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IN-DEPTH CHARACTERIZATION OF MESENCHYMAL STEM CELLS-DERIVED EXTRACELLULAR VESICLES USING QUANTITATIVE PROTEOMICS

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ABSTRACT

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Title: In-Depth Characterization of Mesenchymal Stem Cells-derived Extracellular Vesicles Using Quantitative Proteomics

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As they home in on injured tissue, modulate the immune system and support tissue repairing, mesenchymal stem cells (MSCs) are considered a promising tool for many therapeutic applications. *In vitro,* MSCs have been shown to differentiate into multiple cell types mainly of the mesodermal, and more rarely of the endodermal and ectodermal lineage under appropriate conditions. *In vivo*, however, the beneficial effects mediated by MSCs are mainly attributed to paracrine factors they provide. MSCs are known to secrete large amounts of extracellular vesicles (EVs). EVs are thought to play an important role in intercellular communication, transferring proteins, nucleic acids and lipids to acceptor cells. To identify factors that contribute to the therapeutics roles of MSCs-derived EVs, we characterized the proteins enriched in them. MSCs-derived EVs were isolated from conditioned medium of cultured bone marrow–derived MSCs by ultra-centrifugation steps differentially isolating microvesicles (MVs) and exosomes. We performed LC-MS/MS-based proteomic analysis using reductive dimethylation labeling for quantitation of vesicular proteins against MSC whole cell lysate. In total, we identified 5207 proteins. 4695 and 4386 proteins were quantified in MVs and exosomes, respectively. We further analyzed the up-regulated proteins in both types of vesicles. Functional enrichment analysis was performed and hints at a high contribution of both MVs and exosomes to therapeutic applications of MSCs. EV proteins were linked to

broad biological roles including extracellular matrix organization, cell migration, wound healing and hemostasis. Our findings strengthen the idea that MSCs-derived EVs may be a valuable replacement for MSCs in therapy.

KEYWORDS: Mesenchymal stem cells, microvesicles, exosomes, quantitative proteomics, LC-MS/MS

DEDICATION

To my beloved Mother and Father

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ABBREVIATIONS

Abbreviation	Explanation
AKI	Acute kidney injury
ALI	Acute lung injury
BM	Bone marrow
CM	Conditioned medium
PBS	Phosphate buffered saline
ESC	Embryonic stem cell
EV	Extracellular vesicle
EXO	Exosome
GO	Gene ontology
GOBP	GO biological processes
GOCC	GO cellular compartments
HPLC	High-performance liquid chromatography
iPSCs	induced pluripotent stem cells
KEGG	Kyoto encyclopedia of genes and genomes
LC-MS	Liquid chromatography- Mass spectrometry
MSC	Mesenchymal stem cell
MV	Microvesicle
MVB	Multivesicular body
SDS	Sodium dodecyl sulfate buffer
TE	Total extract
TFA	Trifluoroacetic acid

1. INTRODUCTION

Mesenchymal stem cells (MSCs) are self-renewing multipotent stromal cells (Barry & Murphy, 2004), which can be isolated and cultured from several tissues of adult or fetal origin with the bone marrow (BM) as the most prominent source (Vallabhaneni et al., 2015; Liu & Han, 2008). *In vitro*, MSCs have been shown to differentiate into multiple cell types mainly of the mesodermal lineages, and more rarely of the endodermal and ectodermal lineages under appropriate conditions (Jiang et al., 2002). *In vivo*, engraftment of MSCs has been shown to be low and not persistent. MSCs are considered a promising tool for many therapeutic applications, as, despite their lack of engraftment, they home to injured tissue, modulate the immune system, and support tissue repair. MSCs have been shown to have beneficial effects on cardiac function after myocardial infarction (Amado et al., 2005), improve lung inflammation and survival (Ortiz et al., 2007), and repair damaged bone and cartilage (Noël et al., 2002).

MSCs are known to secrete large amounts of extracellular vesicles (EVs), which are emerging as important players in intercellular communication and are believed to transfer proteins, nucleic acids and lipids to acceptor cells. Microvesicles secreted by MSCs may thus have the potential to mimic the therapeutic effects of MSCs. Moreover, the vesicles may conceptually be engineered to carry molecules that modulate their effects (Tetta et al., 2012). For example, it has been shown that MSC conditioned medium has the same effects as MSCs when injected in mice with acute kidney injury (Bi et al., 2007) and acute hepatic failure (Parekkadan et al., 2007; Van Poll et al., 2008).

1.1. Hypothesis

This is a screening study to profile proteins in MSCs-derived EVs, and generate hypotheses regarding proteinaceous contribution to clinical effects of MSCs in general and MSCs-derived EVs specifically.

1.2. Aims

The aim of this study is the proteomic characterization of MSCs-derived EVs and the comparison of these vesicles to their donor cells to gain insight into the proteinaceous factors enriched in them. Understanding Pathways, for which members are enriched in EVs, may be considered as candidates for EV-modulated pathways, and eventually be employed for therapeutic purposes.

2. LITERATURE SURVEY

2.1. Stem Cells in Cell Therapy

Stem cells are self-renewing and highly proliferative cells that are able to differentiate into specialized mature cells. These characteristics make them an ideal tool in cell and gene therapy, drug delivery, and regenerative medicine (Biffi et al., 2013; Greco & Rameshwar, 2012; Law & Chaudhuri, 2013). Despite the intense attention to embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) due to their advantages in cell therapy application, major challenges have hindered their progression into clinical applications. Ethical issues getting the human oocyte, immune rejection, specifically in graft versus host disease, and potential risk of forming teratomas are some of ESCs and iPSCs challenges in cell therapy (Kingham & Oreffo, 2013; Abdelalim et al., 2014). Unlike ESCs and iPSCs, mesenchymal stem cells (MSCs) overcome these major issues. Comparatively, MSCs have no ethical concerns. They have lower risks of both immune rejection and teratoma formation (Prockop et al., 2010).

2.2. Mesenchymal Stem Cells

MSCs are self-renewing multipotent stromal cells that can be isolated and cultured from several tissues of adult (e.g. adipose tissue, skin, lung, dental pulp, and blood) or fetal (e.g. fetal liver, placenta, and umbilical cord) origin with the bone marrow (BM) as the most prominent source (Barry & Murphy, 2004; Liu & Han, 2008; Vallabhaneni et al., 2015). MSCs were first isolated from the bone marrow iliac crest of guinea pig and cultured by Friedenstein et al. in 1970 (Friedenstein et al., 1970). They

were later isolated and cultured from human bone marrow by Haynesworth et al. (Haynesworth et al.,1992).,where they represent approximately 0.001–0.01% of the nucleated cells present (Pittenger et al., 1999).

According to the minimal defining criteria of the International Society for Cellular Therapy: "First, MSCs must be plastic-adherent when maintained in standard culture conditions. Second, MSCs must express CD105, CD73 and CD90, and lack expression of CD45, CD34, CD14 or CD11b, CD79a or CD19 and HLA-DR surface molecules. Third, MSCs must differentiate into osteoblasts, adipocytes and chondroblasts *in vitro*" (Dominici et al., 2006). Moreover, MSCs have the capability to differentiate *in vitro* and *in vivo* into multiple cell types, mainly to the mesodermal lineages (such as adipocytes, osteocytes, chondrocytes, myocytes and cardiomyocytes), and more rarely to the endodermal and ectodermal lineages (such as hepatocytes and neurons) under appropriate conditions (Jiang et al., 2002).

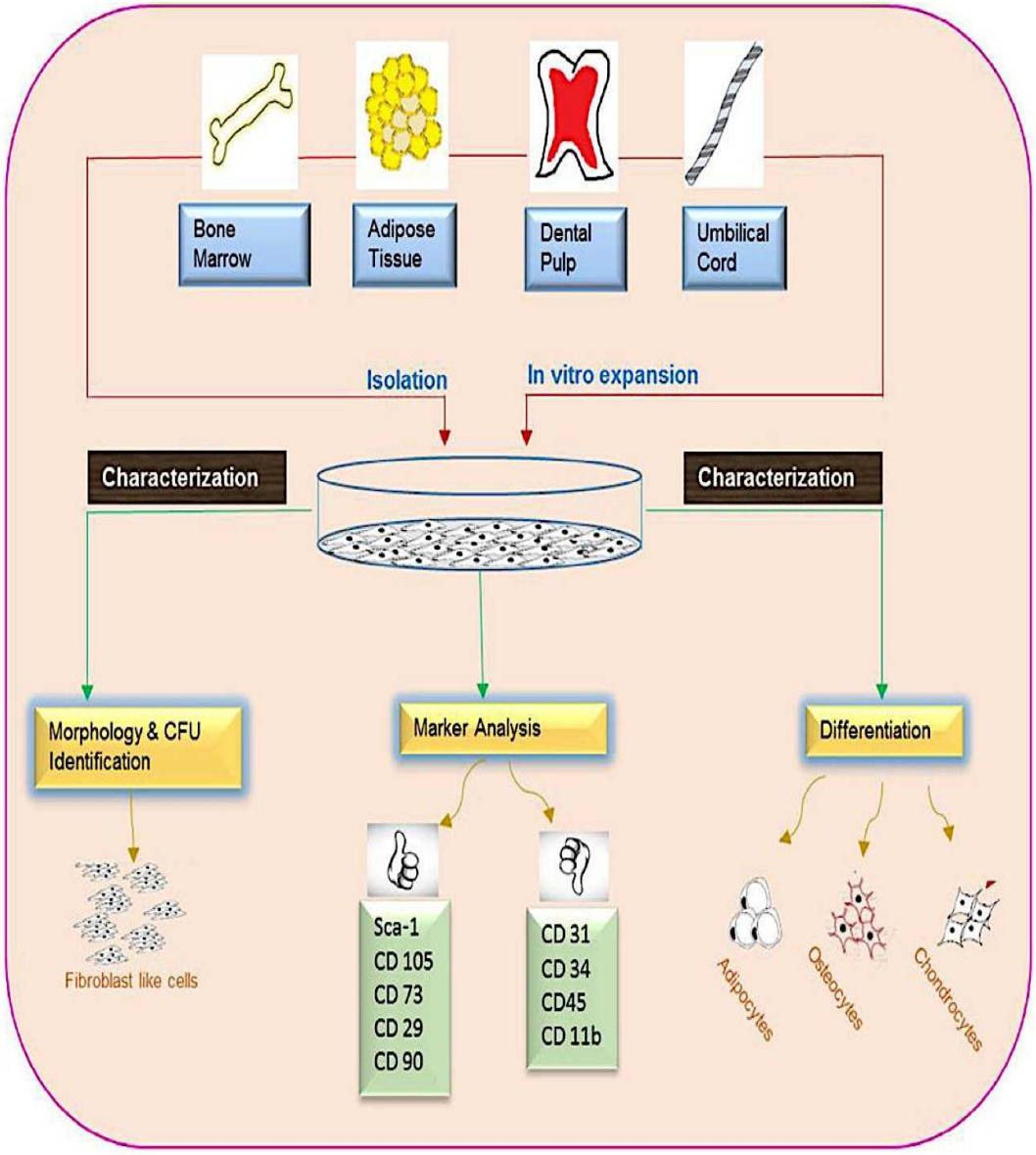

Figure.1: MSCs Isolation, expansion and identification (Konala et al., 2016)

2.3. Therapeutic Application of MSCs

In the recent decade, MSCs are simply isolated and cultured; and their multiple characteristics and potency make them attractive and widely chosen for intense investigations as a therapeutic tool through both cell-based and cell-free (secretion) mechanisms. Currently, more than 646 clinical trials registered within the National Institutes of Health are underway evaluating the potential of MSC-based cell therapy worldwide (Retrieved from http:// clinicaltrials.gov/, 31 st August 2016). Researchers were attracted to MSCs by the context of their ability to home to the injured tissue, modulate the immune response, and consequently support tissue repairing.

2.3.1. Role of MSCs in Tissue Regeneration

Studies and clinical trials of MSC-based therapy indicate the evidence that MSCs participate in tissue repair and regeneration. Many studies have been investigating the effects of MSCs in improving the function of damaged tissues or organs, such as improving cardiac function after myocardial infarction (Amado et al., 2005; Hare et al., 2009), lung inflammation and survival (Ortiz et al., 2007), and repairing damaged bone, cartilage, and skin (Noël et al., 2002; Rasulov et al., 2005).

Several studies have shown that allogeneic or autologous engraftment of MSCs improves the treatment process in patients with an acute myocardial infraction. As has been reported, that MSCs significantly improve heart function, together with providing the clinical safety of the process and providing effectiveness data for further clinical trial phases (Hare et al., 2009; Clifford et al., 2012). Fernández García and co-workers

strongly demonstrated that co-infusion of MSCs improves and facilitates hematopoietic stem cell (HSC) transplantation. Moreover, they claimed that the effect of MSCs is due to its direct interaction with HSC which being observed by *in vivo* and *in vitro* experiments (Fernández-García et al., 2015).

Engraftment of MSCs has been reported to improve bone and cartilage repairing. Encouraging results of improving osteoarthritis and meniscal defect appeared in the knee joint after MSCs injection (Horie et al., 2009; Davatchi et al., 2011; Agung et al., 2006). Horie et al. have shown that MSCs adhered to the injured meniscus after they injected them in the defective knee. They directly differentiated into meniscal cells and enhanced meniscal regeneration (Horie et al., 2009). Similarly, MSCs improve repairing damaged cartilage after differentiated into chondrocytes when injected into a knee joint (Johnson et al., 2012; Wakitani et al., 2002).

Many MSC based therapy studies have been conducted as well as for patients with neurological disorders, such as stroke and multiple sclerosis (Honmou et al., 2011; Karussis et al., 2010; Connick et al., 2011). MSCs are an attractive cellular source for the repair of brain disorders, as they express CD90, PDGF-Rb and CD146, which make them able to differentiate into dopaminergic neural cells *in vitro*. They are also able to assist endogenous neural growth, protect neuron cells against apoptosis, induce synaptic formation, and regulate inflammation (Wolff et al., 2011; Wei et al., 2009). In another study, Wolff et al. demonstrated that the engrafted MSCs into the striatum of the Parkinson disease mouse model can migrate to the foci of cellular injury in substantia

nigra and differentiate there, expressing neuron-like morphology of tyrosine hydroxylase positive neuron cells (TH+) and increased levels of dopamine and its metabolite production (Wolff et al., 2015).

Generally speaking, MSCs have the capacity to differentiate and multiply into various types of cells, after injection into injured tissues clinically, which enhance tissue regeneration.

2.3.2. Role of MSCs in Immunomodulation

MSCs are able to modulate the innate and adaptive immune system. In the adaptive immune system, MSCs impair T-cells maturation and proliferation. For example, MSCs cause an increase in IL-10, as well as decreases in interferon-gamma (IFN- γ) and tumor necrosis factor-□lpha (TNF- α) secretion which alter the microenvironment and make changes in immune actions (Aggarwal & Pittenger, 2005; Yi & Song, 2012). With regard to the innate immune system, they inhibit monocytes, dendritic cells, and natural killer cells maturation and proliferation (Kronsteiner et al., 2011; Keating, 2012). Moreover, MSCs are characterized by low expression of major histocompatibility-II (MHC-II) and other co-stimulatory surface molecules, that make it less recognizable by immune cells (Chamberlain et al., 2007).

The connection between the immune system and MSCs has been reported in different injury models. In an asthma mouse model, a study showed that BM-derived MSCs produce transforming growth factor-b (TGF-b), which suppress allergic responses (Nemeth et al., 2010). In a previous study it has been found that both BM-derived MSCs

and their vesicles were able to prevent renal fibrosis, lymphocyte infiltrates and tubular atrophy *in vivo* model with kidney injury (He et al., 2012). Moreover, they been used to treat graft versus host disease in animal models after bone marrow and HSCs transplantation (Kernan et al., 1993). The immunosuppressive properties of MSCs make them appropriate cells for allo/autologous transplantation, inflammatory and autoimmune disorders (J. Tan et al., 2012).

2.4. MSC Secretomes

The effects of MSCs on re-modulating different pathological conditions are based on their communication with other cells within the microenvironment. Like most cells, MSCs communication is characterized by their capability to produce and secrete paracrine secretions. These secretions can act as a link between MSCs and the target cells to affect on it in different ways and mechanisms. It can regulate the immune response or enhance cell proliferation, which both can have a beneficial effect on tissue repairing.

The use of conditioned medium (CM), collected from MSCs culture, is a potential alternative for the use of MSCs themselves. Conditioned medium or secretomes of MSCs contains secreted biological factors such as proteins, cytokines, chemokines, growth factors and other soluble factors or membrane vesicles derived –MSCs (Makridakis et al., 2013; Collino et al., 2010). Soluble secretions of MSCs and membrane vesicles derived – MSCs are two recent strategies to improve MSCs-based therapies. They could mimic MSCs immunomodulatory regulation and regeneration effects on the hosted cells. Moreover, they both could be more practical and decrease risks of MSCs engraftment,

which is of central concern in cell therapy (Abreu et al., 2016; Stephen et al., 2016).

The beneficial effects of paracrine factors of MSCs were investigated in many studies. MSCs have been shown to be effective in treating various diseases through their CM containing all the paracrine factors. *In vitro*, it has been reported that rat MSCs-CM decreased the rate of cardiomyocyte apoptosis after exposure to hypoxia/reoxygenation (Xiang et al., 2009). Another study found that MSCs-CM significantly prevent decreasing of sodium level in trans-epithelial transport, and preserve epithelial permeability, which could help in treating acute lung injury (Goolaerts et al., 2014). *In vivo*, the paracrine protective effect of MSCs has been investigated in induced diabetic rat models. The study showed that MSCs secretions can effectively prevent renal injury (Park et al., 2012). Other studies have shown that MSCs-CM can mimic the protective effect of MSCs and improve tissue repairing when injected in animal models with acute kidney injury (Bi et al., 2007), acute hepatic failure (Parekkadan et al., 2007; Van Poll et al., 2008), and cornea damage (Roddy et al., 2011).

2.5. Extracellular Vesicles

Many cells, healthy as well as cancer cells have been shown to secrete heterogeneous types of secretion and extracellular vesicles (EVs) into the extracellular microenvironment. Vesicles are present in the majority of biofluids, such as serum, urine, or cerebro-spinal fluid. EVs have been initially regarded merely as cellular debris without biological effects, which discarded the unwanted component of cells. However, many recent studies highlighted EVs as a communication system between cells and tissues. They carry biological information located in their membrane and cytoplasmic content

such as proteins, lipids and genetic material from their parent cells to the target cells, and impact thus tissue repairing and immunoregulation. (Camussi et al., 2011; György et al., 2015; Stephen et al., 2016; Bruno et al., 2016; Abreu et al., 2016).

"Extracellular vesicles" is a broad term that is used to refer to the mixture of phospholipid membranous vesicles, which can be differentiated based on their sizes and intracellular origin. EVs are classified primarily into three main categories: shedding microvesicles (MVs), exosomes and, apoptotic bodies. The sizes of membranous vesicles are varying in diameter from 50 nm to 2 um. EVs originate from cells by different mechanisms, such as direct budding from the plasma membrane in the case of MVs, or by fusion of multivesicular bodies (MVBs) that generates exosomes (Houseley et al., 2006; Théry et al., 2009; György et al., 2015). In addition to size and origin, other properties have been used to describe EVs such as density, lipid and protein composition, as well as sedimentation rate. Furthermore, factors such as age, gender, physical or chemical mediators can affect the nature and number of secreted EVs (Quesenberry et al., 2014; Abreu et al., 2016).

Microvesicles or shedding vesicles are heterogeneous vesicular population ranging from 100−1000 nm in diameter. They are circular fragments that bud by direct cytoplasmic protrusions, and detach from the cell surface, changing the distribution of cell plasma membrane (Muralidharan-Chari et al., 2010; Fierabracci et al., 2015; Stephen et al., 2016). The budding process takes place in resting cells. However, the rate of shedding formation varies. Factors such as cytosolic calcium ion stimulation, and

cytoskeleton reorganization, control the production of shedding vesicles (Cocucci et al., 2009; Stephen et al., 2016). Phosphatidylserines are highly enriched in MVs, as a result of flippase, floppase, and scramblase enzyme activity. For example, increasing level of calcium levels cause scramblase activation and accordingly shift phosphatydilserine from the inner to the outer membrane lipid bilayer (Camussi et al., 2010; Abreu et al., 2016). MVs differ in size and molecular components, according to the origin cell type and the process of biogenesis (Camussi et al., 2010). Practically no specific marker has been characterized to distinguish MVs. Nevertheless, MVs express some markers of their parent cells, and the plasma membrane content reflects the plasma membrane of the original cell (Stephen et al., 2016; Bruno et al., 2016).

Exosomes are a smaller type of EVs, which have a more homogeneous shape than microvesicles, and ranging from 30−100 nm in diameter. Prior to being release from the plasma membrane, exosomes are formed as accumulating intraluminal vesicles inside MVBs in the cytoplasm. When a MVB fuses with the plasma membrane, it secretes the intraluminal vesicles or exosomes into the extracellular space, in a process called exocytosis. Dissimilar to the formation of MVs, exosomes are not depending on calcium stimuli (Fierabracci et al., 2015; Sabin & Kikyo, 2014; Camussi et al., 2010; Stephen et al., 2016; Abreu et al., 2016; Hall et al., 2016). Most exosomes are highly enriched in both Apoptosis Linked Gene -2- Interacting Protein X (Alix) and Tumor Susceptibility Gene 101 (Tsg101), which are involved in the biogenesis of MVBs. The tetraspanins group containing CD9, CD63 and CD81 are also highly enriched in exosomes and has been widely known as exosomal markers. Furthermore, exosomes express many other

markers that reflect their parent cells and help in their identification (Bruno et al., 2016; Hall et al., 2016; Stephen et al., 2016).

Apoptotic bodies are another type of EVs, ranging from 1000 to 5000 nm in diameter. Apoptotic bodies are released from the plasma membrane of dying cells during apoptosis. They contain DNA, noncoding RNAs and cell organelles (Fujita et al., 2015; Akers et al., 2013).

EVs can affect target cells by different mechanisms/pathways. EVs can modify cells through interaction of EVs-surface receptors with the membrane of recipient cells (Cocucci et al., 2009). They can also fuse to the target cells, and transfer their biological contents such as proteins, RNAs, and lipids, which modify the function of the target cell (Mathivanan et al., 2010; Phinney et al., 2015a). The practical effects of the EVs depend on the conditions of the cells they were secreted from. For example, EVs can behave as immunostimulators or immunosuppressants (Bourdonnay et al., 2015). In this context, dendritic cells secrete EVs, which can induce humoral immune responses against antigens (Qazi et al., 2010). On the other hand, lipopolysaccharide-activated monocytes are able to activate caspase pathway and consequently apoptosis of the target cells (Sarkaret al., 2009).

Table.1: Summary of characterization of extracellular vesicles

EVs	Microvesicles	Exosomes	Apoptotic bodies
Size	100 -1000 nm	30 – 100 nm	> 1000 nm
Genesis	Direct budding of the plasma membrane	Exocytosis of multivesicular bodies to the plasma membrane	Blebbing of plasma membrane during apoptosis
Contents	Proteins, lipids, mRNA, micro RNA, rarely DNA, and cell organelles	Proteins, lipids, mRNA, micro RNA, rarely DNA	Fragmented DNA, non coding RNA, and cells organelles
Markers	Tetraspanins (CD63, CD81, CD9), Integrin, selectin, flotillins, and membrane related cell markers	Tetraspanins (CD63, CD81, CD9), heat shock proteins, Alix, Tsg101, integrin, and annexin	Phosphatidyl-serine, and membrane related cell markers

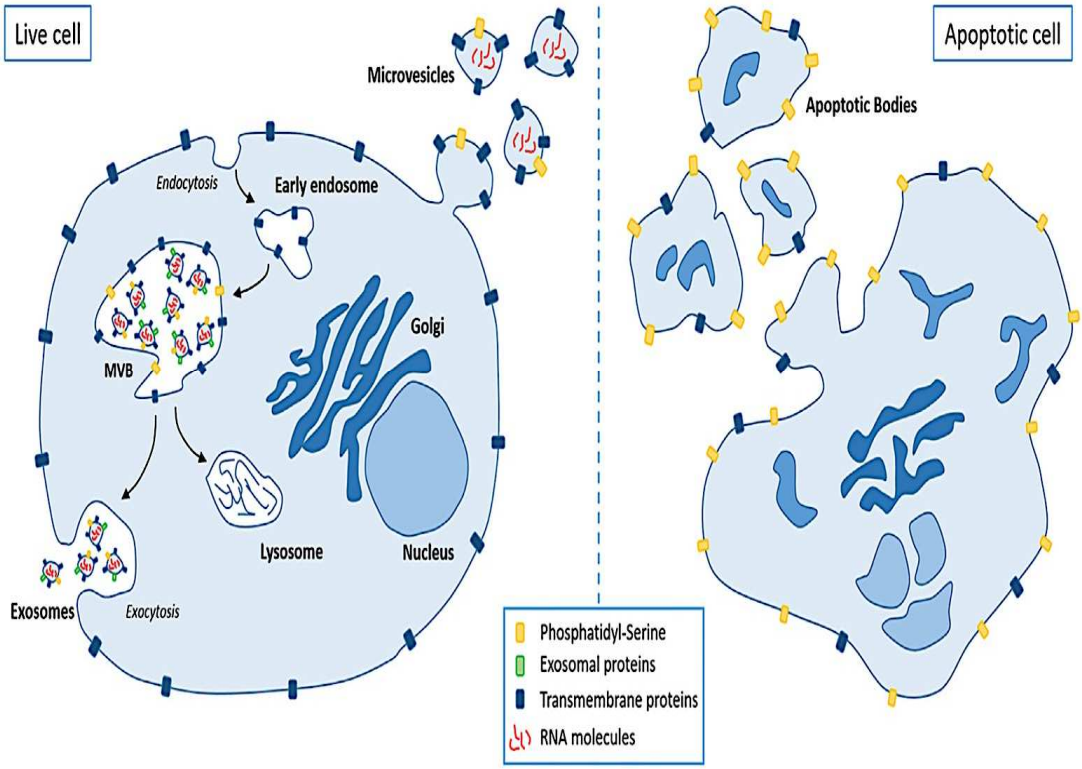

Figure.2: Schematic representation of the origin of EVs. MVs originate from direct budding of the plasma membrane, exosomes originate from exocytosis of MVBs out of the plasma membrane, and apoptotic bodies are as a result of blebbing of plasma membrane of the apoptotic cells. (Bruno et al., 2016)

2.6. Therapeutic Effects of MSCs-derived EVs

MSCs beneficial effects have been attributed to their secretion such as soluble factors and EVs. MSCs are known to secrete large amounts of EVs, which are emerging to have the potential to mimic MSCs in playing an important role in tissue regeneration and immunomodulation. They even could be more beneficial than the MSCs themselves for their therapeutic advantages. MSCs-derived EVs have been most widely studied in recent years. MSCs-derived EVs present surface markers such as CD29, CD44, CD73, CD105 and some molecules, which have been originally described as enriched in the mesenchymal lineage (Bruno et al., 2009; Amiri, et al., 2015; Abreu et al., 2016). They have been shown to be enriched with anti-apoptotic microRNA (miRNAs) and other soluble factors that can improve wound healing and angiogenesis (Amiri et al., 2015). Moreover, these vesicles may conceptually be engineered to carry molecules that modulate their targets (Tetta et al., 2012).

2.6.1. Roles of MSCs-derived EVs in Tissue Regeneration

The therapeutic effects of MSCs-derived EVs have been examined in various disease conditions in *in vivo* and *in vitro* models.

The Heart: The therapeutic effects of MSCs-derived EVs in myocardial injury models have been studied. It has been demonstrated that injected MSCs-derived EVs significantly decrease infarction size and oxidative stress, increase production of ATP and NADH, modulate inflammatory activities and activated the pro-survival signaling (PI3K/Akt pathway), which leads to enhanced cardiac function and reduced ischemic /

reperfusion acute myocardial injury *in vivo* models (Lai et al., 2010; Arslan et al., 2013; Bian et al., 2014). Kang et al. and his colleagues have reported the effect of MSCs-derived EVs on cardiac functions *in vitro* and in *in vivo* myocardial infarction models. They revealed that the CXCR-4 which is overexpressed by exosomes is acting to promote restoration of heart function and reduce left ventricular remodeling (Kang et al., 2015). Genetic information carried by MSCs-derived exosomes such as miRNAs have a cardio-protective role and saves cardiomyocytes against apoptosis and fibrosis (Feng et al., 2014).

Kidney and liver: Similar to MSCs treatment effects, several studies suggest that MSCs-derived EVs could significantly protect against kidney and liver injury through different experimental models. The therapeutic effects of MSCs-derived EVs have been investigated in acute kidney injury (AKI) models which were induced by hypoxia, ischemia–reperfusion, cisplatin, glycerol, gentamicin, and nephrectomy (Monsel et al., 2016). Bruno et al. found that MSCs-derived EVs enhancing the recovery of glycerol-induced AKI in an *in vivo* model of severe combined immunodeficiency mice. It improved the recovery of AKI through preventing apoptosis and increasing renal tubular epithelial cell proliferation (Bruno et al., 2009). In another study, these vesicles were found to assemble in glomeruli and tubules, give rise to a proliferation of tubular cells and consequently protect against both acute and chronic kidney injury development (Gatti et al., 2011). In hypoxic injured kidney tissues, MSCs-derived EVs have been shown to reduce the oxidative stress and apoptosis that result in improving renal function in AKI (G. Zhang et al., 2016).

Some other studies have focused on the role of MSCs-derived EVs in treating induced liver injury *in vivo* models (Li et al., 2013; C. Y. Tan et al., 2014). Li et al. illustrated that MSCs-derived EVs could mitigate liver fibrosis by decreasing the production of collagen, inhibiting TGF-β1/Smad phosphorylation signaling pathway, and restoring the function of aspartate aminotransferase (Li et al., 2013). Moreover, Tan et al. investigated the therapeutic effect of MSCs-derived EVs in induced liver injury. They found that vesicles administration could enhance liver regeneration through increasing hepatic cell proliferation, as a result of higher expression of proliferation proteins (PCNA and cyclin D1), and anti-apoptosis gene (Bcl-XL) (C. Y. Tan et al., 2014).

Brain: The therapeutic effect of MSCs-derived EVs have been studied in models of neurological disorders and shown to mimic the beneficial effects of MSCs. Doeppner et al. study demonstrated that BM-MSCs-derived EVs induced neuro-protection, neuro-regeneration, and improve stroke recovery (Doeppner et al., 2015). MSCs-derived exosomes have been shown to enhance the motor function of neuron axons by transferring microRNAs, from MSCs and load them into the recipient injured neural cells (Xin et al., 2013). Exosomes are non-toxic, have small size and a lipid-bound biogenic nature. These characteristics make them fit to cross through the blood brain barrier to reach the target cells (Xin et al., 2013; Pusic et al., 2014). In a comparison study between the effect of MSCs and their secretome, Lopez-Verrilli et al. observed that neuronal growth was inhibited by MSCs, and enhanced by their secretome, especially exosomes (Lopez-Verrilli et al., 2016). They supported the idea that MSCs-derived exosomes are more associated with beneficial effects and promote regeneration in the central and

peripheral nervous system after nerve injury.

Lung: The anti-inflammatory effects of MSCs-derived EVs were demonstrated in several *in vivo* models of acute lung injury (ALI). In Escherichia coli-induced ALI, Zhu et al. demonstrated that MSCs-derived EVs reduced inflammation and protein permeability in lung, which leads to protection from pulmonary oedema (Zhu et al., 2014; Monsel et al., 2015). In silica-induced ALI, Phinney et al. showed that MSCs-derived exosomes reduce the nodules size and leukocyte number in bronchial lavage fluid of murine lung (Phinney et al., 2015). Similarly, in a mouse model of hypoxia-induced pulmonary arterial hypertension, Lee et al. reported that MSCs-derived exosomes suppress the early hypoxic signal, and act as anti-inflammatory tool by activating the alveolar macrophages (Lee et al., 2012).

Taken together, administration of MSCs-derived EVs can be a powerful therapeutic tool to transport the needed molecules to target tissues, which could lead to treatment of various disease conditions.

2.6.2. MSCs-derived EVs Role in Immunomodulation

Secreted vesicles mimic the immunoregulatory effects of whole cells. Several studies have investigated the various immunomodulatory effects of MSCs-derived EVs in different experimental models of immune-related diseases.

The effects of MSCs-derived EVs against adaptive immune cells (T-cell subsets and B-cells) have also been shown. For example, in a study by Mokarizadeh, the authors showed that BM-MSCs -derived MVs inhibit auto-reactive splenic mononuclear cells activation and proliferation by their tolerogenic molecule contents such as PDL-1, TGF-β, and galecin-1. Furthermore, they showed that when these vesicles are added to splenic mononuclear cells, they Inhibit T-cells differentiation and activation, as well as the interferon-gamma (IFN-γ) secretion. Also, vesicles induce T-cells to secrete anti-inflammatory cytokines such as interleukin-10 (IL-10) and transforming growth factor -β (TGF-β), and activate CD4+, CD25+, Foxp3+ regulatory T cell (Treg) generation and differentiation (Mokarizadeh et al., 2012). Another study investigated that human adipose MSCs-derived exosomes suppress CD4+ and CD8+ T cells activation, proliferation and differentiation *in vitro*, and significantly inhibit their IFN-γ secretion (Blazquez et al., 2014). In addition, microvesicles derived from MSCs were found to modulate T cell anti-inflammatory response *in vitro* model of type-1 diabetes. They have been found to increase T-regulatory cells and their regulatory secretions such as PGE2 and TGF-β, and decrease of Th1 and Th17 cells along with their cytokine secretions (Favaro et al., 2014).

Interestingly, in a study conducted for investigating the effects of vesicles derived from MSCs on glioblastoma cells, both vesicles isolated from BM or umbilical cord - MSCs were found to decrease glioblastoma cell proliferation, while vesicles isolated from adipose tissue were found to increase them (Del Fattore et al., 2015). This suggests that MSCs-derived EVs can either induce or inhibit the immune responses based the tissue of origin, which has to be considered especially in immuno-studies. Researchers suggest that MSCs-derived EVs have a wide range of immunomodulatory effects on different types of the immune system cells and their ability to modulate diseases, which open the way toward new treatment application of autoimmune diseases.

In addition to their effects on adaptive immune system, MSCs-derived EVs may also target innate immune cells. A study by Zhang et al. demonstrated that MSCs-derived EVs activate M2-like monocytes, which subsequently increase regulatory T cell and improve allogeneic skin graft survival in mice (B. Zhang et al., 2014). Another study found that, when macrophages engulf the MSCs-derived EVs, the vesicles transfer their mitochondrial contents and consequently inhibit Toll-like receptor expression and signaling in macrophages. This lead to inhibited macrophage activation and suppresses the inflammation in lung injury models (Phinney et al., 2015). In the same way, Favaro et al. demonstrated that MSCs-derived EVs could target dendritic cells and inhibit their maturation in patients with type-1 diabetes, which delay T cells autoreactive and promote the anti-inflammation(Favaro et al., 2016).

MSCs-derived EVs have also a strong inhibitory effect on B lymphocytes proliferation and differentiation and their immunoglobulin secretion, when they co-culture with peripheral blood mononuclear cells (Budoni et al., 2013).

2.7. Advantages of MSCs -derived EVs over MSCs

Despite the promising strategies to overcome MSCs challenges in cell therapy, some challenges such as their low proliferation rate, and the restricted life span during *in vivo* and *in vitro* expansion, hinder their use in cell therapy. For examples, during *in vivo* transplantation, MSCs undergo apoptosis, resulting in as many as 99 % cell death during the first few days post transplantation. As a result, only a few MSCs reach the target when injected *in vivo*, while the majority remain accumulating in the lungs, spleen and liver (Toma et al., 2002; Boyette & Tuan, 2014).

Such issues may be overcome by cell-free strategies, which have shown beneficial effects under many different pathological conditions. Especially promising agent of such therapies are MSCs-derived EVs, which have many therapeutic advantages over the MSCs themselves: 1) MSCs-derived EVs may decrease some of MSCs engraftment associated risks like MSCs trapping and apoptosis. So, vesicles are able to home to the target tissue, without accumulation in other organs. 2) They also have as lower risk of tumor generation (Abreu et al., 2016). 3) They have less immune rejection than MSCs after injection. This is due to lower cell surface protein content such as major histocompatibility complex molecules compared to parental MSCs, which decrease the organ persistence (Konala et al., 2016). 4) Unlike secreted soluble factors such as

cytokines, growth factors, and RNAs, MSCs-derived EVs are encapsulated, and their content is protected from rapid degradation even after long storage for 6 months at -20 C (Konala et al., 2016).

2.8. MSCs-derived EVs Isolation Methods

Different types of EVs can be isolated and purified from biological fluids or from conditioned media of cell culture. Numerous isolation protocols can be used to purify EVs according to their size, density, biochemical and biophysical properties. The purification of EVs is mainly achieved by three methods, namely ultracentrifugation, ultrafiltration and immunoprecipitation using antibody-loaded magnetic cell beads (Maumus et al., 2013).

The most commonly accepted and used method to isolate EVs is differential ultra-centrifugation. It is a series of repeating centrifugation steps involving gradually increasing speeds, which isolates vesicles according to their size and density. First a low-speed centrifugation removes cells, large apoptotic bodies and other debris, followed by 10,000–20,000 ×g centrifugation to pellet microvesicles (MVs), and finally ultra-centrifugation (100,000–120,000 ×g) to pellet exosomes. After each centrifugation, the supernatant is collected and transferred to another tube, while the pellet is kept. Although ultra-centrifugation is the most commonly used and considered the gold-standard method to isolate EVs, it has certain challenges and limits, which doesn't make it proper method under all conditions. Some of these limits are the length of centrifugation time, the amount of contaminants that may be pelleted with centrifugation, and non-strict vesicle

separation due to overlapping size spectra (Théry et al., 2002; Théry et al., 2006; Witwer et al., 2013; Peterson et al., 2015; Rani & Ritter, 2016). EVs can be purified based on their different density by floatation the sample containing EVs into a sucrose density gradient/ sucrosedeuterium oxide (D_2O) cushions under centrifugation. This protocol is helping to avoid protein co-pelleting with EVs (Théry et al., 2002; Théry et al., 2006).

In addition, ultrafiltration is another commonly used isolation protocol that can be used to eliminate various contaminations based on their hydrodynamic radii. It can be used alone or with other isolation procedures such as ultra-centrifugation or size exclusion chromatography. In ultrafiltration protocol, sample contained vesicles are filtered through a semipermeable membrane. Although this technique has some disadvantages such as blocking of filters and shearing stress that may affect the vesicles characteristics, it has been reported that vesicles yield is more as compared to ultracentrifugation-based isolation (Théry et al., 2006; Nordin et al., 2015; Rani & Ritter, 2016).

Size exclusion chromatography has also been used to isolate vesicles. Chromatography separation is most often used after low speed centrifugation to eliminate the large contaminants and filtration to concentrate the sample. Based on their variety of vesicles size, they can pass through the stationary phase of a chromatography column differentially and are thus separated (Böing et al., 2014; Muller et al., 2014).

Additionally, vesicles can be extracted based on their membranous protein expression. Immunoaffinity purification is a technique that uses micro-beads coated with specific antibodies against proteins on the vesicle surface. So that vesicles can be sorted out by choosing the correct antibodies for the wanted vesicles. This technique has also been used as an additional isolation step after ultracentrifugation or filtration. The limit of this method is that only vesicles that have the selected protein can be isolated, while the rest of vesicles in the sample are neglected (Théry et al., 2002; Théry et al., 2006; Tauro et al., 2012).

In fact, there is no one best method to isolate vesicles. The choice of isolation method differs between laboratories and many factors affect it. Just to mention some: purity and origin of the samples containing vesicles, type of vesicles intended to be isolated, follow-up measurements. Before designing any isolation experiment, such factors must be taken in consideration, and characteristics of isolated vesicles must be precisely analyzed (Cvjetkovic et al., 2014).

2.9. MSCs-derived EVs proteome

Proteomics techniques such as mass spectrometry (MS) and protein microarrays have been emerging as an effective tool to characterize the protein of any complex mixture. Mass spectrometry, the most common proteomics technique, has increased our understanding of the protein complexes and their related biological questions. This technology allows peptide sequencing, which enable peptides and proteins quantification and identification (Han et al., 2008; Mallick & Kuster, 2010).

Quantitative proteomic strategies such as stable isotope dimethyl labeling, and stable isotope labeling with amino acids in cell culture (SILAC) are currently being widely applied. The idea behind stable isotope labeling is creating a mass shift between identical peptides, in order to distinguish and compare the relative abundances of the same protein in different conditions in one MS analysis. Differential protein expression profiling allows signaling pathway analysis, which helps developing protein biomarkers (Han et al., 2008; Kovanich et al., 2012).

In the past few years, proteomic analyses have contributed significantly to clarify the molecular composition of extracellular vesicles and identified proteins that might be associated with fundamental functions and pathways. Few comprehensive mass spectrometry-based proteomic studies of MSCs-derived EVs have been performed (Kim et al., 2012; Lai et al., 2012; Eirin et al., 2016). These previous studies profiled the proteome of MSCs-derived EVs using LC-MS/MS proteomic analyses to understand their potential therapeutic effects. Identified proteins were classified over different biological process groups including vesicles structure, biogenesis, self-renewing, differentiation, proliferation, communication, and tissue repair related proteins that can be associated with the therapeutic effects of MSCs-derived EVs. These proteomic profiling give understanding to their impact on tissues recovery in order to identify candidate proteins associated with their therapeutic potential in various pathological conditions.

3. MATERIALS

3.1. Materials Used for Bone Marrow Mesenchymal Stem Cell Culture

	Tissue Culture	Catalog Number	Company	Country
1	Counting Slides, Dual Chamber For Cell Counter	145-0011	Bio-Rad	USA
2	Dulbecco's Phosphate Buffered Saline (DPBS)	55-031-PB	Corning	USA
3	Eppendorf Centrifuge	5804	Eppendorf AG	Germany
4	Human Mesenchymal Stem Cells	PT-2501	Lonza	USA
5	L-Glutamine,100x	25-005-CI	Corning	USA
6	Mesencult ™ MSC Basal Medium (Human) Mesenchymal stem cell stimulatory supplements (Human)	05401 05402	Stem Cell Technology	Canada
7	Penicillin/streptomycin solution,100 x	30-002-CI	Corning	USA
8	TC^{20} Automated Cell Counter	508BR04557	Bio-Rad	USA
9	Tissue Culture Flask, 75 cm^2	353136	Falcon	USA
10	Trypan Blue Dye, 0.40%	145-0013	Bio-Rad	USA
11	Trypsin EDTA,1 X	25-052-CI	Corning	USA

3.2. Materials Used for Extracellular Vesicles Harvesting

	Extracellular Vesicles Harvesting	Catalog Number	Company	Country
1	100 Ti Rotor, Fixed Angle	363013	Beckman Coulter	USA
2	Avanti J-30I Centrifuge	J-301	Beckman Coulter	USA
3	JA-20 Rotor, Fixed Angle	334831	Beckman Coulter	USA
4	Optima™ L-100 XP Ultracentrifuge	392050	Beckman Coulter	USA
5	Polyallomer bottles 50 ml	357003	Beckman Coulter	USA
6	Polycarbonate Thick Wall Centrifuge Tubes	355645	Beckman Coulter	USA

3.3. Materials Used for Extracellular Vesicles Protein Extraction

	Protein Extraction	Catalog Number	Company	Country
1	96-Well ELISA Plate	353072	Falcon	USA
2	Albumin Standard Ampules	23209	Thermo Scientific	-
3	Benzonase Nuclease	E1014	Sigma	Denmark
4	Chloroform Anhydrons	372978	Sigma	USA
5	CLARIOstar Microplate Reader	-	BMG LabTech	-
6	HPLC-Grade Methanol	34860	Sigma	USA
7	HPLC-Grade Water	34877	Sigma	USA
8	Phosphatase Inhibitors PhosStop	04906837001	Roche	Germany
9	Protease Inhibitors Complete EDTA-Free	11873580001	Roche	Germany
10	Quick Start Bradford 1x Dye Reagent	500-0205	Bio-Rad	USA
11	Sodium Dodecyl Sulfate Solution	05030	Sigma	Switzerland
12	Urea	U5128	Sigma	Germany

3.4. Materials Used for Protein Gel

	Protein Gel	Catalog Number	Company	Country
1	Dl-Dithiothreitol	D0632	Sigma	
2	MES SDS Running Buffer	786-531	G-biosciences	USA
3	NuPAGE® LDS Sample Buffer (4X)	NP0007	ThermoFisher scientific	USA
4	NuPAGE™ Novex™ 4-12% Bis-Tris Protein Gels, 1.0 mm, 15-well	NP0323	Invetrogen	Carlsbad
5	PageRuler™ Plus Prestained Protein Ladder	26619	ThermoFisher scientific	Lithuania
6	Quick Start™ Bradford 1x Dye Reagent	5000205	Bio-rad	USA

3.5. Materials Used for In Solution Digest

	In Solution Digest	Catalog Number	Company	Country
1	Dl-Dithiothreitol (DTT)	D0632	Sigma	USA
2	Iodoacetamide (IAA)	I1149	Sigma	USA
3	Lysyl Endopeptidase-Sequencing Grade From Lysobacter Enzymogenes	129-02541	Wako Chemicals	-
4	Sequencing Grade Modified Trypsin	V511a	Promega	-
5	Triethylammonium Bicarbonate Buffer (TEAB)	T7408	Sigma	Switzerland

3.6. Materials Used for Reductive Dimethyl Labeling for Peptides

	Reductive Dimethyl Labeling	Catalog Number	Company	Country
1	Ammonia Solution (25%)	105428	Millipore Chemicals	Germany
2	Deuterium oxide (D_2O; heavy water; 99.98 atom% ± 0.01 atom% D	364312	Sigma	-
3	Formaldehyde (CH_2O) Solution (37%)	252549	Sigma	Netherlands
4	Formaldehyde-^{13}C-D_2 ($^{13}CD_2O$) Solution (20 Wt. % In D_2O, 98 Atom %D, 99 Atom % ^{13}C)	596388	Sigma	USA
5	Formaldehyde-D_2 (CD_2O) Solution (~20 Wt. % In D_2O, 98 Atom % D)	492620	Sigma	USA
6	Formic Acid (98%)	FX0440-7	Millipore Chemicals	-
7	HPLC-grade light water (CHROMASOLV Plus for HPLC	34877	Sigma	USA
8	Sodium Cyanoborodeutride ($NaBD_3CN$)	Sc-258163	Santa Cruz	-
9	Sodium Cyanoborohydride ($NaBH_3CN$)	156159	Sigma	USA

3.7. Materials Used for Isoelectric Focusing

	Isoelectric Focusing	Catalog Number	Company	Country
1	OFFGEL low resolution kit	5061-0202	Agilent Technologies	Germany
2	G3100 OFFGEL fractionator	DE01500760	Agilent Technologies	Germany
3	Immobiline TM Dry Strips, pH 3-11, NL, 13 cm	17-6003-75	GE Healthcare	Sweden
4	IPG buffer, pH 3-11 NL	17-6004-40	GE Healthcare	Sweden

3.8. Materials Used for STAGE Tipping

	Stage Tipping and Elution	Catalog Number	Company	Country
1	Acetic Acid	33209	Sigma	Germany
2	Acetonitrile	34967	Fluka	Germany
3	Empore Solid Phase Extraction Disks, C18 (Octadecyl)	2215	Empore	USA
4	EpT.I.P.S Reloads 2-200 µl	0030 073.428	Eppendorf	Germany
5	Metal Hub Needle, 17 G, Point Style 3	-	-	-
6	Syringe + Needle Part	-	-	-
7	Trifluoroacetic Acid	T6508	Sigma	USA

4. METHODS

4.1 Bone Marrow Mesenchymal Stem Cell Culture

Three different lots of bone marrow-derived mesenchymal stem cells (BM-derived MSC) from three different human donors were purchased (Lonza). Cryovials of BM-derived MSC were quickly thawed not longer than two minutes in a 37 C° water bath. Thawed cells were cultured and grown in Mesencult ^{TM} MSC Basal Medium (complete medium kit with stimulatory supplements) with 1x of penicillin/streptomycin solution (100x), and 1x of L-Glutamine (100x). During tissues culturing, cells were incubated at 37 C° and 5% CO_2 in a humidified incubator. Cells were seeded every 7 days at 80-90% confluence, while media was changed twice a week. For seeding, cells were washed twice with 1X Dulbecco's Phosphate Buffered Saline (PBS), then detached using 1X Trypsin EDTA. Cell count and viability were evaluated using Trypan blue dye (50% v/v dilution) and automated cell counting. According to the recommended seeding density provided by the company, cells were plated at 5,000 cells/cm^2 (approximately 375,000 cells / 75 cm^2 flasks). Cells used for harvesting of extracellular vesicles (EVs) were between passages 4-8.

4.2. Extracellular Vesicle Harvesting

One week before harvesting EVs, cells were washed twice with 1X PBS; the media with centrifuged mesenchymal stem cell stimulatory supplements were added. The stimulatory supplement was centrifuged at speed 100,000 g for 4 hours at 4 C^o. This is to reduce the possible exosomes and proteins that could be contained in the stimulatory

supplements and interfere with EVs protein. At 80-90% confluence, conditioned medium containing the microvesicles and exosomes secreted from BM–derived MSCs were collected. First of all, the conditioned media was centrifuged at 1,200 g speed for 5 minutes to clean it from dead cells and large debris. The pellet was discarded and the supernatant was centrifuged at 5,000 g speed for 10 minutes to discard large proteins. The pellet was discarded and the supernatant centrifuged to concentrate the EVs. To harvest the large-size vesicles (microvesicles), the supernatant of conditioned medium was centrifuged at 20,000 g speed at 4 C° for 1 hour. Supernatant was pipetted out for further ultra-centrifugation to pellet the small-size vesicles (exosomes), leaving the pellet of microvesicles. The pellet of microvesicles was washed with 1 X PBS buffer and centrifuged at the same centrifugation conditions. The supernatant (containing exosomes) was further centrifuged using the ultra-centrifuge at 100,000 g speed at 4 C° for 1 hour to harvest the exosomes. The pellet of exosomes was washed with 1 X PBS buffer and centrifuged at the same centrifugation conditions.

4.3. Extracellular Vesicles Protein Extraction

Pellets of microvesicles and exosomes were lysed and protein was extracted directly using 10 % Sodium Dodecyl Sulfate buffer (SDS). SDS buffer was supplemented with protease inhibitors, phosphatase inhibitors and benzonase nuclease. Before storing the vesicles lysates in – 80 C^{o} freezer for later use, they were incubated at room temperature for 30 minutes for efficient lysis.

According to Wessel/Fluegge (Wessel & Flügge, 1984), protein was precipitated using methanol/chloroform precipitation method. Precipitation was performed as the following: 4 sample volumes of methanol were added to the samples, followed by 1 sample volumes of chloroform, then 3 sample volumes of dH_2O. The sample was centrifuged for 10 minutes at 20,000 g speed. After discarding the top aqueous phase, 4 sample volumes of methanol were added. The sample was centrifuged again for 10 minutes at 20,000 g speed. Methanol was removed, and precipitated protein was dried. Protein pellet was resuspended in 6 M Urea/2 M Thiourea in 10 mM HEPES buffer, pH 8.0.

Total protein concentration was determined by Bradford assay. Bovine serum albumin was used for the standard curve at 0.5, 1, 1.5, 2, 2,5 and 3 ug/µl. Protein concentration was read using the CLARIOstar Microplate Reader at 580 nm.

4.4. Protein Gel Electrophoresis

The MSCs total extract, microvesicles, and exosomes proteins were separated by protein gel electrophoresis. Prior to running the gel, proteins were mixed with 100 mM dithiothreitol (DTT) and 1X LDS, and then incubated at 95 C^o for 5 minutes. 5 µg of page ruler marker were loaded as a molecular weight ladder, and a total of 3 µg of total cells and vesicles lysate were loaded each lane. The gel was run in MES buffer under the following conditions: 200 volt, 125 mA, and 45 minutes. The gel was washed for 10 minutes 3 times with water before staining overnight.

4.5. In Solution Digest

After 30 minutes reduction with 1 mM of dithiothreitol (DTT) and 20 minutes alkylation with 5 mM iodoacetamide in the dark, proteins were digested by Lys-C while incubated for 3 hours at room temperature. For efficient trypsin digestion, sample containing 6 M of Urea/ 2 M Thiourea buffer was diluted to 2 M Urea using 50 mM Triethylammonium Bicarbonate Buffer (TEAB). Proteins were digested by trypsin overnight at room temperature. Lys-C and Trypsin were used at enzyme to protein ratio of 1:50. The samples were kept in – 80 C^o freezer until further use. In solution digest protocol is based on (Graumann et al., 2008)

4.6. Reductive Dimethyl Labeling for Peptides

After protein digestion, stable isotope dimethyl labeling was performed at the peptide level. Exosome peptides were labeled as "heavy", microvesicles as "medium", and total extract of MSCs as "light". Light, medium, and heavy forms of formaldehyde (4% CH_2O in H_2O, 4% CD_2O in D_2O, 4% $^{13}CD_2O$ in D_2O) were added to exosomes, microvesicles, and MSCs peptides, respectively. Followed by sodium cyanoborohydride (0.6 M of $NaBH_3CN$ in H_2O), which were added to the samples to be light and intermediate labeled, and sodium cyanoborodeuteride (0.6 M of $NaBD_3CN$ in D_2O), which were added to the samples to be heavy labeled. After incubation at 22 C^o for 1 hour while mixing, labeling reactions were quenched by adding 1% of ammonia solution, followed by 5% of formic acid to quench the reaction further. The three differently labeled samples (total MSC extract as light, MVs as medium, exosomes as heavy) were mixed at ratio 1:1:1. The reductive Dimethyl Labeling protocol is based on

(Boersema et al., 2009).

Desalting of labeled peptides was performed using Oligo R2 and R3. R2 and R3 column was prepared using C18 plug by adding around 1 cm of mixed R2 and R3 slurry. Briefly, R2 and R3 beads were conditioned using 100 ul of 0.1 % of trifluoroacetic acid (TFA). After which, the peptides are loaded, the beads are again washed with 100 ul of 0.1 % TFA, then the peptides were eluted using B 60 buffer (60 % acetonitrile, 0.5% TFA). Eluted peptides were dried by vacuum centrifugation prior to fractionation by isoelectric focusing.

4.7. Isoelectric Focusing

Peptide mixes were separated into 12 fractions over a pH range of 3-11 by isoelectric focusing (OFFGEL fractionator, Agilent) according to manufacturer's protocol with some adaptation. In the running buffer, glycerol was reduced from 6 % to 0.3%, and the ampholytes were reduced from 1% to 0.1%. After the run, fractions were harvested with 1% acetonitrile/0.05% TFA and dried down using a vacuum centrifugation. Until STAGE tipping, peptide fractions were stored at −80 C°.

4.8. STAGE Tipping

200 µl eppendorf pipettes tips were packed with 3 layers of C18 disks to make the STAGETips, which were activated with one methanol wash followed by two washes of 2% acetonitrile/ 0.1% TFA. After peptides were loaded, STAGETips were washed with 0.5% acetic acid, and kept in 4 C^{o} until peptide elution. Prior elution, STAGETips were

washed with 0.5 % acetic acid, followed by 2% acetonitrile/0.1% TFA as an additional wash to reduce the ampholytes contamination from IPG buffer. Peptides were eluted twice with 200 µl of 60% acetonitrile/0.5% TFA, and then dried down using a vacuum centrifugation. After that, peptides were re-suspended in 0.5% acetic acid to be run in LC-MS/MS.

4.9. Mass Spectrometry

Each experiment, consisting of a heavy, medium and light mixed labeled conditions was individually submitted to nano liquid chromatography (LC) coupled to mass spectrometry (MS) analysis (Nano LC-MS/MS). The analytical platform consisting of an EASY nLC-II system interfaced to a Q Exactive mass spectrometer MS (Thermo Scientific, Bremen, Germany). Chromatography conditions were defined as follows: H_2O with 0.5% acetic acid for mobile phase A; H_2O: acetonitrile, 20:80 volume ratio, with 0.5% acetic acid for mobile phase B; flow-rate of 250 nL/min; injection volume of 6.0 uL and a maximal loading pressure of 280 bars. LC separation was done on 20 cm long in-house packed emitter columns (ReproSil-Pur 120 C18-AQ 3 µm diameter beads, Dr. Maisch GmbH) using a gradient ranging from 5 to 30% mobile phase B over 90 min, followed by a 25 min wash and column re-equilibration cycle. Precursor scans (MS^1 level) were acquired at a resolution of 70000 (at m/z 300) and an AGC (advanced gain control) target value of 3·106 charges (maximum ion injection time 20 ms). Fragmentation spectra (MS^2 level) were acquired at a resolution of 17500 (at m/z 300) and an AGC target value of 1·105 charges (maximum ion injection time 120 ms).

4.10. Data Analysis

Our 36 mass spectrometer runs were analyzed by MaxQuant version 1.5.2.8 using a Homo sapiens database downloaded from UniProtKB. MaxQuant is a software package used to analyze raw MS data to identify and quantify peptides and aggregate corresponding results at the protein level (Cox & Mann, 2008). It can be freely downloaded from their site (http://www.maxquant.org).

Proteomic data sets were analyzed using the empirical Bayes moderated T test implemented by the limma package <CITE LIMMA> in an in house R analysis pipeline. P values were corrected for multiple hypotheses testing using the Benjamini- Hochberg correction with an adjusted P value cutoff of 5% ($P < 0.05$). Protein quantification was based on two ratio counts. Differential expression analysis was calculated based on log2 normalized ratios. Protein with adjusted P value < 0.05 and an absolute fold change (MVs/TE) or (EXO/TE) > 2 were classified as enriched protein in vesicles, while in case (MVs/TE) or (EXO/TE) < -2 were considered as depleted in vesicles. Gene set enrichment analyses were performed for vesicle enriched proteins using Fisher's exact test with the total dataset as background. All functionalities for data analysis downstream of MaxQuant (normalization, PCA, statistics, gene set enrichments based on GO and KEGG) are combined in an in-house-built R package (autonomics).

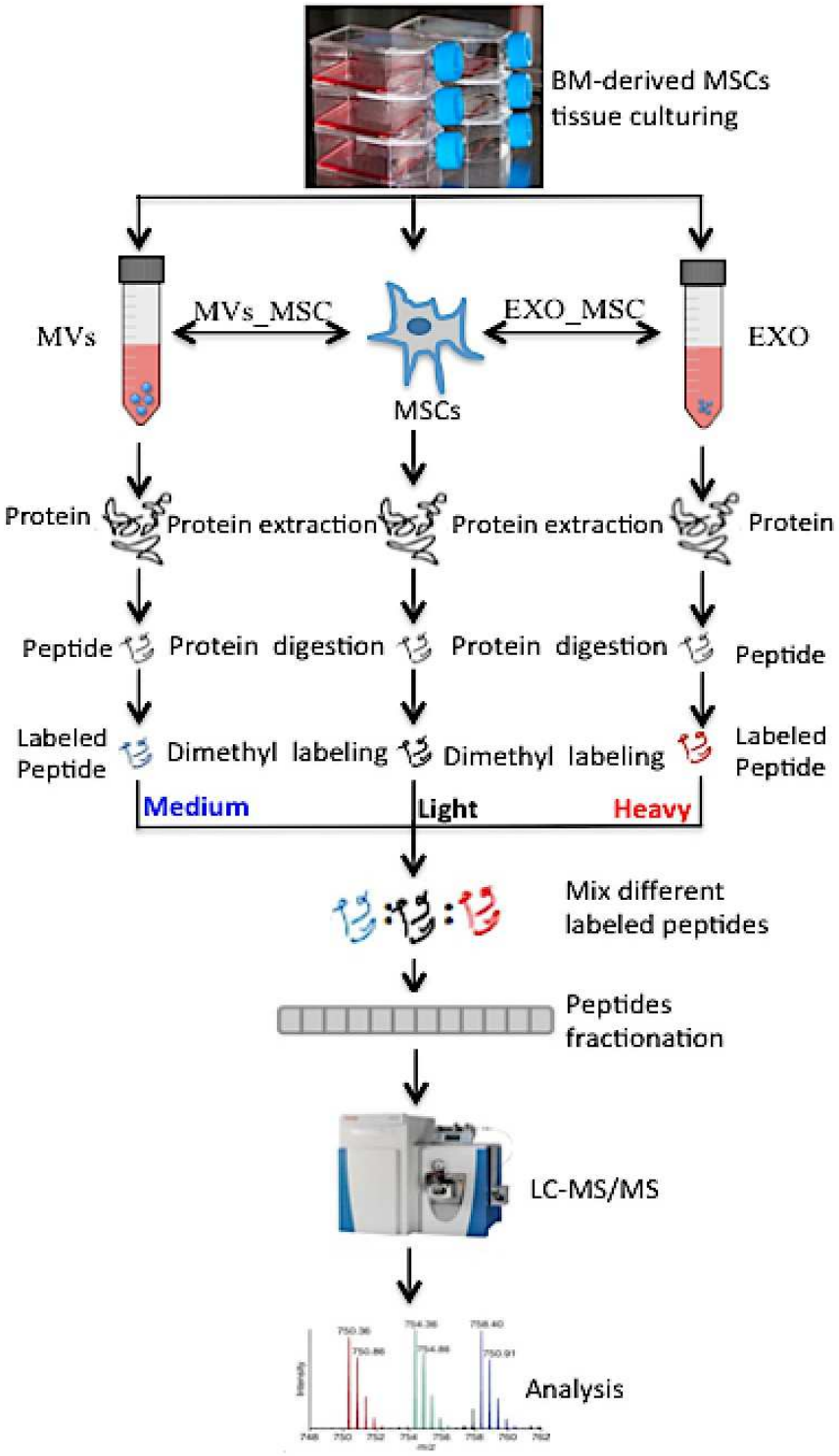

Figure 3: Summary of work plan (prepared using power point document)

5. RESULTS

5.1. Cell Line Culture: BM-derived MSCs

Light microscopic images of BM-derived MSCs from the three different donors are shown in **figure.4**. BM-derived MSCs appear as fibroblast-like cells with elongated spindle shape. MSCs-derived EVs have been obtained from BM-derived MSCs from three donors. Both microvesicles and exosomes have been harvested at 70-90% sub-confluence monolayer at passages 4 to 8.

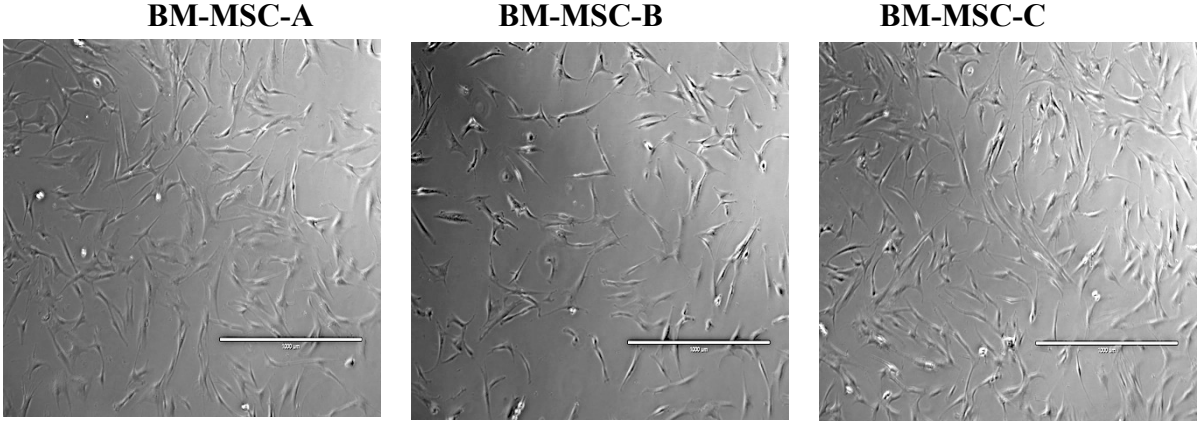

Figure.4: Morphological appearance of BM–derived MSCs while grown in Mesencult media. Light microscopy (Magnification 4 X), scale bar represents 1000 μm.

5.2. Proteins Bands of MSCs Total Extract, Microvesicles and Exosomes

Figure.5 shows the SDS-PAGE gel of MSCs total extract (TE), and their corresponding microvesicles (MVs) and exosomes (EXO) lysates. It shows similar pattern of protein bands for each lysate, supporting reproducibility of the vesicle preparation and samples processing.

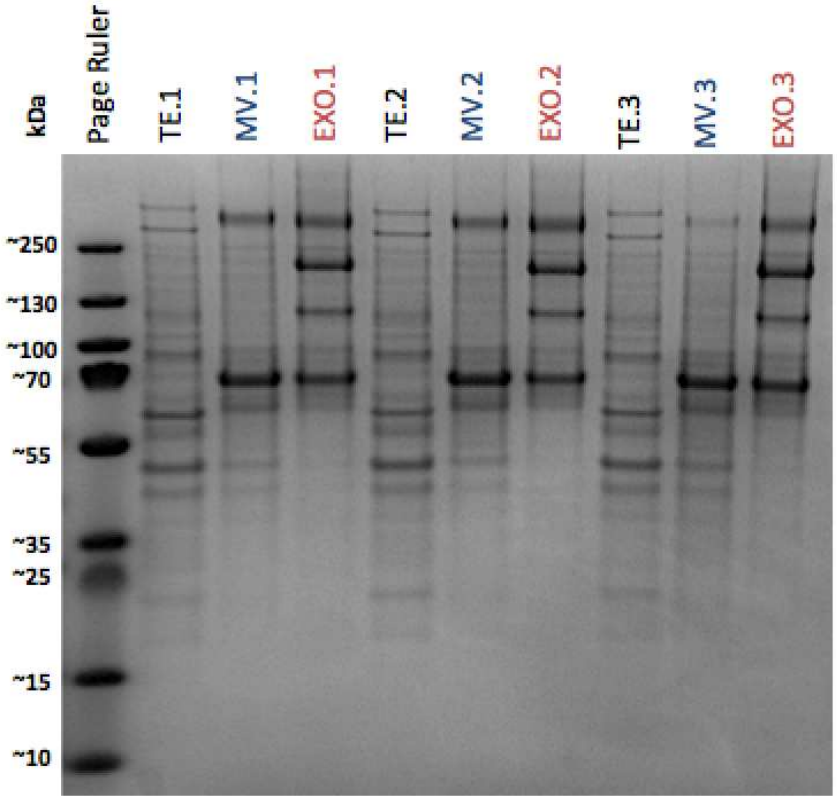

Figure.5: SDS-PAGE (4-12 % gradient gel) of MSCs total lysate (TE), their corresponding microvesicles (MVs) and exosomes (EXO) lysates (in triplicate).

5.3. Principal Component Analysis of the 3 Subgroups

Principal component analysis was applied to provide an overview of the separation pattern and the relationship among the three subgroups EXO_MV, EXO_MSC, and MV_MSC (**Figure.6**). PCA in a 2-dimensional graph scatterplot shows a clear clustering of the subgroups and validates the experimental design. The second component (X2: 16 %) characterizes the proteomes of MVs and exosomes to be much closer related to each other as compared to MSC whole cell lysate.

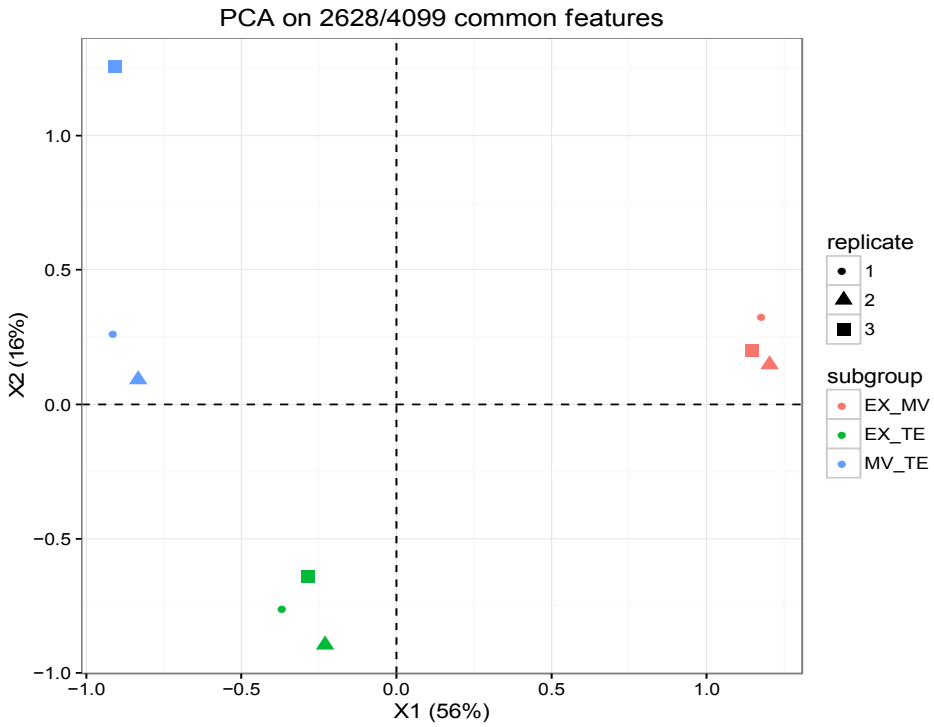

Figure.6: Principal component analysis scatterplot of the 3 comparisons: EXO_MV (Red), EXO_MSC (Green), MV_MSC (Blue).

5.4. Proteins Enriched in MVs and Exosomes

A total of 5207 proteins were identified. Out of 4695 proteins quantified in MVs, and by applying a fold change cutoff value of 2: 477 proteins were enriched in MVs over total extract, 1852 proteins were depleted, and 2366 proteins were equally expressed in both MVs and their parent cells (MSCs) (**Figure.7**). While in the EXO_MSC subgroup, 4386 proteins were quantified. Out of those, 555 proteins were enriched in exosomes, 1807 proteins were depleted, and 2027 proteins were equally expressed when compared to MSCs (**Figure.7**).

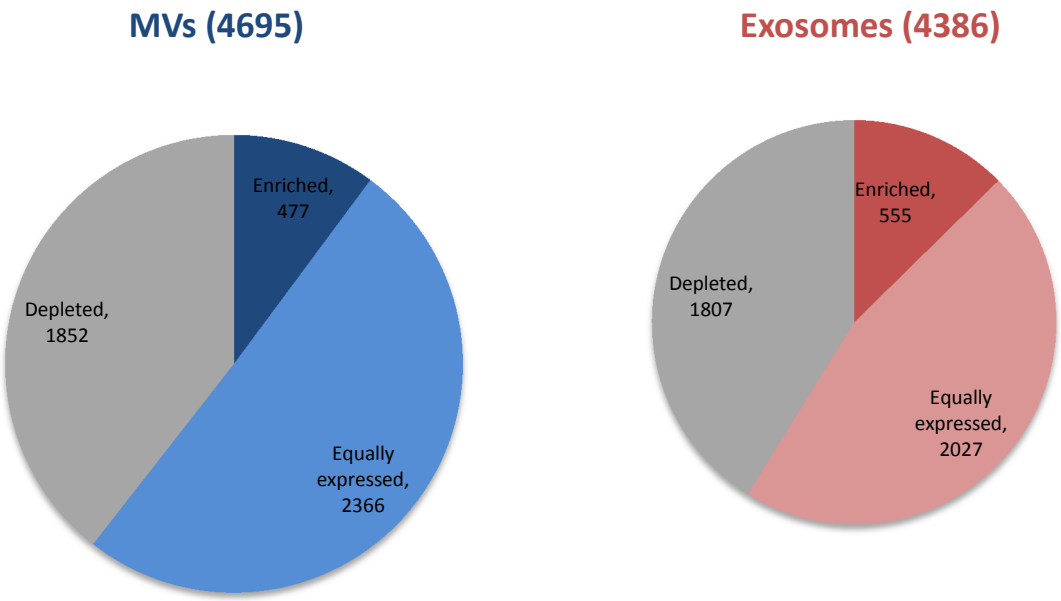

Figure.7: Number of differentially expressed proteins in MVs and exosomes compared to MSCs proteins based on 2 cutoff fold changes.

The majority of proteins identified in the triplicate experiment (4986 out of 5207 proteins) were quantified in all preparations (MSCs, MVs and EXO). Eighty-nine proteins were identified in MSCs total lysate only, while 10 and 11 proteins were identified in MVs and exosomes only, respectively (**Figure.8**).

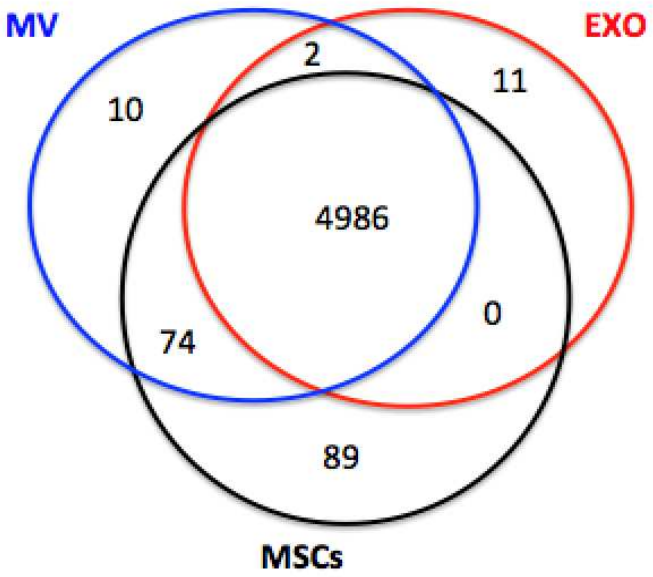

Figure.8: Venn diagram of the proteins identified in MSCs, microvesicles (MVs) and exosomes (EXO). The majority of proteins identified were common to all preparations.

5.5. Analysis of the Most Significantly up-regulated Proteins in Microvesicles and Exosomes Compared to MSCs

The top 36 significantly up-regulated proteins in microvesicles compared to MSCs in MV_MSC subgroup are represented in log2 fold changes as triplicate in **figures.9 and 10**. While **figures.11 and 12** show the top 36 significantly up-regulated proteins in exosomes compared to MSCs in EXO_MSC subgroup. Comparing between the top most significant proteins in subgroup MV_MSC with subgroup EXO_MSC, we can find that all proteins that up-regulated in MVs are also up regulated in exosomes when compared to MSCs (**Figure.9**). Only a small number of proteins (e. g. PXDN, COL6A3, PTX3, LTBP2, and APOB) of the top 36 most significantly up-regulated proteins in exosomes were significantly enriched in exosomes and less so in MVs. **Table. 2** shows the significance values, fold changes increase compared to MSCs, and functions of these proteins.

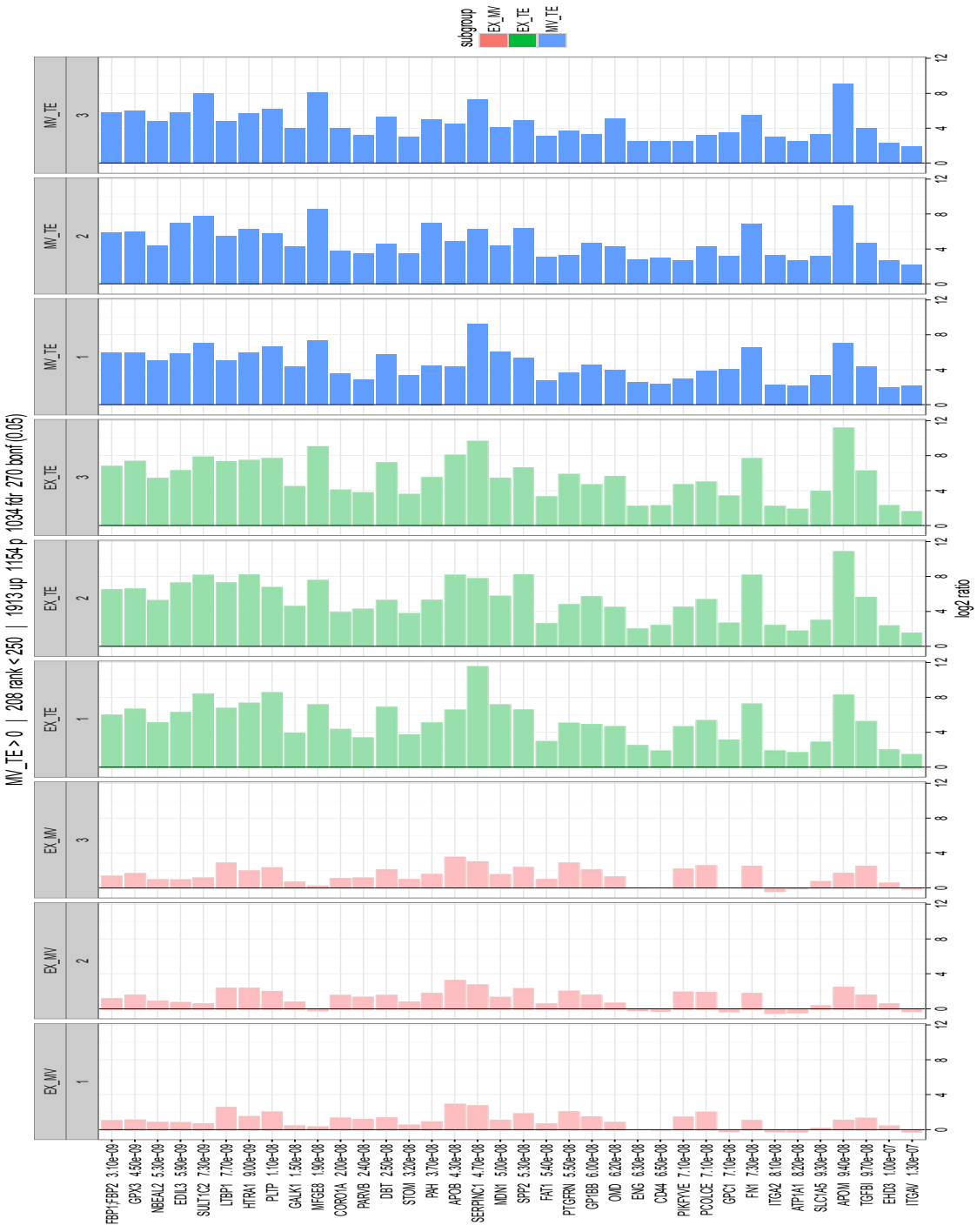

Figure.9: Bar plot of the most 36 significantly up-regulated proteins in MVs compared to MSCs (MV_MSC, Blue), represented in log2 fold changes (in triplicate).

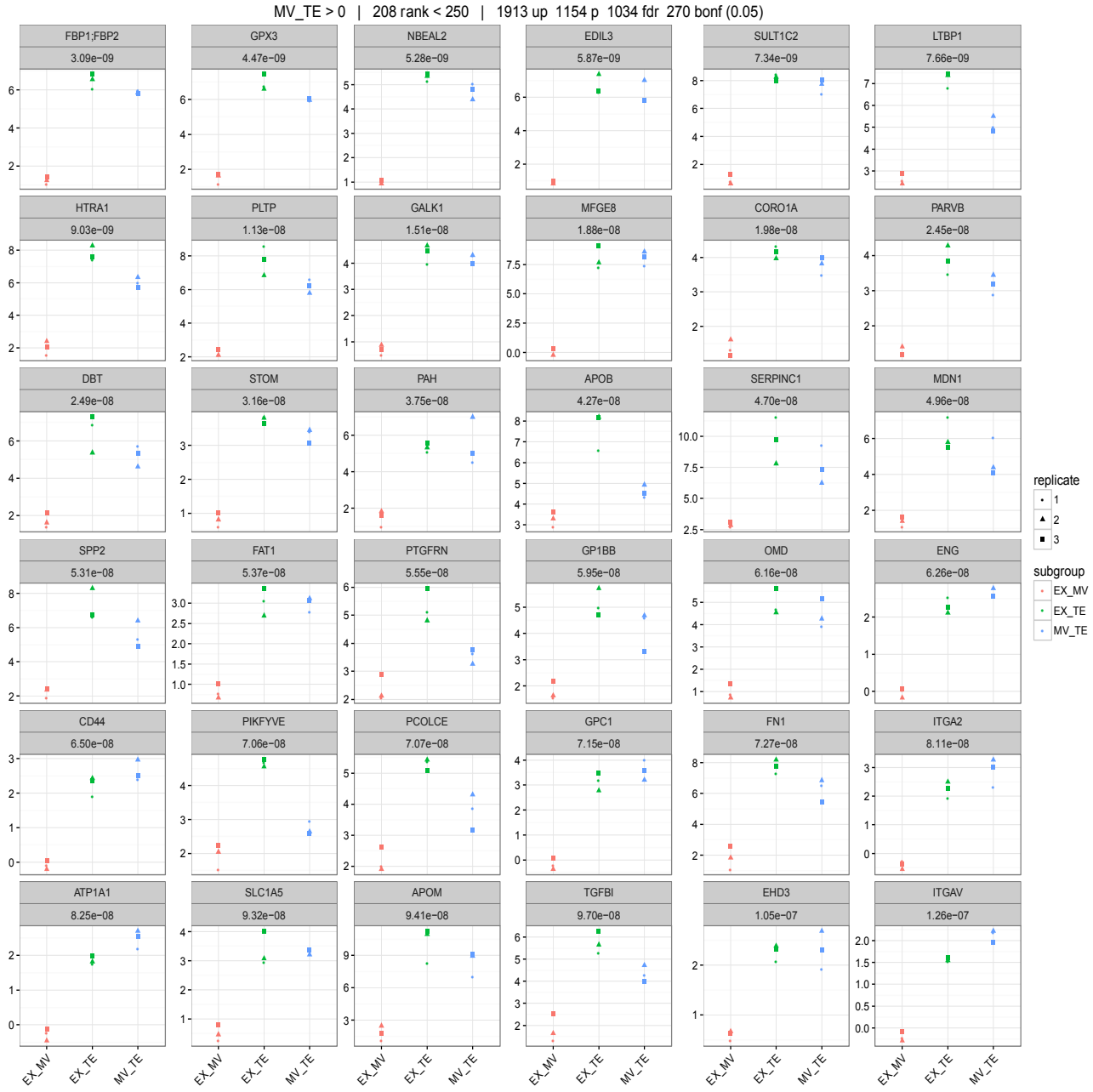

Figure.10: Distribution of the most 36 significantly up-regulated proteins in MVs compared to MSCs (MV_MSC, Blue), represented in log2 fold changes (in triplicate).

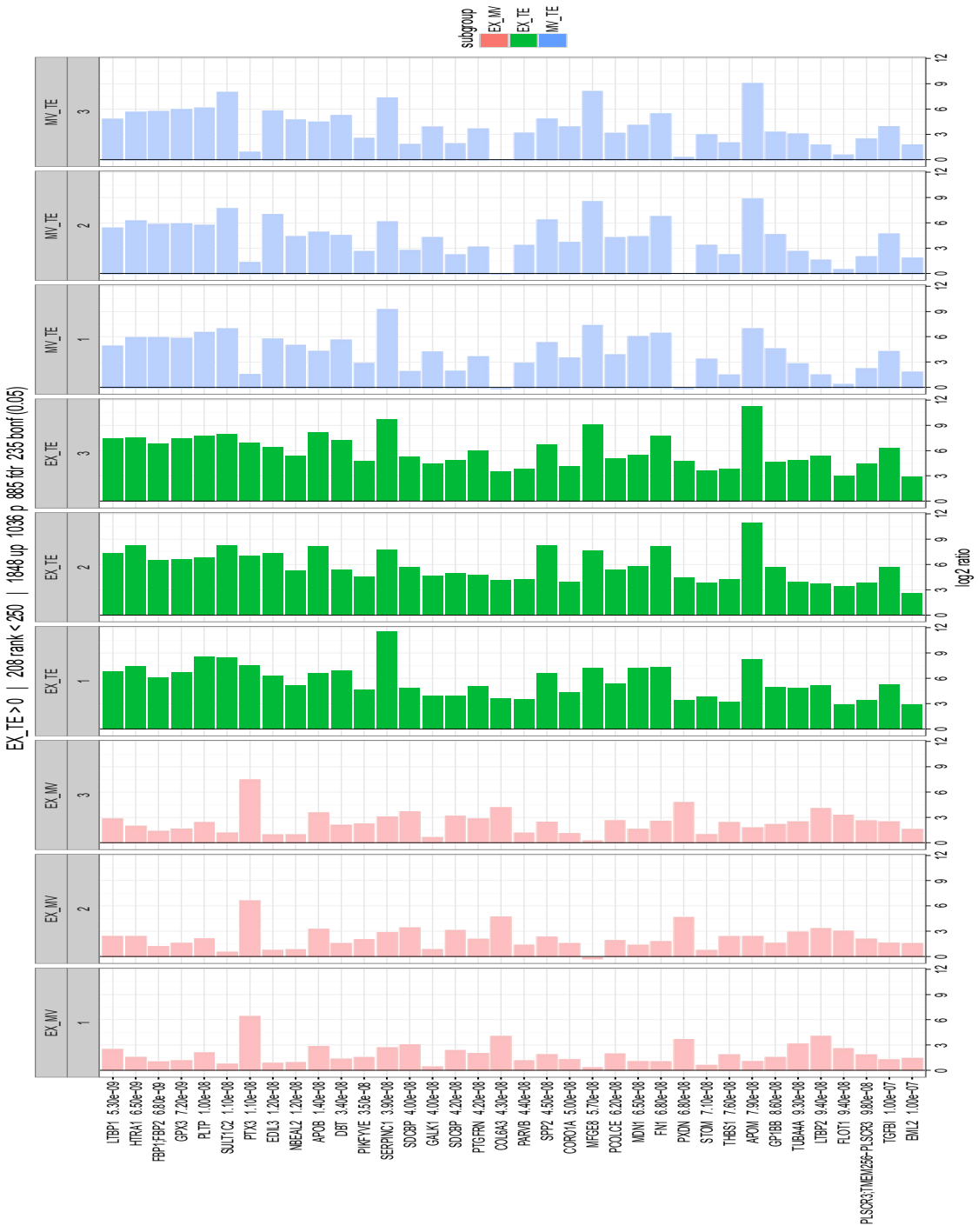

Figure.11: Bar plot of the most 36 significantly up-regulated proteins in exosomes compared to MSCs (EXO_MSC, green), represented in log2 fold changes (in triplicate).

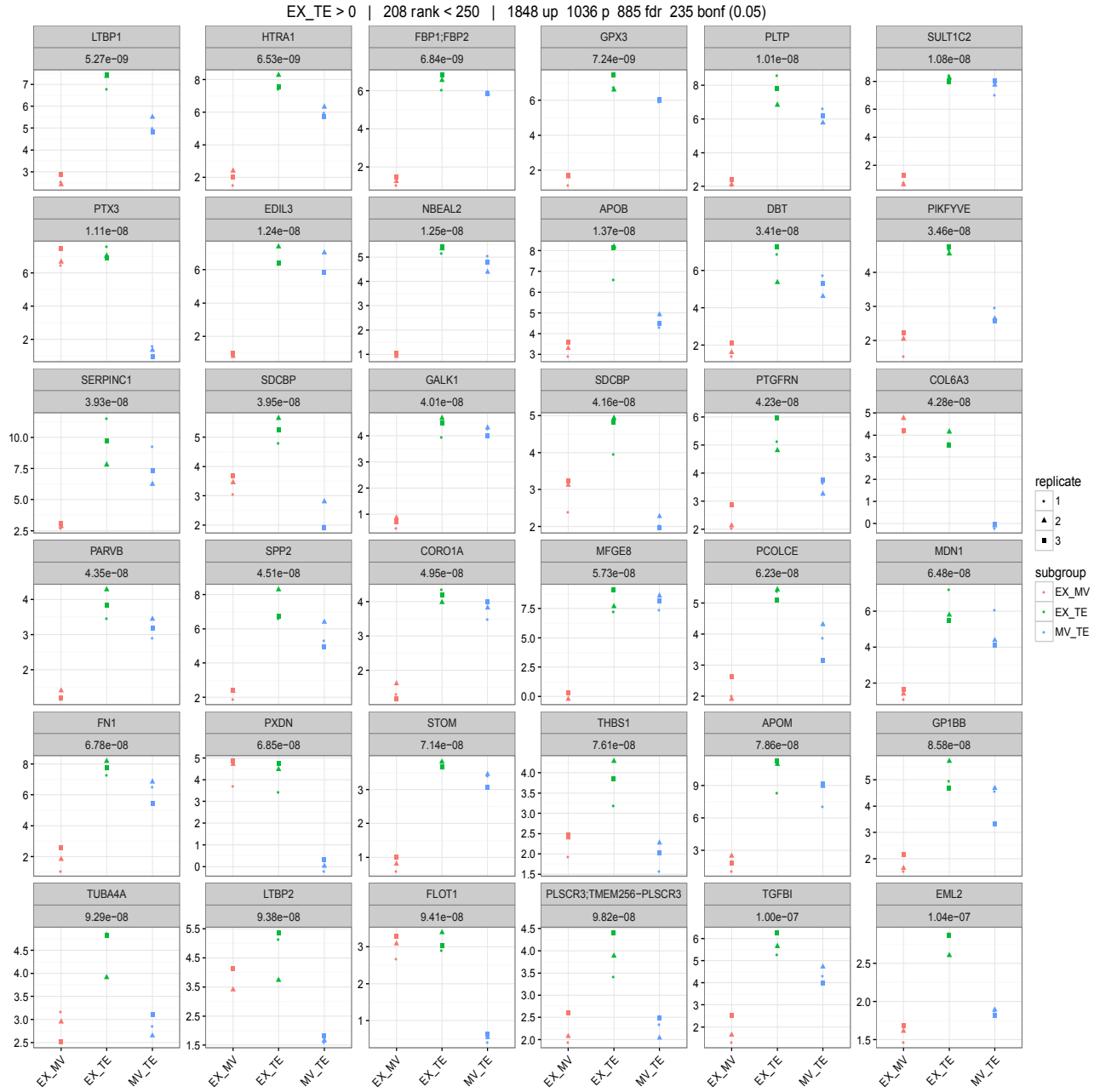

Figure.12: distribution of the most 36 significantly up-regulated proteins in exosomes compared to MSCs (EXO_MSC, green), represented in log2 fold changes (in triplicate).

Table.2: list of selected proteins that are significantly enriched in exosomes

	Exosomes	MVs	Function
PXDN	P = 6.85 –E8 Fold change = 19	P = 0.00012275 Fold change = 1.039	• Extracellular matrix formation (Péterfi et al., 2009)
COL6A3	P = 4.28 –E8 Fold changes = 14	P = 0.00015434 Fold change = 0.9	• Extracellular matrix organization • Cell adhesion
PTX3	P = 1.11-E8 Fold change = 144	P = 2.45 –E6 Fold change = 2.5	• Inflammatory response, • Innate resistance
LTBP2	P = 9.38 –E8 Fold change = 27	P = 1.38 –E6 Fold change = 3	• Extracellular organization
APOB	P = 1.37 –E8 Fold change = 201	P = 4.27 –E8 Fold change = 24	• Cholesterol and Lipid metabolism

FBP1,2 protein was the most significant protein in microvesicles (p= 3.09 E-9), followed by GPX3 (p= 4.47 E-9), and NBEAL2 (p= 5.2 E-9). The top 20 most significant MVs and exosome proteins were sorted by the mean of – log p values and plotted as fold changes in **figures.13 and 14**. Some proteins appear as strong candidates, as they significantly detected and highly expressed in vesicles. To mention some, MFGE8 (264 fold in MVs), and SERPINC1 (196 fold in MVs, 830 fold in exosomes) are significant proteins detected in vesicles, which are also significantly up regulated compared to donor cells.

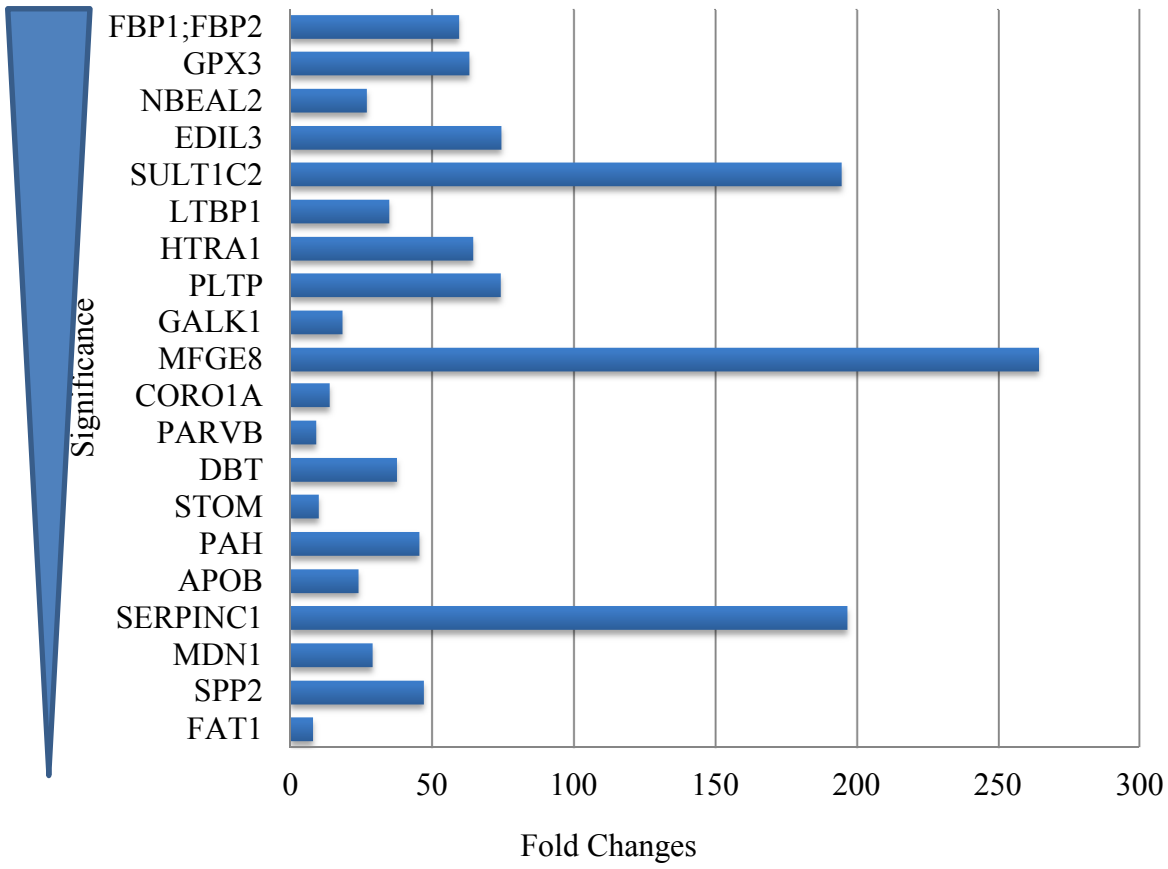

Figure.13: Bar plot of the top 20 significant MV proteins sorted by the mean of –log p values and represented by fold changes.

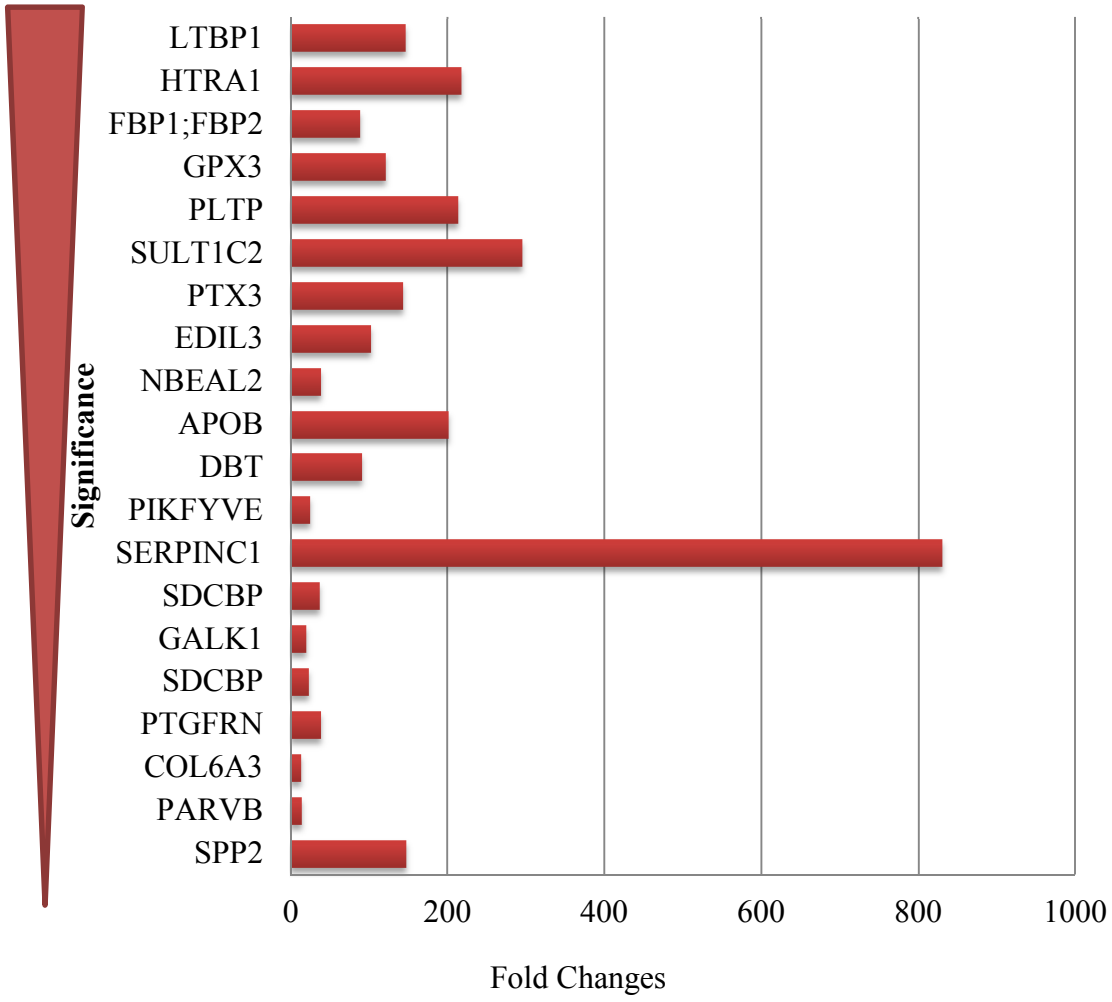

Figure.14: Bar plot of the top 20 significant exosome proteins sorted by the mean of – log p values and represented by fold changes.

As mentioned above, some proteins were differentially detected at significant expression levels in MVs and exosomes compared to MSCs. Fold changes of the top 20 most differentially up-regulated proteins are graphically plotted in **figure.15** (for microvesicles) and in **figure.16** (for exosomes).

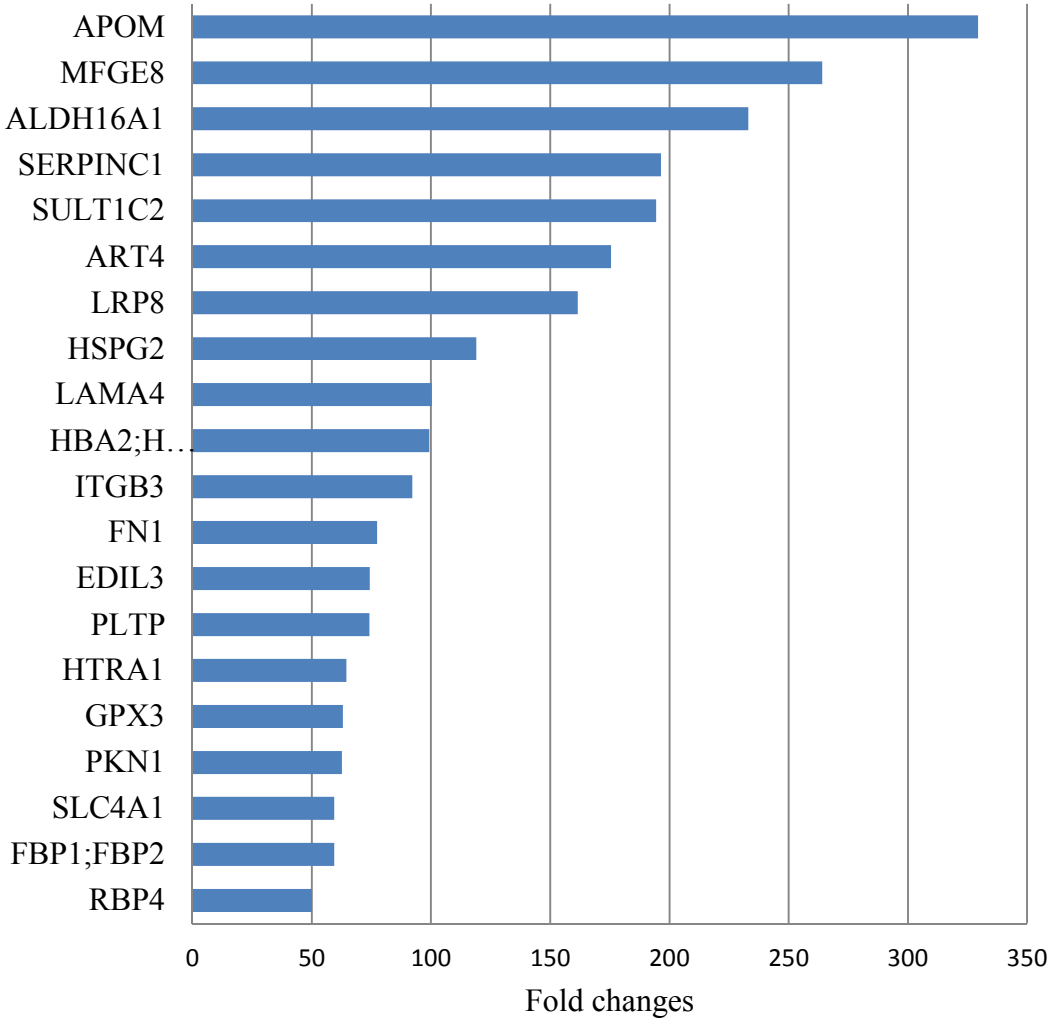

Figure.15: Bar plot of the top 20 proteins of MVs sorted based on fold changes.

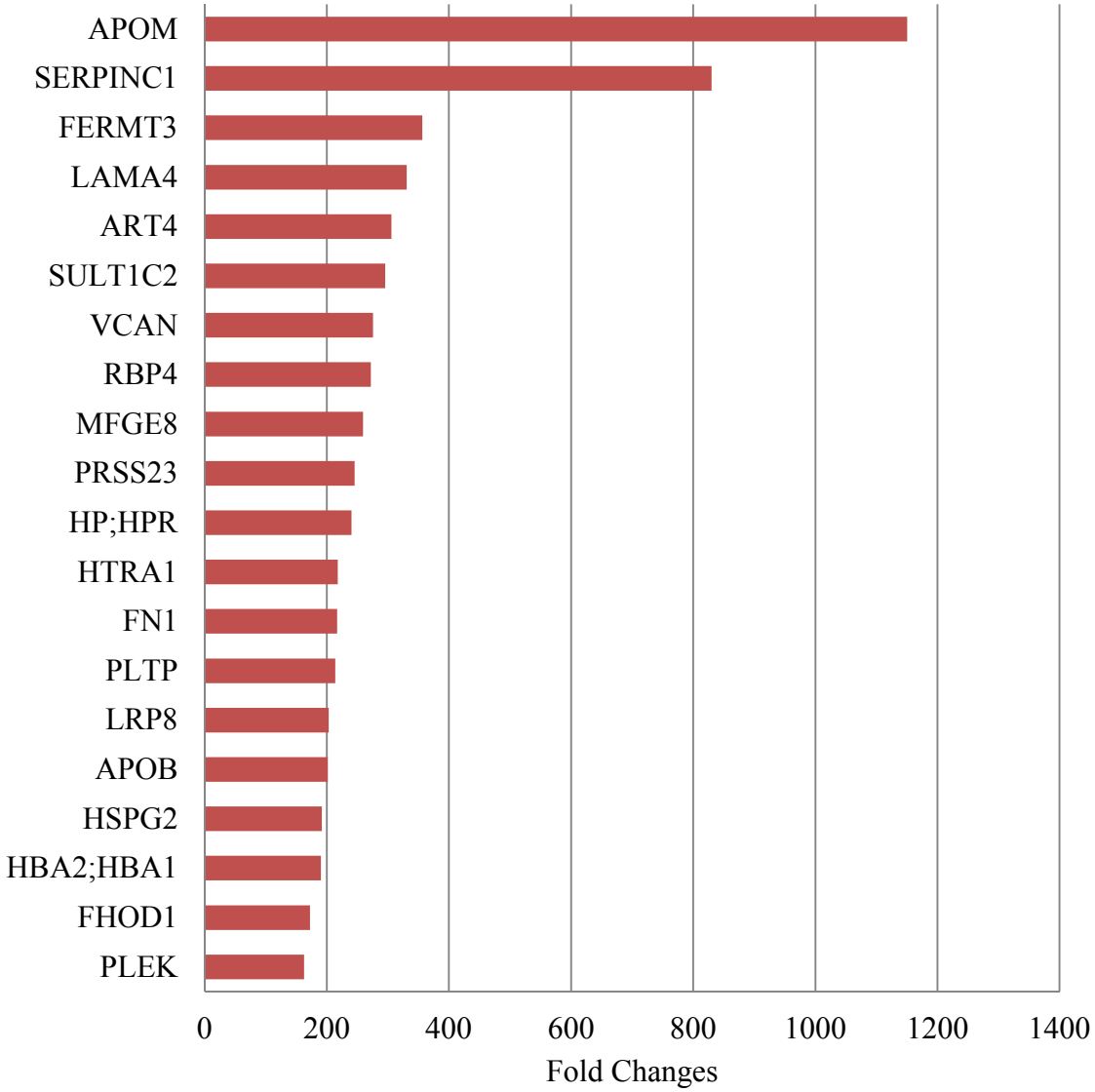

Figure.16: Bar plot of the top 20 proteins of exosomes sorted based on fold changes.

5.6. Gene Ontology Analysis

Gene ontology (GO) enrichment analyses (including biological processes (GOBP), cellular compartments (GOCC), and KEGG pathway) were performed on up-regulated proteins of MVs and exosomes. The top 20 GO enrichment terms are shown in **figures.17- 21**. Top GOBP terms enriched in MSCs-derived vesicles (microvesicles and exosomes) include extracellular organization, cells motion and adhesion, wound healing, and others (**Figure.17**). Common cellular compartment enriched in up-regulated proteins of MSCs-derived vesicles were extracellular region, plasma membrane, cell periphery, exosomes and vesicles (**Figure.20**). The top KEGG signaling pathways that are enriched in up-regulated proteins in MSCs-derived vesicles includes ECM-receptor interaction, cardiomyopathy, focal adhesion, Pl3K-Akt signaling pathway, and hematopoietic cell lineage (**Figure.21**).

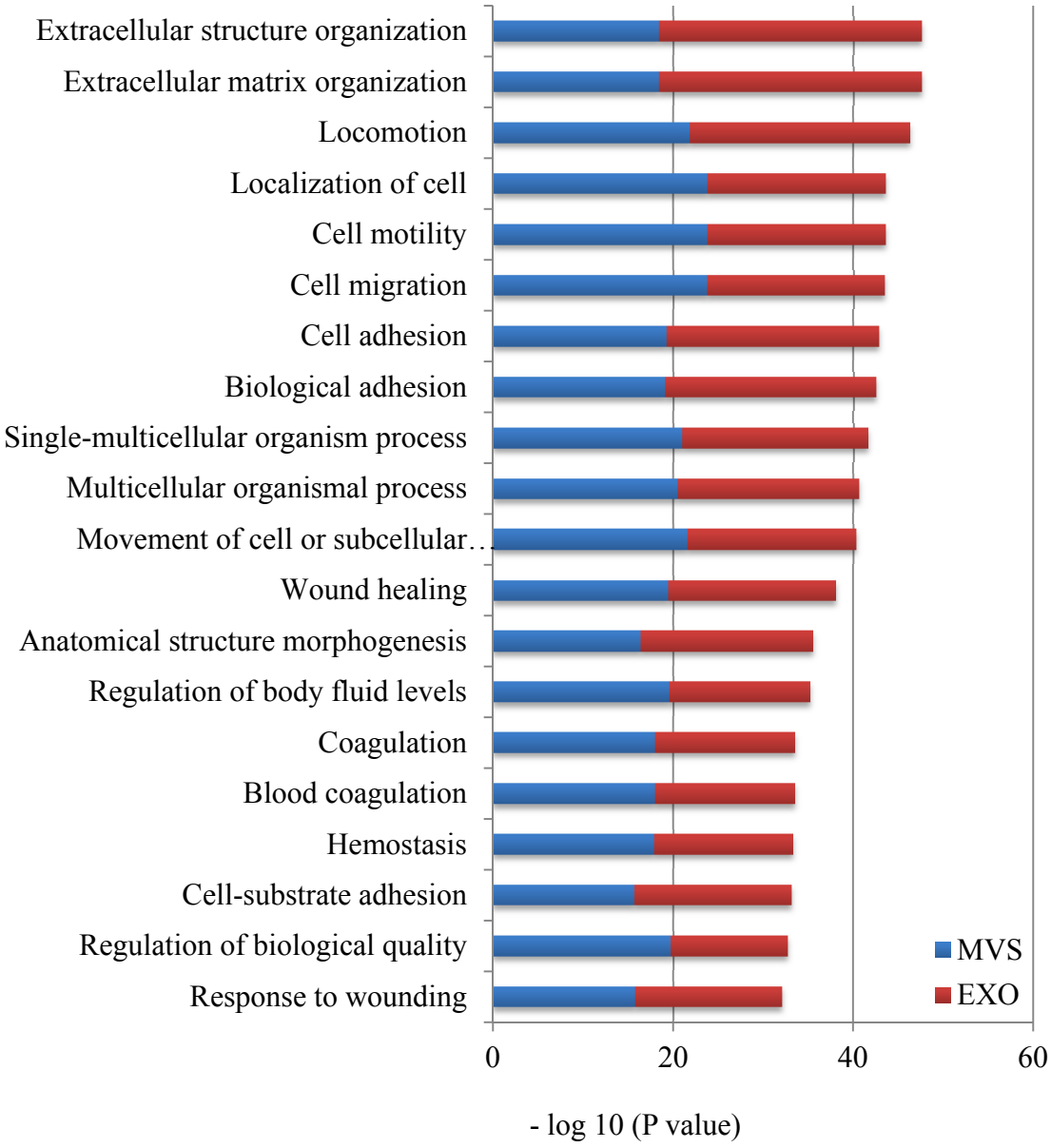

Figure.17: Bar plot of the top 20 significant enrichment analysis based on GO biological process (GOBP) for up-regulated proteins in MVs (blue) and in exosomes (red).

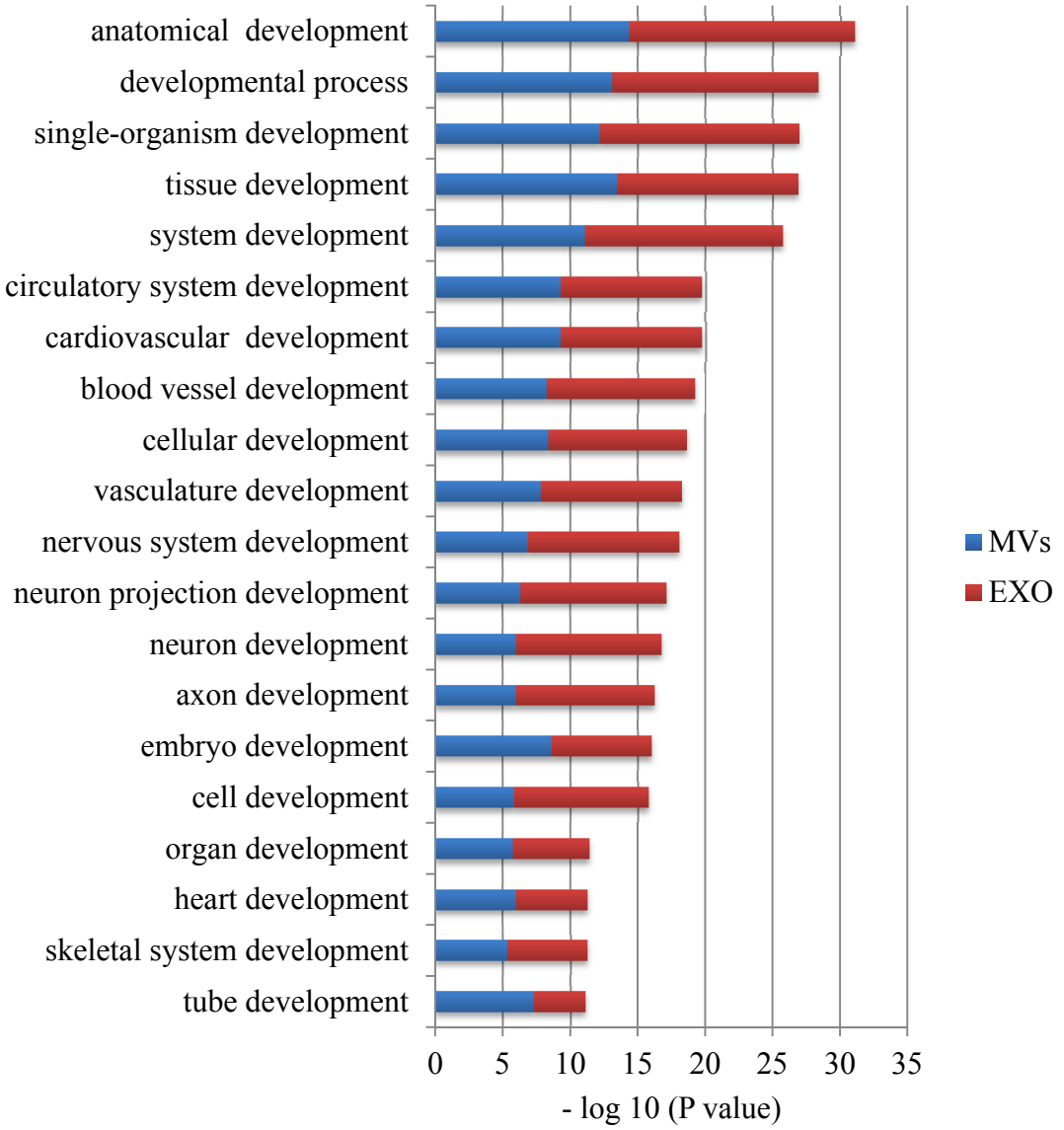

Figure.18: Bar plot of the top 20 significant enrichment developmental terms based on GOBP for up-regulated proteins in MVs (blue) and in exosomes (red).

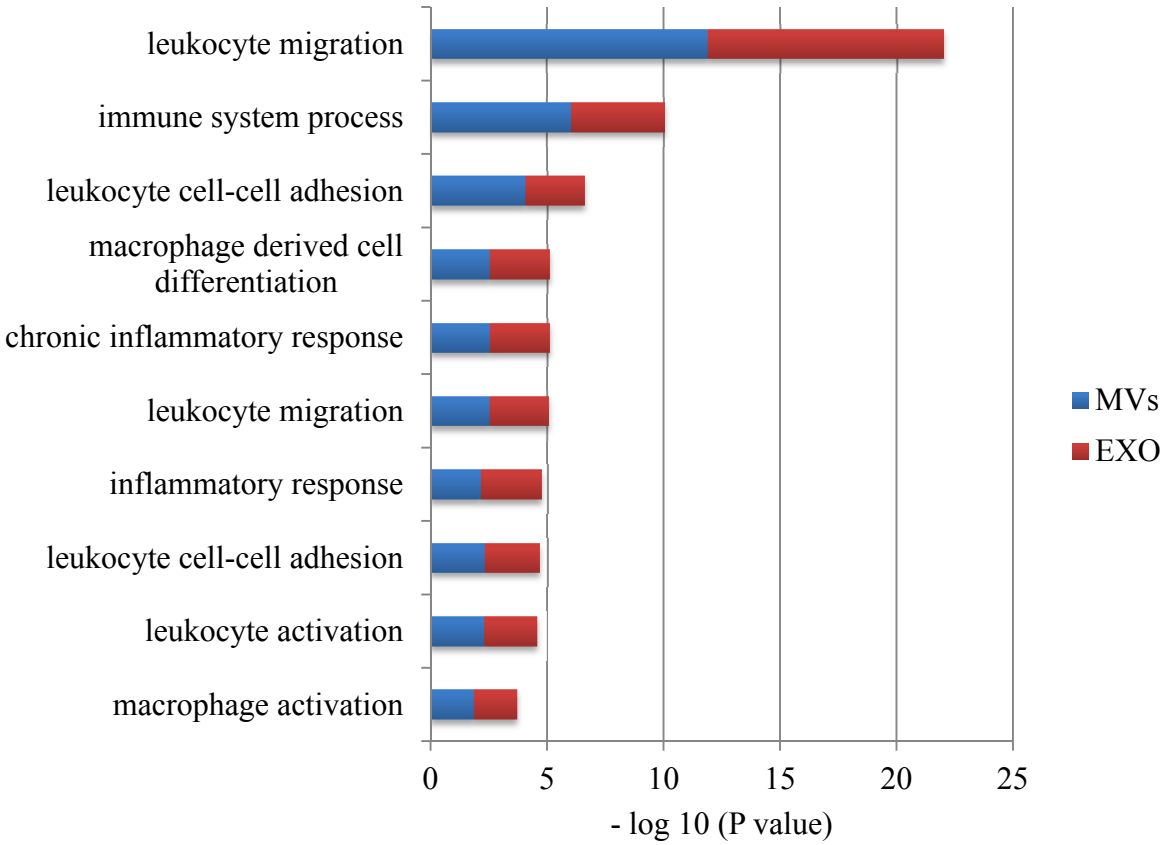

Figure.19: Bar plot of the top 10 significant enrichment immunity related terms based on GOBP for up-regulated proteins in MVs (blue) and in exosomes (red).

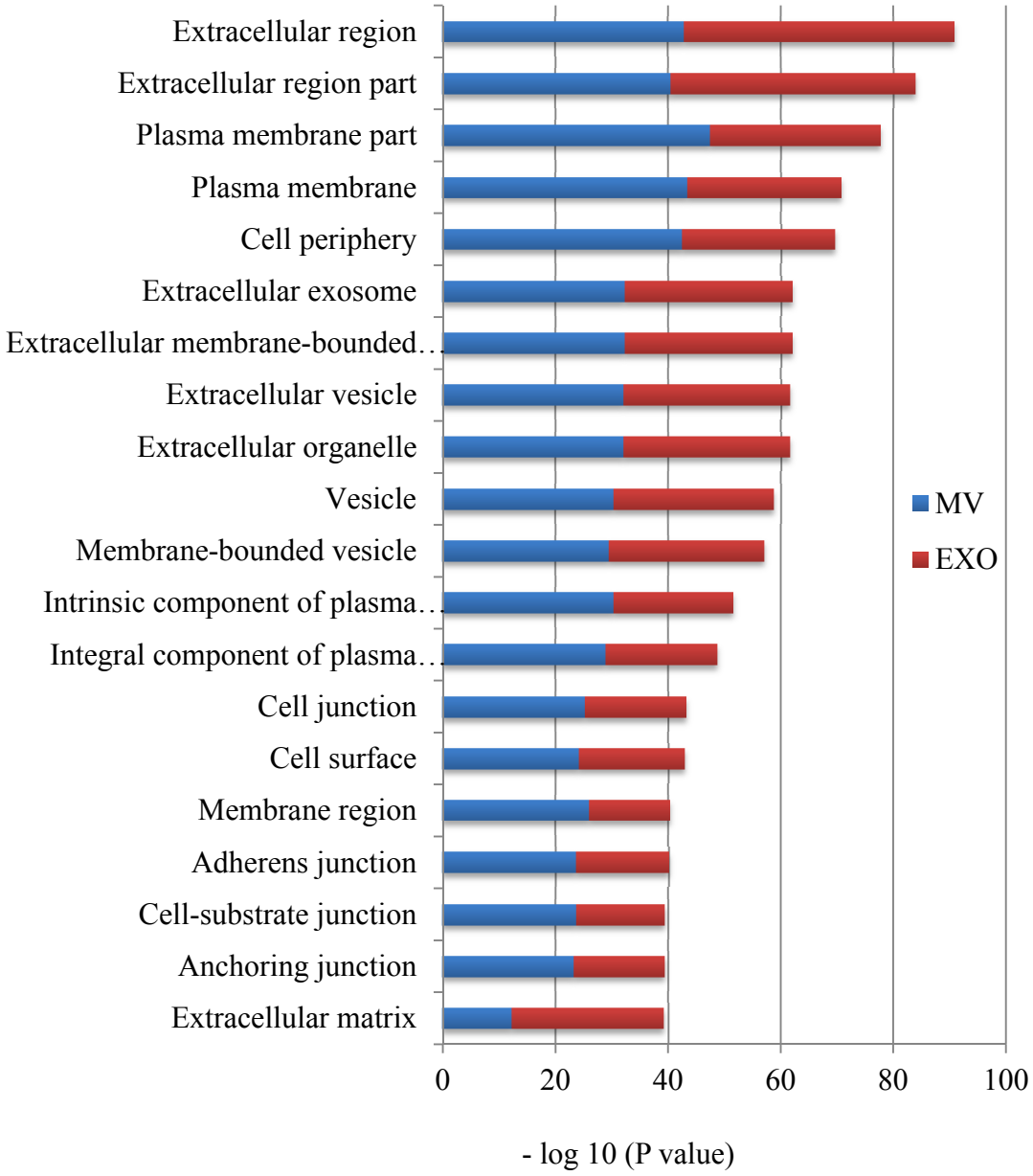

Figure.20: Bar plot of the top 20 significant enrichment analysis based on GO cellular compartment (GOCC) for up-regulated proteins in MVs (blue) and in exosomes (red).

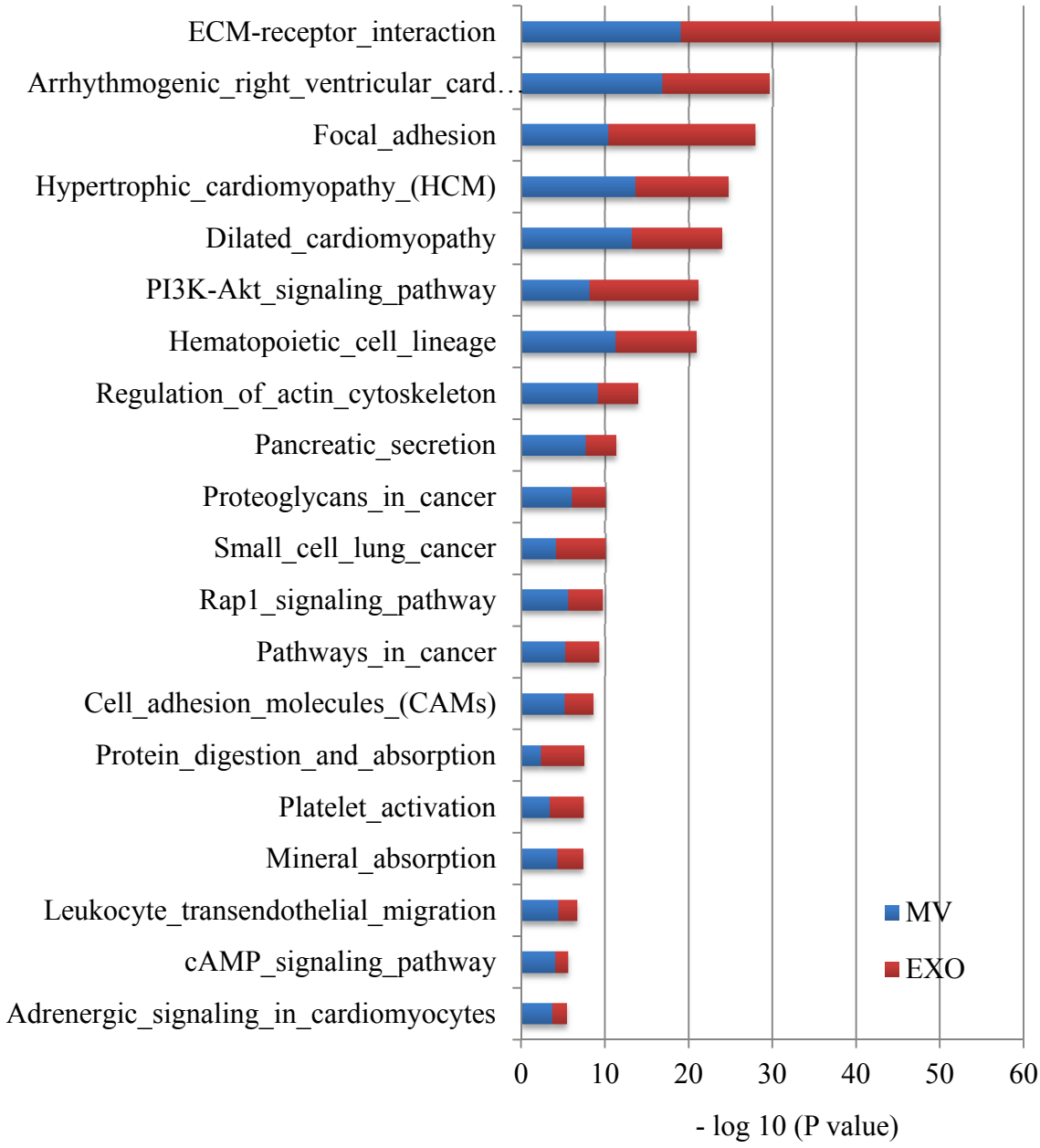

Figure.21: Bar plot of the top 20 significant enrichment analysis based on KEGG pathways for up-regulated proteins in MVs (blue) and in exosomes (red).

6. DISCUSSION

Mesenchymal stem cells (MSCs) have recently been clinically investigated as agents for regenerative and immunomodulation therapy in the treatment of a variety of related diseases. A number of studies that investigated the underlying mechanisms suggested that therapeutic effects are mediated by the paracrine communication between MSCs and their target cells. It is thus necessary to deeply study these paracrine factors including extracellular vesicles (EVs) to elucidate their involvement in the therapeutic activity.

Studying the proteome of MSCs-derived EVs will help understanding their roles and contributions to the therapeutic effects to treat diseases. Exploring the expression profiles of vesicles, and compare them to their donor cells (MSCs) should suggest novel protein candidates that could contribute to improve or establish new models of free cell based therapy. Such proteinaceous factors may, for example, play an important role in damaged tissue recovery. In our current quantitative proteomic analysis, we carried out triple dimethyl labeling LC-MS/MS based analysis, which facilitated detecting differentially expressed proteins between 3 labeled experimental groups: MSCs, microvesicles (MVs), and exosomes.

Recently, Eirin and his colleagues analyzed the proteome of adipose MSCs-derived EVs in general without separation (Eirin et al., 2016). They fractionated and digested MSCs and EVs protein in 6 fractions by in gel digest. Differential protein

expression was determined using a label free approach. In our study, we go beyond that work by separating two MSCs-derived vesicle types: MVs isolated by 20,000 x g centrifugation and exosomes isolated by 100,000 x g centrifugation. Examining each type of vesicles separately allows for deeper proteomic analysis and offers the opportunity to start evaluating functional differences between the studied populations.

The majority of proteins identified in our study (4986 out of total 5207 proteins) were commonly detected in all the experimental groups: MSCs, MVs, and exosomes. Very few proteins were detected exclusively in one group or in two groups only. Regardless of the intensity or differentially expression of these common proteins in each group, this supports the close proteome relationship in the three experimental samples and is explained by the process of vesicle biogenesis from the original cell, resulting in new membranous vesicles carrying components of the parental cell and parts of its plasma membrane.

6.1. Correlation Between MSCs-derived EVs and Their Donor Cell

As previously reported, the secreted vesicles carry most of the donor cell characteristics and components including the protein signatures. MSCs expressed on their surface CD73, CD90, and CD105. As expected, we also identified these MSC protein markers in our MSCs-derived MVs and exosomes. We found significantly enriched in vesicles the most common MSC classical surface markers such as NT5E (CD73: 7 fold in MVs and 6.5 fold in EXO), THY1 (CD90: 4.5 fold in MVs and 6 fold in EXO), and ENG/ endoglin (CD105: 6 fold in MVs and 5 fold in EXO). Other MSC associated

proteins have been quantified as well, such as CD44 (6 fold in MVs and 5 fold in EXO), ALCAM (CD166: 3.5 fold in MVs and 1.5 fold in EXO), Integrin beta-1 (CD29: 4 fold in MVs and in EXO), endosialin (CD248: 10.5 fold in MVs and 5 fold in EXO), and CD276 (4.5 fold in MVs and 4 fold in EXO). Theses findings demonstrate that MSCs-derived EVs do not only share the original cell proteome contents, but also are enriched for a subset of markers considered MSC-specific. This fact may lend credibility to the hypothesis that MSC vesicular secretions may indeed be a valuable replacement for the cells itself in cell-free applications.

6.2. Involvement of MSCs-derived EVs in Therapeutic Applications

A recent study found that MSCs could promote pro-coagulation process which decrease the myocardial infraction size (Gleeson et al., 2015). Consistent with this finding, we observed remarkable expression of proteins in both MVs and exosomes that are involved in blood coagulation and hemostasis including antithrombin-III (SERPINC1), fibronectin (FN1), integrin beta-3 (ITGB3), low-density lipoprotein receptor-related protein 8 (LRP8), neurobeachin-like protein 2 (NBEAL2).

Lactadherin (MFGE8), was previously known to be involved in the phagocytic removal of apoptotic cells and improve mucosal healing in many cells (Raymond et al., 2009) . In our study, we detected MFGE8 significantly with high fold increase in MVs. As a recent clinical trial (trial registration no. NCT01221428) concluded that MSCs infusion in a damaged colonic mucosa might be useful and repair ulcerative colitis (Hu et al., 2016)., MSC-derived EVs may thus.

MSCs directly or via vesicles recruit needed cells in common tissue homeostasis as well as toward sites of injury. They stimulate migration of endothelial or vascular smooth muscle cells, and reduce tissue damage (Liang et al., 2014; Li et al., 2016). In accordance with that work, we observed a significant enrichment of proteins that are associated with motility, migration and adhesion terms. Proteins such as coronin (CORO1A), protocadherin Fat-1 (FAT1), endoglin (ENG), CD44, and integrin alpha group (ITGA) etc. were significantly up-regulated in MVs and exosomes. Moreover, some of these proteins such as fibronectin (FN1), integrin beta-3 (ITGB3), laminin subunit alpha-4 (LAMA4), low-density lipoprotein receptor-related protein 8 (LRP8) etc. were highly enriched in MVs and exosomes when compared to MSCs. Enrichment of these proteins indicates that EVs induce cell migration to the sites of injury, attachment to target cells, and subsequently initiating the process of tissue repairing.

Up-regulation of proteins that enhance tissue development processes were observed in the enrichment analysis of vesicles compared to their donor cell (MSCs). As expected, GOBP enrichment analysis confirms the high expression of MV and exosome proteins that are involved in therapeutic effects of MSCs including tissue regeneration and immune-modulation. More specifically, tissue specific proteins that are involved in angiogenesis, nervous system, heart, renal, retina, cartilage, muscles, and many other tissues were found enriched in EVs. These data may correlate with the large field of therapeutic applications of MSCs, as they are participating in tissue repair by modulating different cellular cascades in the recipient cells.

6.3. Differences Between Microvesicles and Exosomes

Similar GOBP, GOCC, and KEGG terms were found enriched in both MVs and exosomes with minor differences in significance values and hint at close potential functional concordance despite the size difference.

A small number of proteins show different significance and enrichment between MVs and exosomes when compared to MSCs. Peroxidasin homolog (PXDN), Collagen alpha-3 (VI) chain (COL6A3), Latent transforming growth factor beta-binding protein-2 (LTBP2), apolipoprotein B-100 (APOB), and pentraxin-related protein (PTX3) are some selected proteins from the top 36 significantly up-regulated exosome proteins that showed more up-regulation in exosomes compared to MVs. Interestingly, PXDN, COL6A3, and LTBP2 were found to be involved in extracellular matrix generation process, a preliminary sign for the subsequent stages in tissue repairing.

Despite the overall similarity, these findings may reflect minor specialization and complementarity of MVs and exosomes to each other. Those slight differences between the proteomes of each vesicles type should be considered when choosing harvesting methods for potential clinical applications to potentially include all vesicle types and preserve the largest effect potential possible.

7. CONCLUSION

In conclusion, this quantitative proteomic study provides a reference set of MSCs-derived EV proteins and their enrichment as compared to the parental cell. It contributes to our understanding of the potential role of EVs in free cell therapy. Therefore, this study may provide a guide for future studies aiming at replacing MSC in clinical applications. Further work on proteins excluded from secreted vesicles will deepen that understanding and provide more ground for optimization. Ultimately, *in vivo* studies with MSCs-derived EVs, their subpopulation and finally artificial vesicles reconstituted with candidate effector proteins are needed to confirm these data and elucidate how close our *in vitro* findings match the clinical situation and can contribute to cell-free therapies.

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APPENDIX.1: IBC Safety Approval (WCM-Q)

Weill Cornell Medicine-Qatar

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Date: December 8, 2016

Re: Shaymaa Dib
Research Specialist IV
Graumann Lab
Weill Cornell Medicine – Qatar (WCM-Q)

To Whom It May Concern:

This letter is to certify that the laboratory at Weill Cornell Medicine – Qatar where Ms. Shaymaa Dib works is safe to operate and research team members have received the required training to conduct research safely and in accordance with applicable institutional laws, regulations, and best practices.

Please let us know if you have questions or need additional information.

Sincerely,

Thomas L. Doyle

Thomas L. Doyle

Johannes Graumann, Ph.D.