation", and one pathway involved in the regulation of cell survival and proliferation, the "STAT3 pathway", were shared between LP EVs and HP EVs. This result suggested that, although the profile and predicted functional features of the abundant proteins in LP EVs and HP EVs were not identical, they may possess common functions for the regulation of endocytosis and cell proliferation. Indeed, it has been shown that MSC-derived EVs/exosomes can be internalised into homotypic MSCs (Paper I [47]) or heterotypic cells, such as monocytes [228]. The internalisation of EVs/exosomes via various endocytosis pathways has been suggested to be a critical step for EVs/exosomes to release their contents into recipient cells to exert their functions [77]. It is noteworthy that the activation of STAT3 by MSC-derived exosomes has been experimentally validated in previous studies [263, 264]. The exposure of fibroblasts to MSC exosomes increased the activation of STAT3, resulting in the significant induction of the expression of target genes involved in cell cycle progression, including c-myc, cyclin A1 and cyclin D2, and involved in growth factor production, including HGF, IGF1, nerve growth factor (NGF), VEGF and SDF1 [263]. Moreover, it was demonstrated that MSC exosomes carried bioactive STAT3 protein with DNA-binding activity [263]. In line with this finding *in vitro*, the activation of STAT3 by MSC exosomes was also evident *in vivo* [264], where miR-133b-modified MSC-

derived exosomes promoted the activation of STAT3 in injured spinal cord for neuroprotection.

The further quantitative comparison of the protein profiles of LP EVs and HP EVs revealed a set of differentially expressed proteins between HP EVs and LP EVs (Paper II). The differentially expressed proteins significantly enriched several categories of GO biological process, among which the largest category was related to localisation, transport and vesicle formation. This predicted function of the differentially expressed proteins may imply the altered secretion of EVs/exosomes by MSCs undergoing *in vitro* ageing. Indeed, we and others observed the increased secretion of HP EVs in comparison with LP EVs (Paper II, [265]). This assumption that ageing promotes the secretion of EVs is partially supported by the fact that a higher concentration of serum-derived EVs was found in aged healthy volunteers compared with that in the young volunteers [266]. Moreover, it has been reported that the activation of p53, a master gene regulating cellular senescence and apoptosis, promoted exosome secretion [267], which may partially implicate the underlying mechanism of ageing-increased vesicle secretion.

The GO molecular function analysis of the global proteome of Exo1 and Exo2 showed significantly enriched terms mainly related to i) protein binding, receptor binding and activity, enzymatic activity, regulation of translation; ii) nucleoside phosphate binding, transcription regulation; iii) carbohydrate, lipid and ion binding, transporter activity; iv) adhesion; v) structure and morphology; and vi) GF and GFR binding and activity (Paper III). In comparison with two of the earliest proteomic studies of EVs/exosomes secreted from self-renewal human BM-MSCs and human embryonic stem cell (ESC)-derived MSCs [156, 159], more proteins were identified in the present study; they were predicted to possess a broader spectrum of functional features. Nevertheless, the predicted functional features of currently identified MSC exosomal proteins, particularly the enzymatic activity, GF and GFR binding and activity, cell adhesion and transporter activity, are in good agreement with the previous proteomic findings [156, 159]. Indeed, several studies have attributed the therapeutic effects of MSC-derived EVs/exosomes to their exosomal protein contents. Specific MSC exosomal proteins with enzymatic activity, such as NEP [162] and 20s proteasome [159], and proteins functioning as signalling molecules, such as Wnt4 [157, 192], have been experimentally validated. Whereas, the GF and GFR binding and activity may imply the function of MSC exosomes in promoting cell proliferation (Paper

III), however, specific responsible molecules need to be validated experimentally. In addition, in agreement with the predicted function of MSC exosomal proteins in cell adhesion, MSC exosomes were shown to accelerate cell adhesion on titanium surfaces (Paper III). Although the underlying mechanism has yet to be determined, the effects of MSC exosomes in cell adhesion may be contributed to by the presence of a variety of ECM proteins and adhesion-associated receptors, such as integrin and cadherins, in exosomes. Furthermore, a set of proteins were shown to differentially express between Exo2 and Exo1. The differentially expressed proteins significantly enriched pathways regulating a wide array of cellular processes, including cell adhesion, migration, proliferation and differentiation, which will be of great interest in future studies.

5.3 Effects of MSC-derived EVs/exosomes

EVs/exosomes, secreted from MSCs under different conditions, in suspension were used to treat homotypic MSCs to investigate their regenerative effects, in terms of the stimulation of proliferation and osteogenic differentiation (Papers I and II).

5.3.1 Effects on osteogenic differentiation

In the absence of chemical stimuli, a daily supplement of exosomes derived from MSCs undergoing self-renewal or different stages of osteogenic differentiation showed various effects on the osteogenic differentiation of homotypic cells (Paper I). Exosomes secreted from MSCs in the middle and late stages of osteogenic differentiation, but not from self-renewal or early-differentiated MSCs, induced osteogenic lineage commitment indicated by a significant increase in ALP activity. Moreover, evidence obtained from both the quantification of ECM mineralisation and the staining of calcium deposits in the ECM revealed that only exosomes secreted from the late-differentiated MSCs induced mineralisation.

Previously, the completed secretome (or CM) of both self-renewal and differentiating MSCs has been shown to induce and accelerate osteogenic differentiation and mineralisation *in vitro* [268, 269] and to enhance bone regeneration in defect areas [268]. However, this effect was initially attributed to MSC-secreted soluble factors including cytokines, chemokines and GFs, such as BMP2 [269]. Nevertheless, we and others have shown that, in addition

to the soluble factors, MSC-derived EVs/exosomes are also potent to commit MSCs towards the osteogenic lineage [47, 270] and induce mineralisation in a stage-dependent fashion (Paper I [47]). In line with our findings, a recent study showed that osteoblast-derived exosomes, but not self-renewal MSC-derived exosomes promote mineralisation [271], further confirming the notion of stage-dependent effects. The osteoinductive effect of MSC-derived EVs/exosomes was further observed in various *in vivo* models. The local injection of self-renewal MSC exosomes rescued impaired facture healing in CD9 knockdown mice and promoted bone fracture healing in wild type mice [195]. The systemic injection of self-renewal MSC EVs stimulated the proliferation of chondrocytes in the growth plate and improved bone growth in an osteogenesis imperfecta mice model [272]. A combination of porous β-TCP scaffold and self-renewal MSC exosomes accelerated the repair of critical-sized bone defects in both healthy and osteoporotic rat models [193, 194]. These observations together indicate bone regenerative effects of MSC EVs/exosomes in both healthy and compromised tissue conditions. In addition to the secretome, including soluble factors and EVs/exosomes, another component of the microenvironmental milieu, ECM, also plays a pivotal role in promoting the osteogenic differentiation of MSCs and bone regeneration [273]. Interestingly, it was recently shown that the combination of exosomes and ECM from a homo-lineage culture, osteogenic or adipogenic differentiation, further promoted the respective lineage commitment and terminal differentiation of MSCs [271]. Moreover, the supplementation of osteoblast exosomes to MSCs undergoing ECM-mediated adipogenic differentiation stimulated the expression of osteogenic lineage genes and *vice versa*, indicating the capacity of exosomes to override the ECM-mediated instructive signals for the lineage determination and differentiation of MSCs [271]. Taken together, we and others have demonstrated that lineage-specific exosomes play important roles as the microenvironmental cues for MSC differentiation and may thus be a potent tool for tissue regeneration.

Although current observations support the effects of MSC-derived EVs/exosomes in osteogenic differentiation and bone regeneration, dose-dependent effects are often observed irrespective of *in vitro* or *in vivo* studies. One of the outstanding questions is the extent to which the current observed effects reflect the function of MSC-derived EVs/exosomes in physiological conditions. Evidence from the observation of matrix vesicles, recovered using similar isolation method and with a physical appearance similar to that of MSC-derived EVs/exosomes, in the growth plate [2, 274] may indicate the

physiological existence and functional localisation of MSC-derived EVs/exosomes. Furthermore, the finding that matrix vesicles functioned as an initiation site of mineralisation may provide a physiological evidence for the mineralisation induced by the late osteogenic differentiated MSC-derived exosomes [16, 275]. However, it remains to be determined whether the previously observed matrix vesicles are exosomes or other subtypes of EVs and whether MSC-derived EVs/exosomes are a subpopulation of matrix vesicles.

5.3.2 Effects on proliferation

EVs, secreted from both "young" (LP) and "aged" (HP) MSCs, were shown to possess similar potential to improve cell viability and promote cell proliferation in a dose-dependent manner (Paper II).

It has been shown that "young" MSC-derived EVs/exosomes attenuate apoptosis, promote the proliferation of both homotypic [194] and heterotypic cells [177, 263, 264, 276] and induce or accelerate osteogenic differentiation and bone regeneration [47, 193-195, 270, 271]. Very limited knowledge has, however, been obtained on "aged" MSC-secreted EVs. In Paper II, we observed that HP MSCs exhibited senescence-like morphology and the increased secretion of small EVs (mainly < 200 nm). Although HP MSCs showed a significantly decreased proliferation capacity in comparison with LP MSCs, HP EVs were as efficient as LP EVs in improving MSC viability and promoting MSC proliferation in both an autocrine and a paracrine manner. One possible explanation for this finding is that the abundant proteins in HP EVs and LP EVs share some common functional features, such as enriched endocytosis-related pathways and the STAT3 pathway regulating cell proliferation and survival [263, 264], which results in similar EV-protein-mediated effects. Nevertheless, it remains to validate these predicted functional features of identified EV proteins and further demonstrate the underlying mechanisms of HP EV- and LP EV-stimulated MSC proliferation.

Partly in line with our findings, a recent study showed that "aged"/senescent MSCs secreted a higher number of large EVs (200 nm-1,000 nm) compared with "young" MSCs [265]. Both "aged" EVs and "young" EVs promoted osteogenic differentiation but to a varying extent [265]. Moreover, the microRNA and mRNA contents of "aged" EVs were altered in the same direction as their parental cells. It is noteworthy that microRNA, particularly miR-146-5p, associated with senescence profoundly increased in both "aged" EVs and MSCs [265]. It was suggested that the reduced pro-osteogenesis

effect and increased senescence-associated contents of “aged” EVs were propagated from their parental cells. On the basis of this concept, it is reasonable to hypothesise that HP EVs and LP EVs convey distinct signals to promote proliferation. Via the propagation of the features of their parental cells, LP EVs may reflect the self-renewal capacity of their “young” parental cells and serve as proliferation signals, while HP EVs may serve as a survival signal to communicate the compromised state of their “aged” parental cells to the adjacent cells. This assumption could be partly supported by the effects of senescence-associated secretory phenotype (SASP) secretome components. Senescent cells transmit signals and communicate with the surrounding cells via the SASP secretome [277]. In addition to spreading of senescence and stimulating inflammation, SASP components may mediate beneficial effects for tissue repair via stimulation of surrounding cell proliferation [278]. Indeed, it was recently suggested that EVs may represent a new component of SASP [279]. However, further comprehensive study is needed to investigate the roles of EVs in the ageing of MSCs and the way “aged” EVs affect the regenerative capacity of MSCs.

5.4 The behaviour of MSCs on exosome-immobilised titanium surfaces

One of the important findings in the present thesis (Paper III) is that titanium surface-immobilised exosomes influence the behaviour of MSCs, in terms of cell adhesion, spreading/migration and growth, on the titanium surface.

Cell adhesion is one of the first crucial steps for the cell-material interaction and the regenerative process on the implant surface. In Paper III, enhanced MSC adhesion on MSC-derived exosome-immobilised titanium surfaces (im-Exo-Ti) was demonstrated by applying complementary techniques to quantify the number and the surface coverage of the adherent MSCs in comparison to that on control titanium surfaces with or without suspended exosomes in culture (su-Exo-Ti and Ctrl-Ti). This result indicated that the surface-immobilised exosomes promoted MSC adhesion on titanium surfaces. Although the underlying mechanism is yet to be determined, the increase in cell adhesion may be at least partly attributed to the modified surface characteristics by immobilised exosomes, such as surface topography. Exosomes are nanoscale membrane vesicles naturally secreted by cells. The nanovesicle structure provided by surface-immobilised exosomes may modify the surface nanotopography in a similar fashion as the fabrication of

nanopillars or the coating of metallic nanoparticles on surface [280]. Indeed, using SEM, we have observed heterogeneous nanovesicles randomly localised on the im-Exo-Ti surface. It has been demonstrated that surface topography has a varying impact on cell adhesion [281] and the surface microstructure could regulate the expression of different integrin subunits [282], which may in turn result in the regulation of cell adhesion.

Moreover, the enhanced MSC adhesion may be also mediated by bioactive molecules presented on the immobilised exosomes. Indeed, a broad spectrum of molecules mediating adhesion, including ECM proteins, such as FN and a variety of collagens, and cellular transmembrane receptors, such as different integrin subunits and cadherins, were detected in the MSC exosomal proteome, which may contribute to the increase in MSC adhesion. Integrins are heterodimeric receptors that mediate cell adhesion upon ligand binding. A variety of ECM components have been identified to be ligands of integrins. Moreover, integrins can be internalised and recycled through an endosomal pathway [283]. The immobilised exosomes may perform dual roles in enhanced MSC adhesion. On the one hand, exosomal proteins such as FN and collagens may serve as ligands for integrins presented on cell membranes; cells adhere to the surface when the cellular integrins bind to these ligands. On the other hand, the exosomal integrins may also bind to ligands presented in the ECM of adherent MSCs, which may influence the adhesion strength of cells on the surface. This kind of bidirectional interaction between MSCs and immobilised exosomes may consequently result in the observed increase in and acceleration of cell adhesion. However, it remains to validate these proposed interactions. One outstanding question is whether these adhesion-associated proteins localise on the surface or inside the exosomes and the topology of these proteins present in exosomes. The topology of exosomal proteins may play important roles in exosome-mediated functions, as it has been revealed that a number of EV membrane proteins were actually present in a topologically reversed orientation [284].

Another interesting observation is that adherent MSCs were in close proximity to the nanovesicles on im-Exo-Ti surfaces. It remains to be determined whether this co-localisation of adherent MSCs and nanovesicles is a random event or an event driven by a specific signal. However, MSC exosomes have been shown to promote cell migration [192], indicating the possible presence of chemotactic signals, which may induce adherent MSCs to migrate towards surface areas with immobilised exosomes. On the other hand, surface

characteristics, such as surface topography and stiffness, can also be signal cues to promote cell locomotion. A previous study has shown that cells preferred to migrate along a stiffness gradient, termed “durotaxis”, suggesting that cells feel and respond to the difference in rigidity on a substrate or surface [285]. Similarly, adherent MSCs may be able to detect the different rigidity of a titanium surface area without immobilised exosomes, a rigid metal surface, and an area with immobilised exosomes, a less rigid nature cellular membrane surface, thereby preferring to migrate towards an exosome- immobilised area, consequently co-localised with them. However, solid evidence is still needed to provide support for such assumption. For instance, it needs to be demonstrated whether the observed co-localised nanovesicles are the initially immobilised exosomes, which provide the signal cues. Live imaging to observe whether and how the MSCs migrate on the surface may be useful to obtain direct support. Nevertheless, the profound increase of SDF1α expression in adherent MSCs on the im-Exo-Ti surface may indirectly indicate the possibility of MSC migration on titanium surfaces. The SDF1α/CXCR4 axis has been suggested as a predominant pathway promoting MSC migration and homing to tissue injury sites [214]. Furthermore, it was recently demonstrated that the SDF1α/CXCR4 axis promoted the early osteogenic differentiation of MSCs through crosstalk with the signal axis BMP2/Smad/RUNX2/Osterix [217]. In line with this finding, a significantly increase in the expression of RUNX2 was detected in adherent MSCs on the im-Exo-Ti surface at the early time point, indicating the augmentation of osteogenic differentiation.

In parallel with the enhanced MSC adhesion, another major finding was the observation of increased MSC growth on the im-Exo-Ti surface after both three days and six days. The increase in cell growth can be partly attributed to the initially enhanced cell adhesion, which resulted in an optimised cell density to promote the subsequent growth. Indeed, the proliferation and osteogenic differentiation of MSCs are density sensitive. The *in vivo* transplantation of MSCs at low density failed for new bone formation [86]. In addition, the possible changes of surface characteristics caused by the immobilised exosomes may also have a positive impact on the cell proliferation [286]. Moreover, another possible mechanism is that the presence of bioactive molecules in immobilised exosomes triggered the increase of cell growth. Such assumption is supported by the identification of a variety of GFs and GFRs in the MSC exosomal proteome.

Interestingly, the immobilised exosomes and suspended exosomes appeared to promoted cell growth via different modes. The immobilised exosomes induced the rapid and profound increase of cell growth. On the other hand, exosomes in suspension stimulated an increase in the gene expression of the common proliferation marker, Ki67, and, BMP2 that has been shown to promote MSC proliferation and osteogenic differentiation, at the early time point, day 3. Moreover, the proliferation stimulated by suspended exosomes was increased between three to six days. This observation indicates that the delivery routes of exosomes may affect the biological effects of exosomes due to different action modes. Indeed, a previous study has shown that, in contrast to the soluble form, the matrix-bound mode of presentation of GFs, particularly VEGF, improved efficacy and elicited the prolonged activation and clustering of VEGFR2 [287]. Moreover, in comparison to soluble VEGF, only matrix-bound VEGF induced the association of integrin β1 with focal adhesion and activated VEGFR2 in an integrin β1-dependent manner. This distinct molecular mechanism, in terms of the recruitment of receptor pattern and the activation of downstream effectors, resulted in a difference in cellular response and a varying degree of biological effects. This "solid induction mode" was also validated on other GFs, including BMP-2 [288] and IGF1 [289]. Matrix-bound BMP-2 significantly increased early cell adhesion, spreading and migration compared with soluble BMP-2 [288]. It has been suggested that the immobilised form of GFs represents a model closer to physiological conditions as evidence has been shown that many GFs bound to ECM proteins and were presented in an ECM-bound manner to cells *in vivo* [290, 291]. The enhanced effects mediated by immobilised GFs are probably due to the crosstalk between GFRs and adhesion receptors, leading to a combined activation of the downstream signalling network [287, 288]. This may also explain the difference of the current observation between immobilised and suspended exosomes (Figure 6). Indeed, one of the earliest found EVs, namely matrix vesicles, were observed to be located in the ECM of growth plate and functioned as an initiation site of mineralisation [2, 275]. This finding indicates the existence and functions of immobilised EVs/exosomes in physiological condition.

Nevertheless, the assumption that surface-immobilised exosomes modify the titanium surface features and in turn influence the cellular behaviour on the surface requires further more comprehensive investigation. It is necessary to characterise the surface properties of im-Exo-Ti, such as the surface roughness, stiffness and charge. Moreover, it is of critical importance to further

evaluate the exosome immobilisation protocol. One interesting question to address is the surface coverage of the immobilised exosomes and whether it can be increased, along with increasing the initial concentration of exosome solution for titanium discs immersion. In addition, it would also be interesting to determine whether the distribution of immobilised exosomes is random or follows a specific pattern is also of interest to examine. On the other hand, the validation of the bioactive molecules in exosomes that are potentially responsible for the observed effects in influencing the cellular behaviour on titanium surfaces is important in order to identify the underlying mechanisms.

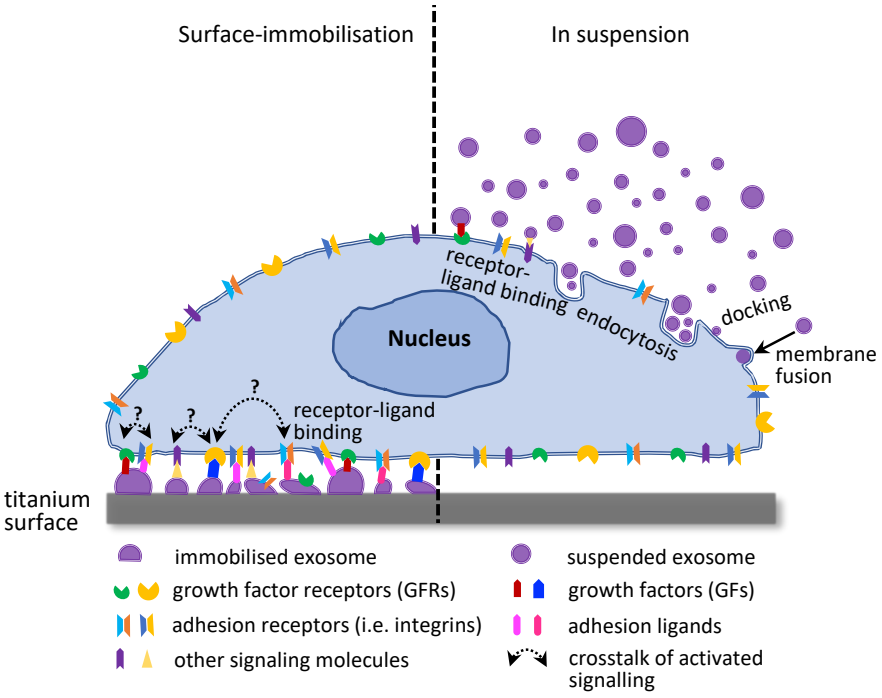


Figure 6. Schematic illustration of the different action modes of immobilised exosomes and suspended exosomes. The surface-immobilised exosomes may mediate cell-to-material interaction mainly via receptor-ligand binding and the subsequent activation of signalling pathways. Moreover, the activated signalling may crosstalk to one another and thereby generate a cooperative effect. The suspended exosomes may interact with cell via receptor-ligand binding or via various endocytosis pathways. However, the interaction may be limited by the diffusion of ligands and/or receptors.

5.5 Potential application of MSC-derived EVs/exosomes

Overwhelming evidence of MSC-derived EV-/exosome-mediated therapeutic effects in various disease/injury models has shed light on their potential applications as a novel cell-free therapeutic tool. Given the observation that MSC-mediated therapeutic effects were partly attributed to the paracrine effects mediated by their secreted EVs/exosomes and that MSC EVs/exosomes harbour cargoes reflecting their parental cells, MSC EVs/exosomes could perhaps be used as an alternative to MSC-based therapy. In some contexts, MSC EV-/exosome-based therapy may offer several advantages due to its unique physical and biological features in comparison with MSC-based therapy. The nano-size of EVs/exosomes provides the ability to cross biological barriers, such as the blood-brain barrier and the blood-cerebrospinal fluid barrier, and passively diffuse through tissues. The nature of the EV/exosome membrane, originating from the cellular membrane, is of benefit for their efficient internalisation by the target cells. The bioactive molecules harboured on the surface may create an opportunity to orientate the interaction towards specific target cells. The intravesicular cargoes, protected by the membrane structure, gain access into target cells via fusion with the PM or via internalisation and thus create the opportunity to regulate the intracellular signals. In this section, the potential application of MSC-derived EVs/exosomes will be discussed on the basis of the current literature and observations in the present thesis.

In light of the functions of MSC-derived EVs/exosomes summarised in Section 1.3.2, MSC-derived EVs/exosomes can be proposed as a novel therapeutic tool due to their regenerative and immunomodulatory effects. Through local or systemic administration, the majority of the current studies attributed the observed trophic effects to the heterogeneous population of EVs/exosomes naturally secreted by unmodified MSCs. However, it was recently suggested that different subpopulations of EVs from a single cell type might elicit different biological effects, positive, negative or no effects, in the recipient cells [7]. It was therefore suggested that the therapeutic effects of EVs may be enhanced by enriching a specific subpopulation of EVs. Nevertheless, to our knowledge, no study has demonstrated such hypothesis partly due to the limitation of current techniques to isolate a pure subpopulation of EVs. Alternatively, several other strategies have been applied to modify MSC-derived EVs/exosomes in an attempt to improve their functional effects. In general, the modification of MSC-derived EVs/exosomes

can be achieved indirectly via the modification/engineering of the parental MSCs or directly via the modification/engineering of MSC-secreted EVs/exosomes. EVs/exosomes secreted by preconditioned MSCs via stimulation with proinflammatory cytokines [207, 231], injured tissue extract [82] or hypoxia [204, 247] have been shown to significantly enhance the trophic effects, which may be contributed to by the altered EV/exosome contents because of preconditioning. We have also shown that the microRNA contents of exosomes altered when the parental MSCs underwent osteogenic differentiation, which may be partly responsible for the different effects of MSC exosomes in osteogenic differentiation and mineralisation. So, from a bone regeneration perspective, EVs/exosomes secreted from MSCs undergoing osteogenic differentiation may be more potent than those from self-renewal MSCs (Paper I). Gene modification can also be used to modify MSC-secreted exosomes. EVs/exosomes derived from gene-modified MSCs, for example, transfected MSCs to overexpress a specific microRNA, showed to propagate the enriched expression of the specific molecule and in turn result in improved effects [239-245]. On the other hand, the modification can be directly conducted on MSC EVs/exosomes. The direct loading of exogenous nucleic acid can be achieved by electroporation, while protein loading can be mediated by protein-protein interaction [7]. In addition to loading of intravesicular contents, a recent study made an effort to improve the targeting capacity of MSC exosomes by functionalising the exosome surface with conjugated c(RGDyK) peptide [292].

In addition to applying MSC EVs/exosomes, mainly administrated in suspension, as a delivery vehicle for the transfer of unmodified or modified functional contents, MSC EVs/exosomes can be immobilised on a substrate or material surface, for example, a titanium implant surface, to generate a functionalised surface favourable for cell-to-material interaction and consequently benefit for bone regeneration. It has previously been shown that MSC exosomes lyophilised on a β-TCP scaffold promoted bone regeneration in a critical-sized bone defect model [193, 194]. In the present thesis, we showed that titanium surface-immobilised exosomes accelerated MSC adhesion, promoted MSC growth and potentially augmented the osteogenic differentiation of MSCs (Paper III). Although further studies are needed to optimise the exosome immobilisation protocol, to demonstrate the underlying mechanism and to obtain evidence from an *in vivo* model, the current observations encourage us to propose the potential application of MSC EVs/exosomes for the surface modification of titanium implants (Figure 7). The im-Exo-Ti surface may have several potential advantages. The nature-

derived bioactive molecules presenting on the surface and/or inside of the immobilised exosomes provide bio-signals on the im-Exo-Ti surface. The exosomal surface molecules may mediate direct and rapid cell-to-material interaction to regulate cell adhesion on implant surfaces, while the intravesicular molecules may mediate a secondary interaction via internalisation into the surface adherent cells and further regulate other cellular behaviours such as proliferation and differentiation. In addition, the surface-harboured contents and intravesicular contents of immobilised exosomes can be modified as described above to further improve the biological effects or to provide the flexibility to customise functionalised implant surfaces for specific microenvironments. Compromised tissues with reduced regenerative capability are examples of such environments. Moreover, the physical features of immobilised exosomes may modify the titanium implant surface properties such as surface nanotopography. Taken together, immobilised exosomes could potentially modify surface bio-signals and surface nanotopography and can thus be suggested as a novel tool to functionalise implant surfaces.

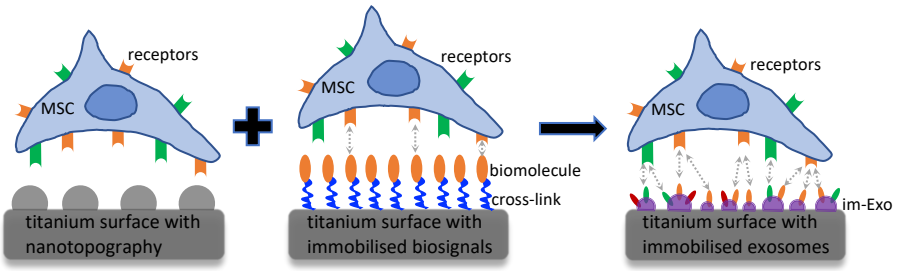


Figure 7. Functionalised titanium implant surface by immobilisation of exosomes. The exosome-immobilised titanium surface may offer combined advantages to modify the surface nanotopography and meanwhile provide bio-signals on the surface by the bioactive molecules presented on the immobilised exosomes. im-Exo, immobilised exosomes.

6 Summary and Conclusion

In Paper I, the results showed that MSC-derived exosomes possess different biological properties depending on the differentiation stages of their parental cells. Exosomes secreted from MSCs in various stages of osteogenic differentiation, particularly the mid and late stage of osteogenic differentiation, committed homotypic cells to differentiate towards the osteogenic lineage, but only exosomes from the late stage of osteogenic differentiation induced the mineralisation of the ECM. Exosomes, irrespective of the status of their parental MSCs, were internalised by a subpopulation of homotypic MSCs. Furthermore, microRNA profiling revealed a large overlap of the top 50 abundant microRNAs but altered expression level of a set of microRNAs in exosomes and their respective parental cells. The comparison of microRNAs from various groups of exosomes showed a specific set of differentially expressed exosomal microRNAs from the late stage of osteogenic differentiation. These microRNAs were osteogenesis related and predicted to enrich pathways involved in the regulation of osteogenic differentiation and general mechanisms by which exosomes exert their functions.

In Paper II, an *in vitro* ageing model was established by continuous passaging to investigate the impact of ageing, particularly *in vitro* ageing, on the release of EVs. The results showed that *in vitro* ageing retained the phenotypic signature of MSCs, but resulted in senescence-like morphology and reduced regenerative capacity, in terms of proliferation and osteogenic differentiation. *In vitro* ageing did not alter the characteristics of EVs in terms of size and typical exosomal protein markers. However, *in vitro* ageing promoted the more numerous secretion of EVs and, in some respects, altered the protein profiles of EVs. The top 200 abundant proteins shared similar but not identical functional features. A small portion of the top abundant proteins were shared between HP (“aged”) and LP (“young”) EVs, with an overlap of the enriched pathways related to endocytosis and the regulation of cell proliferation and survival. The differentially expressed proteins in HP EVs were predicted to enrich GO biological process terms related to transport and secretion and pathways involved in the regulation of cell morphology, growth/proliferation and development. Functional validation revealed that both HP EVs and LP EVs promoted the proliferation of MSCs in an autocrine and a paracrine manner and in a dose-dependent fashion.

In Paper III, exosomes from both self-renewal (Exo1) and the mid/late stage (Exo2) of osteogenic differentiated MSCs were immobilised on titanium surfaces and interacted with the surfaces in a similar manner. The immobilised exosomes, irrespective of their parental cell source, accelerated and increased MSC adhesion on the titanium surface, influenced the early morphology of adherent MSCs and promoted the growth of MSCs on titanium surfaces. Exosome treatment promoted the osteogenic differentiation of MSCs to a lesser extent. The delivery routes of exosomes, in terms of suspension or surface immobilisation, influenced the behaviour of MSCs on titanium surfaces differentially. The proteomic analysis of the exosomal protein cargoes revealed a profile of proteins with the predicted GO molecular function related to adhesion, structure and morphology, and GF and GFR activity. Differentially expressed proteins in Exo2 were predicted to enrich pathways related to the regulation of cell morphology, adhesion, migration and differentiation.

In conclusion, the present thesis demonstrates that MSC-derived EVs/exosomes possess regenerative effects, in terms of stimulating proliferation and osteogenic differentiation. MSC-derived exosomes are internalised by a subpopulation of homotypic cells. The titanium surface-immobilised exosomes influenced the behaviours of MSCs, in terms of adhesion, early morphology and proliferation on the titanium surface. The investigation of the microRNA and protein contents of MSC-derived EVs/exosomes revealed altered expression of exosomal cargoes during osteogenic differentiation and *in vitro* ageing. Predicted functions of the exosomal cargoes were shown to partially correspond to the observed effects, indicating that the internalisation of exosomes and the transfer of exosomal cargoes are at least partly responsible for the biological effects of MSC-derived EVs/exosomes.

7 Future perspectives

The therapeutic effects of MSC-derived EVs/exosomes on tissue regeneration and immunomodulation have attracted a great deal of attention in an attempt to develop a novel cell-free regenerative tool. The findings presented in this thesis demonstrate the regenerative effects of MSC-derived EVs/exosomes, in terms of stimulating proliferation, osteogenic differentiation, titanium surface-adhesion and growth of MSCs, which may be attributed to the internalisation of EVs/exosomes and the transfer of exosomal cargos including microRNAs and proteins. These findings contribute to our understanding of the functions of MSC-derived EVs/exosomes and their potential application as a regenerative tool specifically for bone regeneration. Nevertheless, several questions are of great interest for future investigation.

The predicted functions of exosomal microRNA and proteins would be of interest to validate experimentally. The strategy of loss-and-gain function can be applied to demonstrate the function of specific exosomal microRNAs and their contribution to MSC-derived EV-/exosome-mediated effects. To further boost the effects of MSC-derived EVs/exosomes, genetically modified MSCs can be applied to produce exosomes with overexpressed contents that are beneficial to regeneration.

Although in the present thesis we observed similar effects of "aged" (HP) EVs and "young" (LP) EVs on the stimulation of MSC proliferation, it is necessary to further study the respective underlying mechanisms. Moreover, other aspects of the biological effects mediated by HP EVs and LP EVs, i.e. effects on the osteogenic differentiation of MSCs and effects on the immunomodulation of monocytes/macrophages, remain to be determined. It is of particular interest to validate whether HP EV is a new component of SASP and to investigate the impact of HP EVs on the change of regeneration capacity of MSCs during ageing.

It is yet to determine the mechanisms by which the surface-immobilised MSC exosomes promote cell adhesion on titanium surface and whether any specific exosomal components are responsible for the observed effects. The effects of exosomal surface proteins on cell adhesion are of particular interest. Meanwhile, it would be interesting to investigate the impact of the immobilised MSC exosomes on other cell types, such as the other important cell types involved in bone regeneration, i.e. monocytes/macrophages. On the

other hand, it is also of interest to immobilise EVs/exosomes secreted from other cell types, i.e. monocytes/macrophages, on titanium surfaces and further examine the effects of these surfaces, in order to deepen our understanding of whether the current observation is an effect unique to MSC-derived exosomes or to immobilized exosomes, irrespective of the cellular source of these exosomes. On the basis of the observation that the delivery routes of exosomes, in suspension or surface immobilisation, appear to affect the biological effects of exosomes, it is of great interest to study whether the delivery routes of exosomes mediate the biological effects via different action modes or via activation of different pathways. Furthermore, from a materials science point of view, it is necessary to evaluate the distribution and coverage of immobilised exosomes on the surface and the way the immobilised exosomes affect the titanium surface properties, such as surface nanotopography, stiffness and charge, and the consequences of these changes on the cellular behaviour on the titanium surfaces. Methodologically, it is valuable to optimise the exosome immobilisation protocol or coating technique to control the effects of immobilised exosomes prior to *in vivo* studies.

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