

**Extracellular Vesicle Preparations from Mesenchymal Stem Cell-Derived
Pre-Adipocytes
Enhance Endothelial Cell Migration and Angiogenesis**

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Hiermit erkläre ich, dass ich diese Arbeit zuvor weder bei der Heinrich-Heine-Universität Düsseldorf noch bei einer anderen wissenschaftlichen Einrichtung eingereicht habe. Weiterhin erkläre ich, dass ich die vorliegende Arbeit selbstständig und ohne andere Hilfsmittel und Quellen als die hier angegebenen verfasst habe.

Patrick-Sven Babczyk

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Abstract

Cardiovascular diseases (CVDs) account for the majority of deaths in industrialized countries. The underlying cause of CVDs is atherosclerosis. Also, it is linked to the metabolic syndrome. The metabolic syndrome refers to the co-occurrence of several known cardiovascular risk factors, such as insulin resistance, obesity, atherogenic dyslipidemia, and hypertension. Obesity is one major cause for the development of atherosclerosis. During the early stage the medium-sized and large blood vessels (arteries) are blocked by the development of plaques leading to severe life-threatening consequences like heart attack or stroke. The process of gaining weight is crucial for the development of atherosclerosis due to soluble factors secreted from mesenchymal stem cells (MSCs) differentiating towards adipocytes. These so-called adipokines have an impact on endothelial cells (EC) which are forming the inner layer of blood vessels. It is known that during early atherosclerosis inflammatory processes play a crucial role for the plaque formation, however activation of endothelial cells is not clearly understood. This work focuses on the secretome of MSCs with major focus on extracellular vesicles (EVs). EVs can carry a variety of bioactive components like cytokines, cytosolic fluids, proteins, DNA, RNA, and others. In this study MSCs differentiating towards adipocytes and extracellular vesicle preparations (EVPs) obtained during the differentiation process are utilized. The EVPs were added to ECs in potency assays to investigate their ability to activate ECs, to reveal processes in early atherosclerosis due to the development of obesity. The results showed that extracellular vesicles derived from adipogenic differentiating MSCs indeed activated ECs as demonstrated by increased migration and angiogenesis.

Zusammenfassung

Kardiovaskuläre Erkrankungen sind die Haupttodesursache in den Industrienationen. Zugrunde liegende Ursachen für diese kardiovaskulären Erkrankungen sind die Atherosklerose und das damit verbundene metabolische Syndrom. Das metabolische Syndrom ist das gleichzeitige Auftreten verschiedener Risikofaktoren, wie Insulinresistenz, Übergewicht, atherogene Fettstoffwechselstörungen und Bluthochdruck. Übergewicht ist ein Hauptrisikofaktor für die Entstehung der Atherosklerose. Während der frühen Phase werden mittelgroße und große Blutgefäße (Arterien) durch sogenannte Plaques blockiert, welche zu lebensbedrohlichen Konsequenzen wie Herzinfarkten und Schlaganfällen führen. Der Prozess der Gewichtszunahme ist dabei sehr kritisch für die Entstehung der Atherosklerose, da mesenchymale Stammzellen (MSC) sich zu Fettzellen entwickeln und dabei Faktoren sekretieren. Diese Faktoren, Adipokine genannt, haben einen Einfluss auf die Endothelzellen, welche die innere Schicht der Blutgefäße bilden. Es ist bekannt, dass während der frühen Atherosklerose entzündliche Prozesse eine entscheidende Rolle bei der Entwicklung der Plaques spielen, aber die vorausgehende Aktivierung der Endothelzellen ist noch nicht ganz verstanden. Die vorliegende Arbeit konzentriert sich auf einen weiteren sezernierten Faktor von mesenchymalen Stammzellen, den extrazellulären Vesikeln (EVs). Diese EVs können eine Vielzahl von biologisch aktiven Komponenten beinhalten, wie zytosolische Flüssigkeiten, Proteine, DNA, RNA, Zytokine, usw. In der Studie wurden Präparationen von extrazellulären Vesikeln (EVPs) aus sich in Richtung Fettzellen differenzierenden MSCs gewonnen und in Potenz-Assays auf Endothelzellen gegeben, um ihre Fähigkeit im Hinblick auf die Aktivierung der Endothelzellen zu untersuchen. Die Ergebnisse zeigen, dass diese adipogenen EVPs aus MSCs rasch von Endothelzellen aufgenommen wurden und zu einer

erhöhten Migration und Angiogenese der Endothelzellen führten, was für ihre Aktivierung spricht.

1. INTRODUCTION

1.1 The Metabolic Syndrome

The metabolic syndrome refers to the co-occurrence of several known cardiovascular risk factors, including insulin resistance, obesity, atherogenic dyslipidemia and hypertension. These conditions are interrelated and share underlying mediators, mechanisms and pathways. There has been a recent controversy about its definition and utility. It identifies a subgroup of patients with shared pathophysiology who are at high risk of developing cardiovascular disease and type 2 diabetes. The metabolic syndrome is a common metabolic disorder that results from the increasing prevalence of obesity. The disorder is defined in various ways, but in the future a new definition will be applicable worldwide. The pathophysiology seems to be largely attributable to insulin resistance with excessive flux of fatty acids implicated. A pro-inflammatory state probably contributes to the syndrome. The increased risk for type 2 diabetes and cardiovascular disease demands therapeutic attention for those at high risk. The fundamental approach is weight reduction and increased physical activity (Huang *et al.*, 2009). The metabolic syndrome and its cluster of conditions that occur together, increase the risk of heart disease, stroke and type 2 diabetes. These conditions include increased blood pressure, high blood sugar, excess body fat around the waist, and abnormal cholesterol or triglyceride levels (Eckel *et al.*, 2005). The following factors increase the chances of having the metabolic syndrome:

- **Age:** The metabolic syndrome probably increases with age.
- **Ethnicity:** In the United States, Hispanics, especially Hispanic women, appear to be at greatest risk of developing the metabolic syndrome. The reasons for this are not entirely clear.

- **Obesity:** Overweight, especially in the visceral fat, increases the risk of developing the metabolic syndrome.
- **Diabetes:** It is more likely to develop the metabolic syndrome if diabetes occurred during pregnancy (gestational diabetes) or if there is a family history of type 2 diabetes.
- **Other diseases:** The risk of metabolic syndrome is higher with nonalcoholic fatty liver disease, polycystic ovary syndrome, or sleep apnea (Mayo Clinic, 2023).

Since the metabolic syndrome refers to the co-occurrence of several known cardiovascular risk factors, it can result in cardiovascular diseases (CVDs). A common reason for CVDs is atherosclerosis. Obesity, also a factor in the metabolic syndrome, is one major risk factor for atherosclerosis.

1.2 Cardiovascular Diseases – Atherosclerosis as Underlying Cause

Cardiovascular diseases are the leading cause of death in industrialized countries (WHO; 2020). Atherosclerosis, a chronic inflammatory disease of large and medium-sized arteries, is the underlying cause of events like stroke, heart attack and other thrombotic complications. Risk factors like smoking or obesity trigger the development. The early atherosclerosis is characterized by a progressive accumulation of lipids, necrotic cell debris, and extracellular matrix proteins in the vessel intima. Atherosclerotic plaques start at regions of local endothelial dysfunction. Deposition of cholesterol, primarily packaged in low-density lipoproteins (LDL), leads to the accumulation of monocyte-derived macrophages with the aim to clear the lipid deposits. The inflammatory response drives the formation of a microenvironment rich in oxidized LDL (oxLDL), cytokines, growth factors, and wound-associated matrix proteins like fibronectin (Kingsley *et al.*, 2002; Chahine *et al.*, 2009; Orr *et al.*, 2009; Autieri *et al.*, 2012; Finney *et al.*, 2017). Activated by those stimuli, smooth muscle cells from the media

migrate into the intima and their accumulation leads to blockage of the vessel (Stary *et al.*, 1995; Owens *et al.*, 2004; Virnabi *et al.*, 2006).

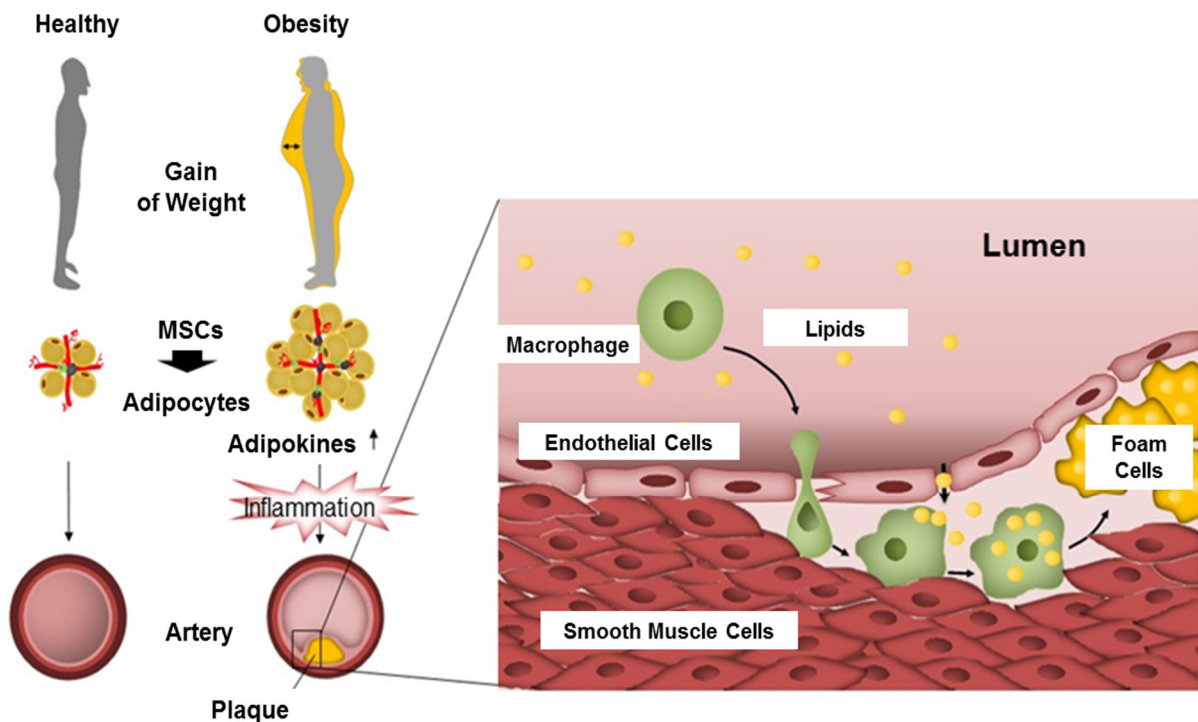


Fig. 1: Development of Early Atherosclerosis during Gain of Weight. During the process of gaining weight, MSCs differentiate towards adipocytes within the body. The developing adipocytes secrete adipokines, which can evoke inflammation leading to atherosclerosis. During the early stage of atherosclerosis, a formation of plaques leads to the blocking of blood vessels (left picture). On the cellular level, endothelial cells are activated by adipokines and recruit macrophages out of the lumen of the vessel. These macrophages migrate through the dense barrier formed by the endothelium and incorporated lipids are becoming foam cells underneath the endothelium within the smooth muscle cell tissue and they are building up plaques.

The success of current therapies such as the insertion of stents, or the treatment with statins is low since patients develop re-blocking of the vessel (restenosis) within five years in two-thirds of the cases (Libby *et al.*, 2005). However, the activation of endothelial cells (ECs) due to mediating risk factors, especially obesity, and the resulting dysfunction of the endothelium is not clearly understood yet and might lead to new approaches for drug development.

1.3 Vascular Structure

1.3.1 Blood Vessels

Blood vessels within the body are mainly classified into arteries and veins. The transportation of oxygen-rich blood from the lungs *via* the heart into the tissues and organs and therefore away from the heart is conducted *via* arteries while oxygen-depleted blood is transported back to the heart and later back to the lungs.

Both vessel types consist of three layers: *tunica intima* (inner layer), *tunica media* (middle layer) and *tunica adventitia* (outer layer). In arteries the *tunica media* is thicker than in veins due to the high amount of smooth muscle cells, which control the caliber of the vessels and contribute to the blood pressure. The *tunica adventitia* consists of connective tissue mainly containing fibroblasts and additionally serves as stem cell niche. The *tunica media* and *tunica adventitia* are separated by a thick elastic band called external elastic lamina (Cines *et al.*, 1998). The inner layer, *tunica intima*, is composed of a monolayer of endothelial cells (ECs) resting on extracellular matrix. These endothelial cells are the only cells in direct contact with the blood and function as a barrier between blood and tissue. Therefore, they play a crucial role in the regulation of blood pressure, protection against vascular injury, and maintenance of blood fluidity (Potente *et al.*, 2011).

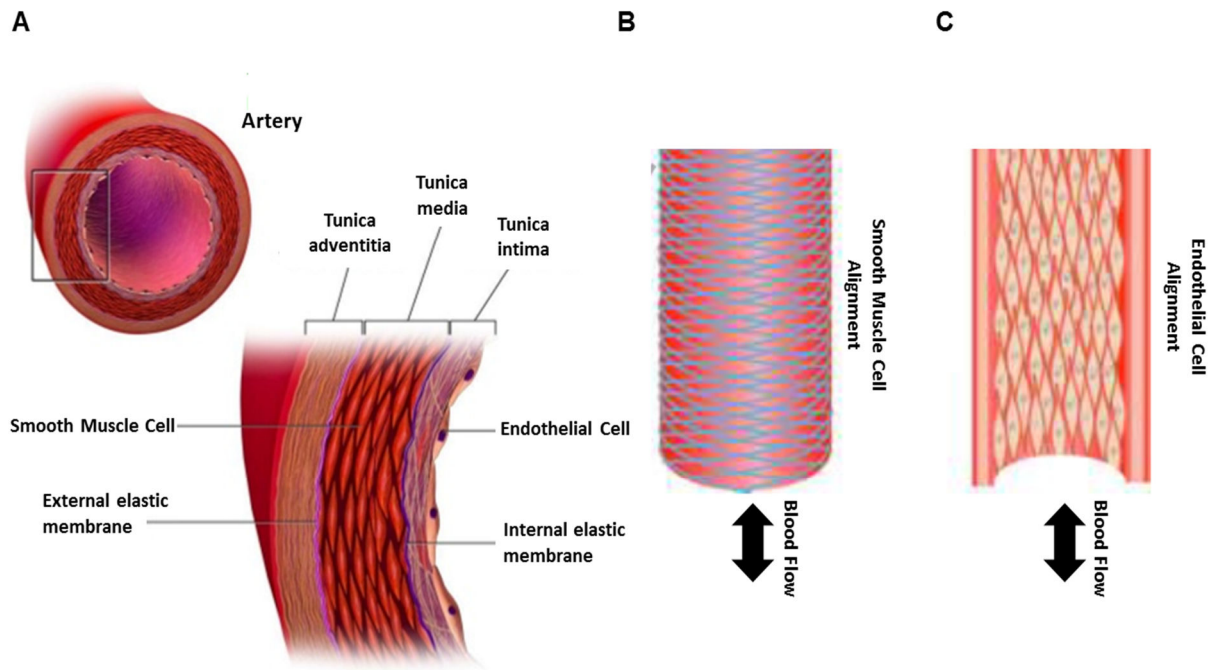


Fig. 2: Structure of Blood Vessels. (A) Blood vessels consist of three layers: the *tunica adventitia* surrounding the vessel, the *tunica media* consisting mainly of smooth muscle cells and the *tunica intima* formed by a monolayer of endothelial cells on an elastic membrane. (B) The smooth muscle cells align in a 90° order to the blood flow. (C) Due to the blood flow, the endothelial cells line the vessel and form a cobblestone pattern via alignment (adapted from Zuyong Wang *et al.*, 2019).

1.3.2 Endothelial Cells

The thin monolayer of ECs lining the blood vessel form, known as endothelium, plays a complex role in vascular biology. ECs exhibit a regular cobblestone morphology and were termed “phalanx cells” (Carmeliet *et al.*, 2009). The endothelium regulates vessel tone, hemostasis, neutrophil recruitment, hormone trafficking, and fluid filtration (Liao, 2013). It forms a semi-permeable barrier that separates the bloodstream from organs and tissues. During inflammation and infection, endothelial cells undergo remodeling processes that allow leukocytes, fluids, and small molecules to enter the surrounding tissue. Endothelial barrier function is mediated by cell–cell adhesions, including tight junctions, adherens junctions, and other adhesion molecules, particularly PECAM-1 (CD31) and

nectins, which are linked to the actin filaments of the endothelial cytoskeleton (Cerutti *et al.*, 2017).

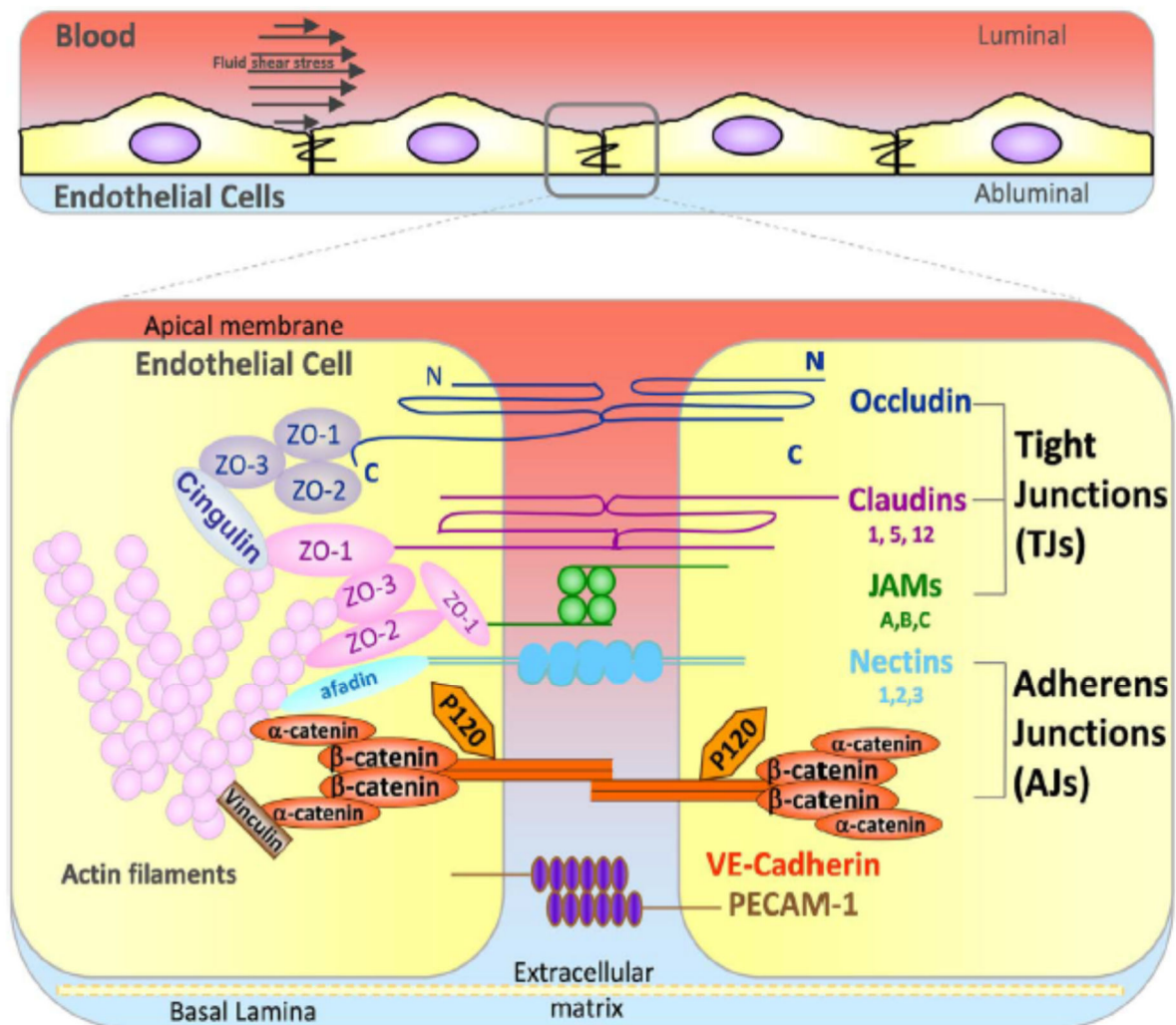


Fig. 3: The Main Transmembrane Proteins in Endothelial Cell-Cell Junctions. Endothelial cells line the blood stream and are constantly exposed to fluid shear stress (top panel). The bottom panel shows the main transmembrane proteins in endothelial cell-cell junctions (right). They are associated with tight junctions or adherens junctions as indicated, with the exception of PECAM-1, which is not associated with either type of junction. Three different claudin, JAM and nectin genes are reported to be expressed in endothelial cells (numbers/letters indicated under a protein name). Intracellular proteins link transmembrane proteins to the actin cytoskeleton. There are additional junctional molecules, such as CD99 and ESAM, which are omitted for clarity (from: Cerutti *et al.*, 2017).

Tight junctions are formed by hemophilic cell-cell adhesion molecules (occludin, claudins, and junctional adhesion molecules (JAMs)). They regulate diffusion of ions, and polar solutes while blocking large macromolecules. Adherens junctions

like VE-cadherin (unique to ECs) together with PECAM-1 hold ECs together and give mechanical support to the endothelium (Dejana *et al.*, 2009; Lertkietmongkol *et al.*, 2016). ECs also express endothelial nitric oxidase (eNOS) which is a key regulator for vascular tone and blood pressure. A lack of eNOS has been linked to a variety of disease states such as atherosclerosis, diabetes mellitus, coronary artery diseases, hypertension, and hypercholesterolemia (Liao, 2013).

1.3.3 Endothelial Cell Migration

The vast majority of ECs in blood vessels are quiescent with little migratory and proliferative activity. Nevertheless, like nearly all cell types, ECs also migrate in certain stages of development or during other events such as injuries. The mechanism of cell migration consists of four steps: induction, polarization, protrusion formation and the last step, adhesion formation and forward movement. Cells not only migrate individually but also collectively during many biological processes, moving as chains, sheets, or clusters (Michaelis, 2014). EC migration is essential for embryogenesis and angiogenesis. Additionally, EC migration also occurs in vessel repair. The primary network of blood vessels is formed by the process of vasculogenesis, the *de novo* formation of blood vessels, in which endothelial precursor cells (angioblasts) aggregate. After a primitive network has been formed, angiogenesis leads to the formation of a complex, functional vascular system (Ferguson *et al.*, 2005; Swift *et al.*, 2009). Angiogenesis is not only a process during embryogenesis but also important for the regeneration of newly formed tissue (wound healing). When nutritional and oxygen demand within a tissue exceed the supply, the tissue secretes signals to stimulate the formation of new blood vessels (Carmeliet *et al.*, 2009). The ECs change into an “activated” phenotype and start migration and proliferation, which includes sprouting, intussusceptive growth, splitting, remodeling, stabilization, and differentiation into arterioles, venules and capillaries. In a new angiogenic

sprout, endothelial cell migration is guided by so-called guidance cues provided by surrounding cells like astrocytes. The most prominent mechanism of EC migration is stimulated by VEGF. A “tip cell” leads the following ECs toward the stimulus either in sheets or in an angiogenic sprout (Carmeliet, 2003; Germain *et al.*, 2010). Endothelial cell migration during vessel repair is essential to maintain the integrity of the monolayer. In the case of damage, cells migrate into the gap to fill it. The process is promoted by growth factors like FGF (bFGF). The cells at the boundary function as leader or pioneer cells and pass the migratory signal to the follower cells (Vitorino *et al.*, 2011).

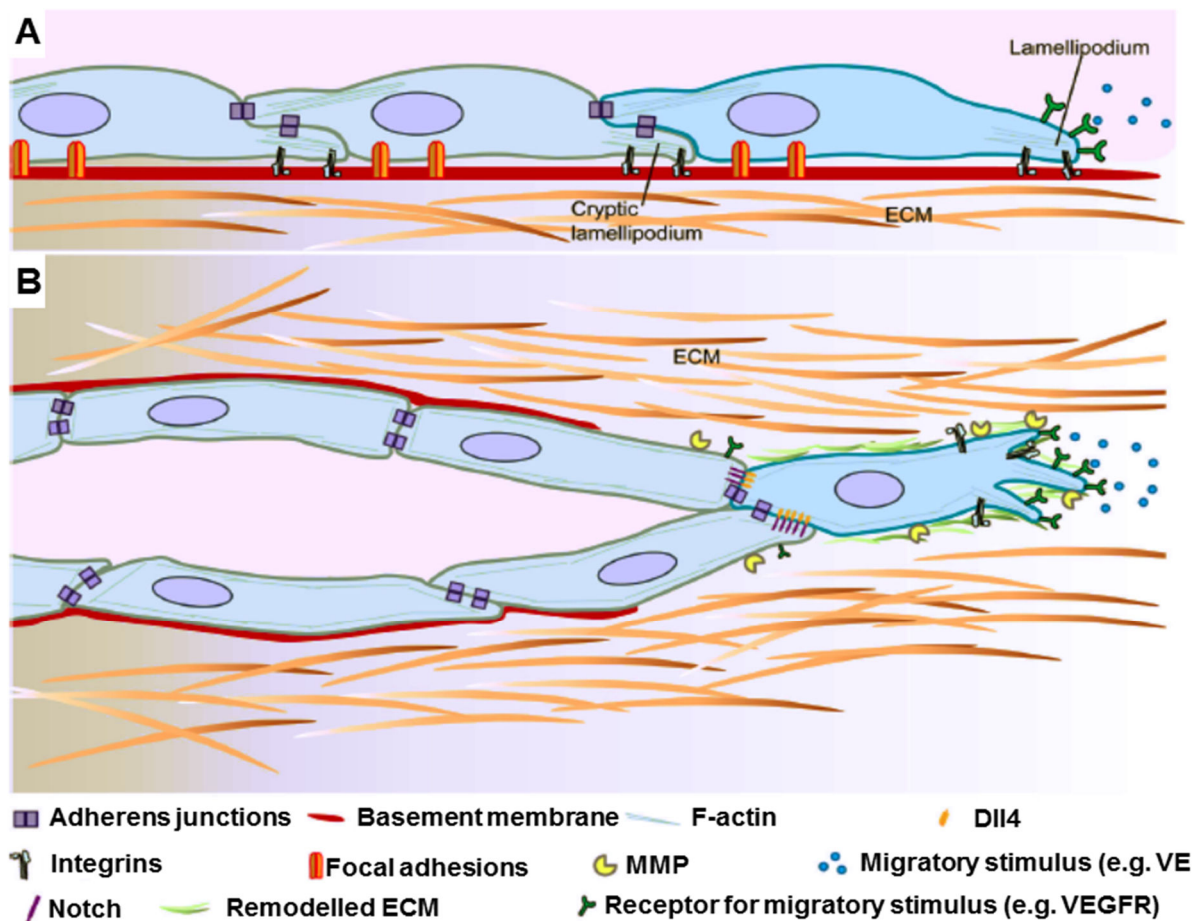


Fig. 4: Endothelial Cell Migration. Collective endothelial cell migration. (A) Endothelial sheet migration. If the endothelial monolayer is damaged, endothelial cells migrate to fill the gap. Leader cells are formed sensing the gradient of a chemoattractant and forming a lamellipodium. At the leading edge new focal contacts are formed. Stable focal adhesions in the center adhere the cell to the basement membrane. The following cells are characterized by cryptic lamellipodia. (B) Endothelial cell migration in an angiogenic sprout. In a newly forming blood vessel a tip cell is formed sensing the chemotactic gradient. It is characterized by

filopodia and high activity of metalloproteases. Induction of Notch signaling in the following stalk abolishes the formation of additional tip cells (from Michaelis, 2014).

Very important mediators of EC migration are integrins. Nine of them have been described to be expressed in ECs and are implicated in angiogenesis: $\alpha1\beta1$, $\alpha2\beta1$ (collagen receptors), $\alpha3\beta1$, $\alpha6\beta1$, $\alpha6\beta4$ (laminin receptors), $\alpha4\beta1$, $\alpha5\beta1$ (fibronectin receptors), and $\alpha v\beta3$, $\alpha v\beta5$ (αv receptors) (Hynes *et al.*, 1999; Serini *et al.*, 2006). The integrins mediate adhesion at the leading edge and thereby regulate directional movement. Most of them require VEGF or PDGF as inducing signals (Kiosses *et al.*, 2001). In general, EC migration depends on both the environment and the specific migratory context.

1.3.4 Endothelial Cell Dysfunction and Activation

Endothelial cell dysfunction was first defined as decreased synthesis, release, and activity of eNOS leading to vasoconstriction, smooth muscle cell proliferation, platelet aggregation, leukocyte adhesion, LDL oxidation and the activation of matrix metalloproteases (MMP). Endothelial cell activation in response to non-laminar flow as an early mediator of leukocyte trafficking within the vascular wall was first described in 1997 (Gimbrone *et al.*, 1997). EC activation is defined by the expression of cell-surface adhesion molecules such as VCAM-1, ICAM-1, and ELAM (E-selectin) and is typically induced by inflammatory cytokines (e.g. IL-1, TNF- α) mediating the recruitment of circulating leukocytes to the vessel wall (Libby *et al.*, 2002). It was shown that EC dysfunction and activation are linked, as the inhibition of eNOS could increase neutrophil adherence to mesenteric venules (Kubes *et al.*, 1991). In further studies, it was shown that NO donors suppressed the expression of VCAM-1, ICAM-1 and ELAM in response to pro-inflammatory cytokines by preventing endothelial cell activation through inhibition of NF- κ B (De Caterina *et al.*, 1995). Later the mechanism of EC activation prior to the recruitment of inflammatory cells was shown to be linked

to hypercholesterolemia via an atherogenic diet resulting in VCAM-1 expression and inhibition of eNOS activity (Li *et al.*, 1993; Ohara *et al.*, 1993; Liao *et al.*, 1995). All these findings provided a mechanistic link by which mediators of EC dysfunction (hypercholesterolemia, smoking, oxidative stress) could enhance EC activation. The results also suggested that areas of turbulent flow within the vessel wall with less endothelium-derived NO is more prone to EC activation and atherosclerosis (Kuhlencordt *et al.*, 2001).

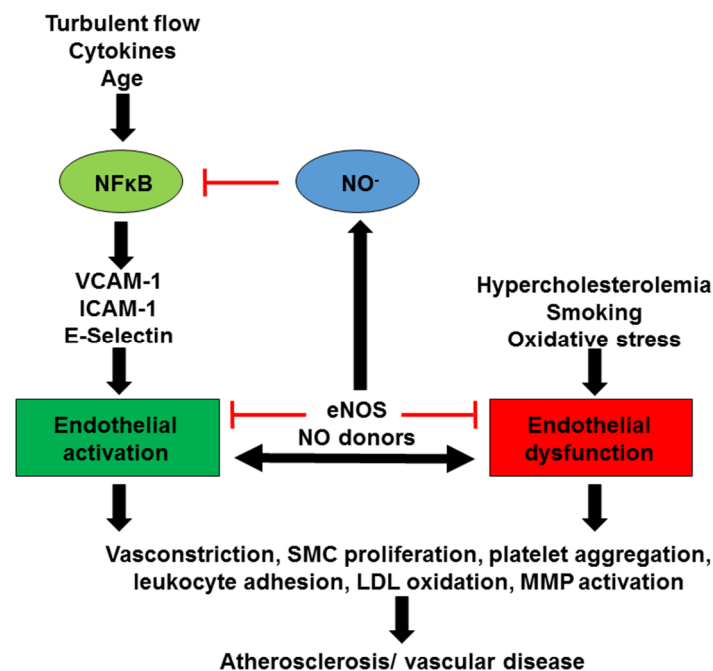


Fig. 5: Role of Endothelial Dysfunction and Endothelial Cell Activation in Vascular Diseases. Cardiovascular risk factors, such as hypercholesterolemia, smoking, and oxidative stress are important mediators of endothelial dysfunction, while pro-inflammatory cytokines, turbulent flow, and advanced glycation end products (AGEs) are important mediators of endothelial cell activation via activation of the transcription factor NF-κB. NO from eNOS or NO donors reduces endothelial cell activation through inhibition of NF-κB. Loss of NO leads to increased endothelial cell activation. Likewise, endothelial cell activation can cause endothelial dysfunction. Both, endothelial dysfunction and endothelial cell activation lead to atherosclerosis and vascular disease by increasing vasoconstriction, SMC proliferation, platelet aggregation, leukocyte adhesion, LDL oxidation, and MMP activation (adapted from Liao, 2013).

1.4 Stem Cells

Stem cells are regarded as a promising cell source for regenerative medicine and tissue engineering (Lund *et al.*, 2009). They are cells with the capability of self-renewal and a variable but specific potential to differentiate towards cell types of the human body. Stem cells can be classified into embryonic and adult stem cells, the latter comprising hematopoietic, neural crest-derived, and mesenchymal stem cells among others. Since 2006, induced pluripotent stem cells and recently cancer stem cells have expanded the spectrum of recognized stem cell types.

1.4.1 Mesenchymal Stem Cells

Mesenchymal stem cells (MSCs) are able to repair tissue and maintain the normal cell turnover of regenerative organs and tissues and are multipotent. They can be obtained from several tissues including bone marrow, peripheral blood, umbilical cord, and umbilical cord blood, adipose tissue, skeleton muscle, wisdom teeth, and placenta (Zuk *et al.*, 2002; Kern *et al.*, 2006; Pansky *et al.*, 2007; Tobiasch, 2010; Hass *et al.*, 2011; Khan *et al.*, 2012). Since numerous markers for the characterization of MSCs have been suggested, most researchers agree and rely on the minimal criteria defined by the International Society of Cellular Therapy in 2006. According to these criteria, MSCs must adhere to plastic under standard culture conditions, and be able to differentiate towards adipocytes, osteoblasts and chondrocytes, *in vitro* which must be confirmed via specific staining. Additionally, a positive expression pattern (>95 % of the cell population) of CD73, CD90, and CD105 is required as well as the absent expression (> 98% of the cell population) of CD45, CD34, CD14 or CD11b, CD79 or CD19, and HLA-DR (Dominici *et al.*, 2006).

1.4.2 Mesenchymal Stem Cell Differentiation

As mentioned above, the minimal criteria require the differentiation of MSCs towards adipocytes, osteoblasts, and chondrocytes *in vitro*. Next to this, they are able to differentiate towards other cell types of the mesodermal cell lineage, which makes them a favorable source for regenerative medicine approaches. MSC could be successfully differentiated towards skeleton muscle cells (Wakitani *et al.*, 1995; Ferrari *et al.*, 1998; Gronthos *et al.*, 2001), cardiac muscle cells (Planat-Bénard *et al.*, 2004) and cells of the vasculature, endothelial cells and smooth muscle cells (Wosnitza *et al.*, 2007; Zhang *et al.*, 2020). Interestingly, MSC also have the capacity to differentiate into cells derived from other germ layers. These include neuron-like cells (Scuteri *et al.*, 2011), astrocytes (Kopen *et al.*, 1999), islet-like cells (Liu & Han, 2008), and hepatocyte-like cells (Seo *et al.*, 2005; Banas *et al.*, 2007; Pournasr *et al.*, 2011).

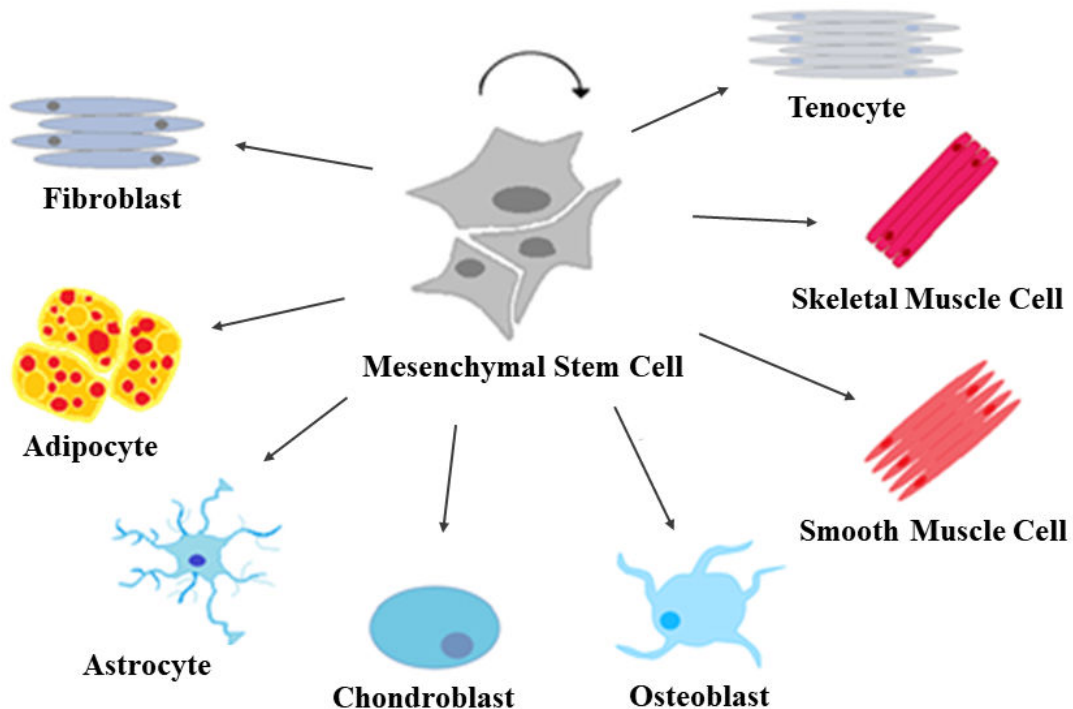


Fig. 6: Multi-lineage Differentiation Capacity of Mesenchymal Stem Cells. Mesenchymal stem cells can differentiate towards tenocytes, fibroblasts, adipocyte, chondroblasts, osteoblasts, astrocyte as well as cardiac-, skeletal-, and smooth muscle cells.

1.4.3 Mesenchymal Stem Cells from Adipose-Tissue

To date, bone marrow-derived MSCs (BM-MSCs) and adipose tissue-derived MSCs (AT-MSCs) are the most frequently investigated types of MSCs. Since the isolation of BM-MSCs is an invasive procedure with low yields (Kitagawa *et al.*, 2006; Ding *et al.*, 2011), adipose tissue gained the interest of research. The isolation of AT-MSCs can be easily collected from adipose tissue like liposuction material which is a waste product. It was shown that AT-MSCs express a similar pattern of mesenchymal stem cell markers compared to BM-MSCs (Zuk *et al.*, 2001; Lee *et al.*, 2004). The difference within the expression pattern includes the surface markers CD34, CD54, CD49d, the latter one only found in AT-MSCs and CD106 only expressed in BM-MSCs (Zuk *et al.*, 2002; De Ugarte *et al.*, 2003). AT-MSCs show a higher proliferative activity (Lee *et al.*, 2004; Peng *et al.*, 2008), a higher lifespan, and no decreasing potential with increasing donor age which has been observed in BM-MSCs (Mueller *et al.*, 2001; Stenderup *et al.*, 2003).

1.4.4 Adipogenesis

Adipose tissue is comprised of a mixture of blood vessels and different cell types including adipocytes, stromal cells, and immune cells. Its sole function was once regarded as energy storage. However, it has since been shown that adipocytes in particular are endocrine cells that produce and secrete bioactive molecules known as adipokines (Galic *et al.*, 2010). These molecules include adiponectin, leptin, TNF α , interleukins and hormones like estrogen (Rajala & Scherer, 2003; Hauner *et al.*, 2005; Sethi & Vidal-Puig, 2007; Robertson *et al.*, 2009). Adipose tissue expansion influences circulating adipokine levels. A disturbed secretion of adipokines has been identified as a contributor to the negative consequences of adipose tissue expansion, including cardiovascular disease, diabetes and certain types of cancer (Rutkowski *et al.*, 2009). Elevated serum levels of free fatty acids,

as observed in obesity, reduce the activity of key proteins of the insulin signaling pathway and thus contribute to the development of insulin resistance. Additionally, they increase hepatic gluconeogenesis and shift the substrate preference from glucose to free fatty acids in muscle, both of which contribute to hyperglycemia. Low adiponectin concentrations are linked to adipositas, diabetes and cardiovascular disease (Ziemke & Mantzoros, 2010). Moreover, in obese patients a high secretion of TNF α from the adipose tissue has been linked to insulin resistance. Beneath other effects, TNF α impairs insulin signaling through the decreased expression of the GLUT4 glucose transporters (Mingrone *et al.*, 2002; Fernández-Veledo *et al.*, 2006) In addition, TNF α has been described to promote calcification of atherosclerotic plaques (Fadini, 2007). Adipokines can also influence the secretion of biological active factors from other cell types. Secretory products from adipocytes stimulate proinflammatory cytokine secretion from endothelial cells (Sommer *et al.*, 2009). Receptors for adipokines are present on multiple cell types, which are linked to atherosclerosis *i.e.*, vascular cells such as endothelial cells and smooth muscle cells, and thus having an impact on cell migration, proliferation, differentiation, and thereby angiogenesis (Wolk *et al.*, 2005).

Adipocytes appear to be endocrinologically very active during their differentiation into mature cells. A detailed examination of the differentiation process is therefore an important preliminary step for the investigating the relationship between dysfunctions associated with metabolic syndrome and the development of new therapeutic approaches. In the body adipocytes develop from mesenchymal stem cells, which reside in the vascular stroma of adipose tissue (Lin *et al.*, 2010). The differentiation is a multistep process. In an initial commitment step, cells become restricted to the adipogenic lineage but do not yet express markers of mature adipocytes. A subsequent activation program involving

various transcription factors results in the adipogenic phenotype (Otto & Lane, 2005).

Commitment is the process by which a stem cell responds to signals that induce determination toward the adipocyte lineage. This process is initiated by BMP4 (Ahrens *et al.*, 1993; Butterwith *et al.*, 1996). Pre-adipocytes become density-restrained and arrest at the G₀/G₁ cell cycle boundary. The growth arrested pre-adipocytes can be induced to differentiate with factors such as insulin or insulin-like growth factor 1, glucocorticoids and agents that enhance cAMP levels.

Treatment with the differentiation factors causes an increased phosphorylation of CREB, which in turn transcriptionally activates C/EBP β . The pre-adipocytes reenter the cell cycle to undergo two rounds of mitotic clonal expansion. It has been shown that growth arrest rather than cell-cell contact at confluence, triggers pre-adipocyte differentiation during terminal differentiation, while a multitude of adipogenic genes is transcriptionally activated. Coinciding with entry into S phase, C/EBP β is phosphorylated by MAPK and GSK-3 β . C/EBP β transcriptionally activates the two key adipogenic transcription factors C/EBP α and PPAR γ . These factors, in combination with ADD1/SREBP-1c, coordinatively activate the genes responsible for maintaining the adipogenic phenotype (Otto & Lane, 2005). An overview, how MSCs transform into adipocytes and which pathways, noncoding RNAs and epigenetic effects on DNA methylation and acetylation are responsible is described by Liu (Liu *et al.*, 2024). Bhatta and colleagues identified adipogenesis markers on human umbilical cord-derived mesenchymal stem cells (UC-MSCs) through holotomography, a label-free live-cell imaging technique in 2024 (Bhatta *et al.*, 2024).

As described above, secreted adipokines have an impact on endothelial cells and thus are linked to atherosclerosis. As developing adipocytes secrete a lot of adipokines during their differentiation process it, is not surprising that gaining weight and not being obese is associated with a greater risk for the development of atherosclerosis. The activation of endothelial cells therefore can be investigated

by using conditioned medium of developing adipocytes in which these bioactive molecules have been secreted.

1.5 Extracellular Vesicles

Extracellular vesicles (EVs) are secreted by many cell types and are classified into four types according to their size:

- (I) endosomal exosomes (50 - 100 nm)
- (II) microvesicles (MVs) (20 - 1.000 nm)
- (III) membrane particles (50 - 600 nm)
- (IV) apoptotic bodies (1.000 - 5.000 nm) (van der Pol *et al.*, 2012).

Because some groups only distinguish two types of EVs (exosomes and ectosomes), the International Society for Extracellular Vesicles stated the following in the “Minimal Information for Studies of Extracellular Vesicles 2018” (MISEV2018), in relation to the EV nomenclature: “EV is the preferred generic term for the subject of our investigations, and subtypes should be defined by physical and biochemical characteristics and/or conditions/sources. When other terms are used, careful definition is required” (Théry *et al.*, 2018).

1.5.1 Exosomes

Exosomes are a subtype of extracellular vesicles (EVs), which are defined as a spheroid structure composed of a lipid bilayer with various cargo or content. They are not able to replicate by themselves, but they are released from eukaryotic and prokaryotic cells and are involved in intercellular communication. EVs were first reported by Chargaff and West in 1946 when they observed procoagulant platelet-derived particles in blood plasma and called them “platelet dust” (Chargaff and

West, 1946; Wolf, 1967). Almost 40 years later two research groups reported the release of vesicles generated after the formation of multivesicular bodies in reticulocytes (Pan and Johnstone, 1983; Harding *et al.*, 1984). EVs have further been found in other biological fluids, such as saliva, semen, urine, and breast milk (Yáñez-Mó *et al.*, 2015).

The standard isolation procedure of EVs from cell supernatant is a multistep centrifugation procedure consisting of several steps, starting from 300g (to remove cells and debris), over 10.000g (to remove proteins) up to 100.000g (ultracentrifugation). Over the past years several additional methods have been developed, such as density gradient ultracentrifugation (to isolate specific populations), antibody-based techniques like capture-beads in magnetic-activated cell sorting, precipitation using polymers or size-exclusion chromatography (Gurunathan *et al.*, 2019).

To determine which type of EVs was isolated their biochemical properties and biological functions have to be revealed. This can be achieved by determination of size, shape, content, and surface markers. Methods for these characterizations are:

- atomic-force microscopy (AFM) or transmission electron microscopy (TEM), both for visualization and characterization of their structure, morphology, and size
- nanoparticle-tracking analysis (NTA) or tunable-resistive pulse sensing (TRPS), for determination of the size and concentration of particles
- polymerase chain-reaction (PCR), microarray and second-generation sequencing (SGS) and third-generation sequencing (TGS), for their content
- Western blotting and flow cytometry, for the characterization of surface biochemical markers; and fixation for *in situ* imaging (Choi *et al.*, 2013; Kreimer *et al.*, 2015; Gupta *et al.*, 2019; Gurunathan *et al.*, 2019).

1.5.2 Biogenesis of Exosomes, Cargo and Characteristics

The term exosomes was introduced by Johnstone (Johnstone *et al.*, 1987). The biogenesis of exosomes is based on the exocytosis of multivesicular endosomes, also called multivesicular bodies (MVBs). The biogenesis process is divided into three stages. The first stage is the formation of endocytic vesicles from pits in the plasma membrane. The second stage is the formation of MVBs by inward budding of endosomal membranes. During this stage, the MVBs are loaded with their cargo. In the third stage, these MVBs can then be degraded by the lysosome or fuse with the membrane of the cell and thus be released as exosomes (Colombo *et al.*, 2014). The best-known mechanism is carried out by the endosomal sorting complex required for transport (ESCRT). This ESCRT is composed of four complexes: ESCRT-0, -I, -II, -III with associated proteins. The ESCRT-0 complex recognizes ubiquitinated proteins in the endosomal membrane. ESCRT-I and ESCRT-II complexes are responsible for membrane deformation into buds, with sequestered cargos. ESCRT-III drives vesicle scission (Juan and Fürthauer, 2018). Next to ESCRT-dependent pathways, other ESCRT-independent mechanisms for EV biogenesis have been described. These involve the hydrolysis of sphingomyelin into ceramide, or proteins like tetraspanins, such as CD63 (Andreu and Yáñez-Mó, 2014; Colombo *et al.*, 2014). Tetraspanins are also involved in cargo secretion of EVs, as well as the process of uptake by receptor cells (Andreu and Yáñez-Mó, 2014).

The contents of the MVBs are often degraded by hydrolases when they fuse with lysosomes. However, in some cases, MVBs may fuse with plasma membrane. This allows the release of their contents into the extracellular environment. Specific MVB features include the presence of tetraspanins (CD9, CD63, and CD81), as well as other molecules generally found in late endosomes, such as major histocompatibility complex (MHC) class II, in antigen-presenting cells (Raposo *et al.*, 1996; Colombo *et al.*, 2014).

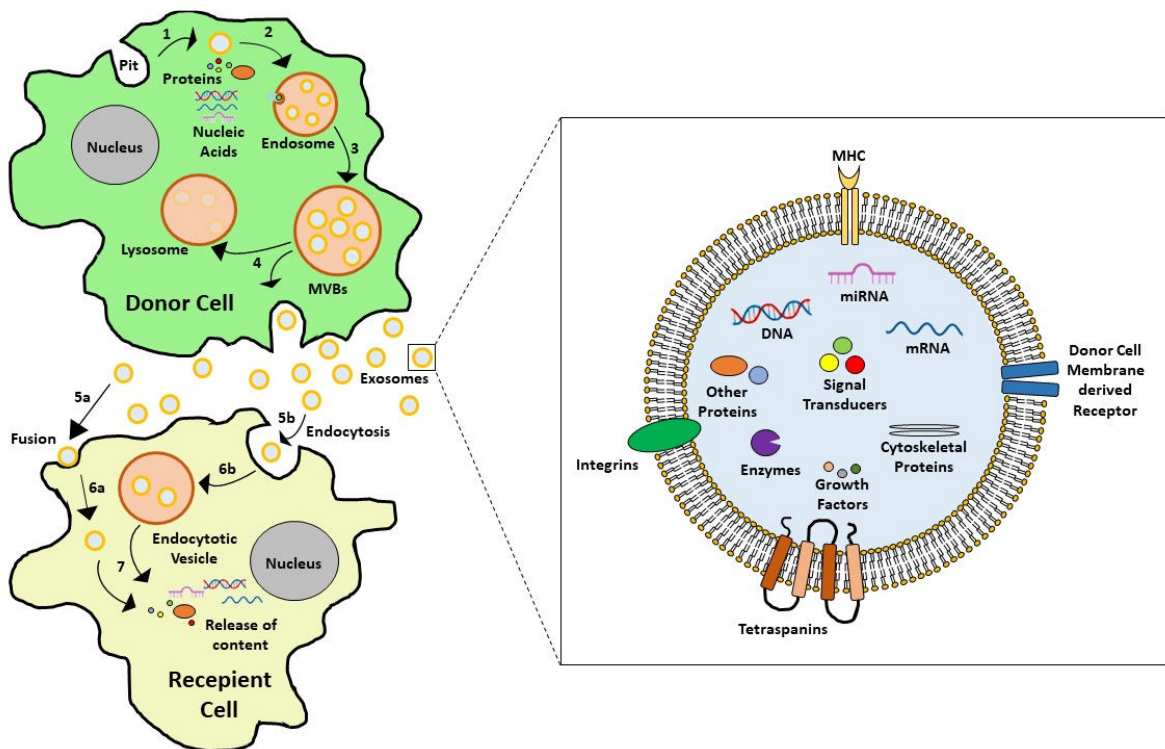


Fig. 7: Biogenesis and Cargo of Exosomes. Left panel: After formation of a pit (1) within the donor cell membrane, intracellular endosomes are loaded with cytosolic cargo (2). The endosomes form multi-vesicular bodies (3), which are either degraded by the lysosome (4) or the exosomes are secreted into the intercellular space (4). The exosomes can be uptaken by a recipient cell via fusion (5a) or endocytosis (5b). Within the recipient cells exosomes stay within the cytosol after direct fusion with the membrane (6a) or an endocytotic vesicle is formed (6b). The cargo is then released into the cytosol of the recipient cell (7). Right panel: Exosomes consist of a double membrane in which receptors and proteins of the donor cell are incorporated. The cargo of exosomes can be numerous biological compounds including big proteins such as cytoskeletal proteins, enzymes, signal transducers, growth factors or nucleic acids like DNA, mRNA and miRNA.

The cargo of EVs and thus exosomes depends on the cell type (donor cell) and their physiological condition. They show a specific differential selection of proteins when generating such vesicles. The main content found in the EVs includes proteins from the endosome itself, plasma membrane, and cytosol. Proteins from the nucleus, mitochondria, endoplasmic reticulum, and Golgi complex are usually absent in EVs. In addition to proteins and lipids, nucleic acids, especially mRNA and microRNA, can be found in high amounts (Colombo *et al.*, 2014). Since the discovery that EVs carry nucleic acids, numerous studies

have described the presence of different RNA types in such particles, including messenger RNA (mRNA), miRNA, and non-coding RNA (ncRNA) (Ratajczak *et al.*, 2006; Valadi *et al.*, 2007).

The lipid composition of EVs depends on the cell origin. The lipid bilayer mainly contains components from the plasma membrane (Fig. 7). They may be enriched with other lipids including phosphatidylserine, disaturated phosphatidylethanolamine, disaturated phosphatidylcholine, sphingomyelin, GM3 ganglioside, and cholesterol (Choi *et al.*, 2013).

Comparative analyses of nucleic acids between cells and EVs may show differential contents. Some biomarkers like tumor susceptibility gene 101 (TSG101), charged multivesicular body protein 2a (CHMP2A), Ras-related protein Rab-11B (RAB11B) in association with CD9, CD63, and CD81 proteins are used for exosome characterization (Gruntham *et al.*, 2019; Console *et al.*, 2019; Jeppesen *et al.*, 2019; Hu *et al.*, 2020).

1.5.3 Exosomes from Mesenchymal Stem Cells as Therapeutic Agents

After the paracrine effect of MSCs has been described in 1996, interest in exosomes and their therapeutic potential increased considerably. Consequently, various disease models have been investigated using MSC-derived exosomes. The studies showed that in acute myocardial infarction models the injection of MSC-derived exosomes lead to a reduced infarct size and the recovery of cardiac function. In acute kidney injury models, exosome treatment ameliorated kidney dysfunction and renal injury and increased cell proliferation *in vitro*. In other studies, MSC-derived exosomes showed a positive effect in liver injury, skin injury, and even a stroke injury model by restoration of neurogenesis (Rani *et al.*, 2015; Suzuki *et al.*, 2017). Exosomes derived from AT-MSCs also showed a positive effect in cardiac injury, liver injury, skin injury, kidney injury and

amyotrophic lateral sclerosis (reviewed in Fang *et al.*, 2019). Clinical trials mainly target cardiovascular diseases like acute myocardial infarction, ischemic heart disease and even some types of cancer (Joo *et al.*, 2020; Tsao *et al.*, 2014). *In vitro* studies showed that exosomes from MSC promoted angiogenesis (Merino-González *et al.*, 2016) and lineage-specific exosomes could override extracellular matrix-mediated MSC differentiation (Narayanan *et al.*, 2018).

1.5.4 Impact of MSC-derived Exosomes on Endothelial Cells

Angiogenesis is a complex process in which both pro-angiogenic and anti-angiogenic factors are coordinated. This includes the degradation of the basement membrane during activation, the proliferation and migration of endothelial cells, and the reconstruction of new blood vessels and vascular networks, involving multiple molecules from a variety of cells (Klagsbrun and D'amore, 1991; Parithimarkalaignan and Padmanabhan, 2013; Lugano *et al.*, 2020).

In numerous works, the angiogenic activity of MSC-derived exosomes has been investigated with regard to their impact on different cell types, especially endothelial cells. HUVECs were treated *in vitro* with exosomes obtained from MSCs, derived from adipose tissue by Ren and bone marrow-derived MSC by Shabbir. The results showed that endothelial cells engulfed exosomes, increased proliferation, migration, and enhancing their angiogenic capacity (Shabbir *et al.*, 2015; Ren *et al.*, 2019). Many studies focusing on angiogenesis used wound healing models because the setup is simple compared to other models.

Regarding the activated pathways in endothelial cells, it has been reported that BMSC-derived exosomes may independently activate the VEGF and Hippo signaling pathways by regulating cell-to-cell contact and actin cytoskeleton dynamics (Wang *et al.*, 2021). The pro-angiogenic effect of MSC-derived exosomes has been shown by several research groups. The main results always showed an improved angiogenesis (Xiong *et al.*, 2018; Hu *et al.*, 2022) or an

enhanced angiogenesis *via* hypoxia-treated donor cells or exosomal cargos like miRNA (Xue *et al.*, 2018). Ning and colleagues demonstrated that miRNA-153-3p reduced EC apoptosis and improved angiogenesis (Ning *et al.*, 2021). The group of Pan showed that miRNA-126 from MSCs enhanced angiogenesis *via* the PI3K/AKT/eNOS pathway (Pan *et al.*, 2019).

To enhance the pro-angiogenic effects, several studies have investigated chemicals, drugs, and scaffolds. This preconditioning can be done by inducing hypoxia or applying chemical compounds. Hypoxia is a well-known angiogenic inducer and can be achieved by culturing cells with deferoxamine (DFO) (Han *et al.*, 2019; Templeton and Liu, 2003). ECs treated with DFO showed increased proliferation, migration, and angiogenesis. An important angiogenic factor within exosomes is miR-126 which activates the PI3K/AKT signal

ing pathway (Ding *et al.*, 2019). In addition to hypoxia, the production of exosomes can be increased by preconditioning. The working group of Liang used low doses of dimethylxaloylglycine (DMOG) in BM-MSC cultures and showed that these exosomes activated the AKT/mTOR pathway to stimulate angiogenesis in human umbilical vein endothelial cells (HUVEC) (Liang *et al.*, 2019). Yu and colleagues treated BM-MSCs with Atorvastatin and showed that the exosomes (ATV-exos) promoted proliferation, migration, tube formation, and increased the VEGF level in HUVEC. Additionally, they found an upregulation of miR-221-3p after ATV-exo stimulation (Yu *et al.*, 2020).

Another way to enhance angiogenesis via exosomes is to use genetic engineering or bioengineering. Overexpression of proteins or miRNAs could also lead to pro-angiogenic effects in ECs. For example, overexpression of islet-1 (ISL1) enhanced the paracrine effect of MSCs and promoted angiogenesis in a myocardial infarction model (Hu *et al.*, 2022). On the other hand, Chen and colleagues transfected MSCs with a miR-150-5p expression plasmid and showed reduced migration and tube formation in HUVECs (Chen *et al.*, 2018).

Next to preconditioning and bioengineering, physical conditions may also have an impact on exosomes. Gao cultured MSCs in 2D or 3D and investigated HUVEC after exosome treatment. The cells treated with 3D-exos showed stronger effects in proliferation, migration, tube formation, and *in vivo* angiogenesis (Gao *et al.*, 2020).

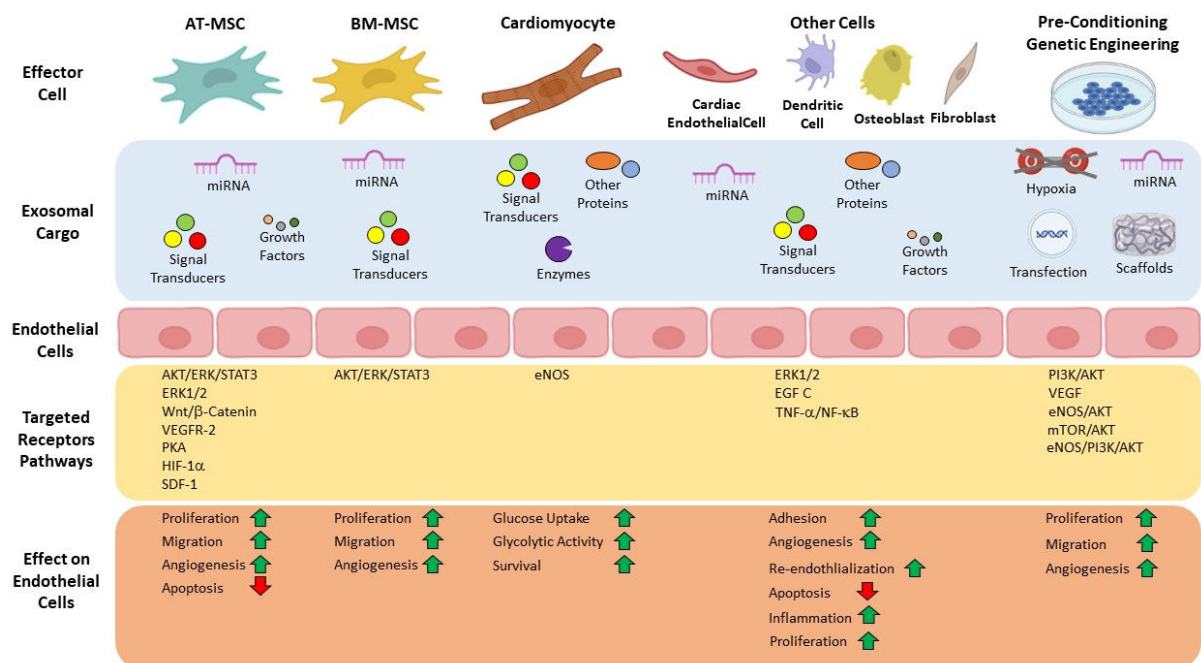


Fig. 8: Exosomes in Angiogenesis. Several types of exosomes from different cell types influence endothelial cells. These exosomes can also be enriched or manipulated by genetic engineering or pre-conditioning of the effector cells. The cargo of exosomes varies from proteins to nucleic acids. Within the endothelial cells the content of exosomes, which can also be encapsulated in scaffolds, trigger different pathways. These pathways activate numerous reactions of the endothelial cells such as proliferation, migration, adhesion, angiogenesis, inflammation, or apoptosis.

1.6 Aim of the Study

The aim of this study is to investigate the activation of porcine aortic endothelial cells (PAECs) by MSC-derived EVs during the differentiation of MSCs towards adipocytes. The key aims are:

- to investigate differences between adipogenic-induced extracellular vesicle preparations and non-induced extracellular vesicle preparations.
- to determine the uptake of induced extracellular vesicles into endothelial cells.
- to investigate the impact of adipogenic-induced extracellular vesicle preparations on endothelial cell migration as marker for endothelial cell activation.
- to investigate the impact of adipogenic-induced extracellular vesicle preparations on angiogenesis as marker for endothelial cell activation.
- to uncover possible signaling pathways triggered by adipogenic-induced extracellular vesicle preparations.

2. MATERIALS & METHODS

2.1 List of Abbreviations

Tab. 1: List of Abbreviations

Abbreviation	Description
°C	Degree centigrade
Akt (= PKB)	Protein kinase B
AM	Adipogenic differentiation medium
APS	Ammonium persulfate
ATCC	American Type Culture Collection
ATP	Adenosine triphosphate
hAT-MSC	Human adipose tissue-derived stem cell
BMP	Bone morphogenic protein
BM-MSC	Bone marrow-derived mesenchymal stem cell
bp	Base pairs
Ca ²⁺	Calcium ions
CD	Cluster of differentiation
cDNA	Complementary deoxyribonucleic acid
CH ₃ COOH	Acetic acid
C ₂₆ H ₂₄ N ₄ O	Oil Red O
CM	Conditioned medium
cm ²	Centimeters squared
CO ₂	Carbon dioxide
DMEM	Dulbecco's modified Eagle medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTPs	Deoxyribonucleosides
ECM	Extracellular matrix
EDTA	Ethylene diamine tetra-acetic acid
EGTA	Ethylene glycol tetraacetic acid
eNOS	Endothelial nitric oxidase syntheses
ERK1/2	Extracellular signal-regulated kinase 1/2
EtBr	Ethidium bromide
EV(s)	Extracellular vesicle(s)
EVP(s)	Extracellular vesicle preparation(s)
F+	DMEM with 10% FBS standard conditions
F-	DMEM with 10% FBS extracellular vesicles reduced
GSK-3	Glucogen synthase kinase 3

HMEC-1	Human microvascular endothelial cells
HU	Hydroxyurea
HUVEC	Human umbilical vein endothelial cells
IF	Immunofluorescence
IL	Interleukine
iPS	Induced pluripotent stem cells
IP3	Inositol 1,4,5-triphosphate
ISCT	International Society for Cellular Therapy
KCl	Potassium chloride
KHCO ₃	Potassium hydrogencarbonate
KH ₂ PO ₄	Potassium dihydrogenphosphate
LDL	Low density lipoprotein
MAPK	Mitogen-activated protein kinase
μg	Microgram (1 x 10 ⁻⁶ g)
μL	Microliter (1 x 10 ⁻⁶ L)
M	Molar
min	Minutes
mL	Milliliter (1 x 10 ⁻³ L)
mM	Millimolar (1 x 10 ⁻³ M)
MMP	Matrix-metallo-protease
mRNA	Messenger ribonucleic acid
MSC	Mesenchymal stem cell
NaCl	Sodium chloride
Na ₂ HPO ₄	Disodium hydrogen phosphate
Na ₂ S ₂ O ₃	Sodium thiosulfate
NH ₄ Cl	Ammonium chloride
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
pH	Pondus hydrogenii; pH = -log [H ⁺]
PI3K	Phosphoinositide 3-kinase
PIP2	Phosphatidylinositol 4,5-bisphosphate
PKA	Protein kinase A
PKC	Protein kinase C
PLC	Phospholipase C
PPAR γ	Peroxisome proliferator-activated receptor γ
RNA	Ribonucleic acid
RT	Room temperature
RT-PCR	Reverse transcriptase polymerase chain reaction
SDS	Sodium dodecylsulfate
TEMED	N,N,N',N'-Tetramethylethylene-diamine
TGF- β	Transforming growth factor- β

TNF α	Tumour necrosis factor alpha
U	Units
v/v	Volume per volume
WB	Western blot
WHO	World Health Organisation
w/v	Weight per volume
g	Relative centrifugal force (RCF)

2.2 Materials

2.2.1 Equipment

Tab. 2: Equipment

Appliance	Supplier
Automated tissue processor	Leica TP1020, Leica, Wetzlar, Germany
Boyden Chamber	Neuroprobe, Warwick, UK
Boyden Chamber Membranes 8 μ m	Neuroprobe, Warwick, UK
Camera, Canon Powershot A570IS	Canon, Tokyo, Japan
Centrifuge, Z 383 K	Hermle, Gosheim, Germany
Centrifuge, Z 233 MK-2	Hermle, Gosheim, Germany
CO2-Incubator, MCO-18 AIC	Sanyo, Munich, Germany
FACS, CYTOMICSTM FC 500	Beckman Coulter, Krefeld, Germany
Freezer, Liebherr Premium	Liebherr, Ochsenhausen, Germany
Gel Chamber, ComPhor L Midi	BIOplastics BV, Landgraaf, Netherlands
Gel Documentation System, Chemi Doc	Bio-Rad, Richmond, USA
Gel Electrophoresis, Power Supply 1001	Amersham Biosciences, Piscataway, USA
Heater and Magnetic Stirrer	ALC International, Milan, Italy
Immunofluorescent microscope, Axio Observer D1	Carl Zeiss, Göttingen, Germany
Inverted Light Microscope	Hund, Wetzlar, Germany
Laminar Flow Cabinet, biowizard	KOJAIR Tech Oy, Vilppula, Finland
Microwave, Daewoo Electronics	Daewoo Electronics Europe, KOR-63A5 Butzbach, Germany

Neubauer Counting Chamber	LO-Laboroptik GmbH, Friedrichsdorf, Germany
NOVOstar® Plate Reader	BMG LabTechnologies, Offenburg, Germany
pH Meter inolab® Serie 720	WTW, Weilheim, Germany
Scale, KERN 770	Bio-Rad, Richmond, USA
Shaker, Heidolph UNIMAX 1010	Heidolph Instruments GmbH & Co. KG, Schwabach, Germany
Spectrophotometer, Gene Quant	Amersham Biosciences by Biochrom Ltd., Cambridge, UK
The Essential Microtome, RM2125	Leica Biosystems, Wetzlar, Germany
Thermal Cycler, PTC-200	MJ Research (now Bio-Rad), Watertown, USA
Ultra Centrifuge	Beckmann Coulter, Krefeld, Germany
Ultrasonic Homogenizer	VTSYIQI, China
Vortexer, Autovortex SA6	Stuart Scientific, Redhill, UK
Water Bath with Agitator, SWB 25	Thermo Fisher Scientific, Waltham, USA

2.2.2 Consumables

Tab. 3: Consumables

Consumable	Supplier
Biotinylated Protein Ladder	Cell Signaling Technology, Danvers, USA
Collagenase / Dispase	Biochrom AG, Berlin, Germany
Cotton Swabs	Ekos, Hamburg, Germany
CD81-labeled Micro Beads	System Biosciences, Palo Alto, USA
DMEM	Biochrom AG, Berlin, Germany
dNTPs (2 mM; 100 mM)	Invitrogen, Carlsbad, USA
EGM-2 Medium	PromoCell, Heidelberg, Germany
Exo-Check Exosomes Array	System Biosciences, Palo Alto, USA
FCS	Biochrom AG, Berlin, Germany
Immobilon Western Chemiluminescent HRP Substrate	Merck Millipore, Billerica, USA
innuSOLV RNA Reagent	Analytik Jena AG, Jena, Germany
L-Alanyl-L-Glutamine (200 mM)	Biochrom AG, Berlin, Germany
GeneRuler 100 bp DNA Ladder	Fermentas GmbH, St. Leon-Rot, Germany

Growth Factor Reduced Matrigel (GFR-Matrigel)	BD Biosciences, CA, USA
Mounting Media	Sigma-Aldrich, Steinheim, Germany
Human Phospho-Kinase Antibody Array (ARY003B)	R&D System, Minneapolis, USA
Human Cytokine Array (ARY022B)	R&D System, Minneapolis, USA
Human Adipokine Array (ARY024B)	R&D System, Minneapolis, USA
Prestained protein marker broad range	Cell Signaling Technology, Danvers, USA
Primer, RT-PCR	Metabion, Martinsried, Germany
PVDF Membrane	Amersham Biosciences, Bucks, UK
DreamTaq DNA Polymerase	Thermo Fisher Scientific, Rockford, USA
Restore Plus Western Blot Stripping Buffer	Thermo Scientific, Rockford, USA
RevertAid™ H Minus First Strand cDNA Synthesis Kit	Fermentas GmbH, St. Leon-Rot, Germany
Collagenase Type I (6 U/mg)	Biochrom AG, Berlin, Germany
Whatman Paper	VWR International Ltd, Lutterworth, UK

Cell culture flasks, plates, multi-well plates, centrifuge tubes, PCR tubes, pipette tips, cell strainer and other plastic items were obtained from TPP (Trasadingen, Switzerland), Santa Cruz (Santa Cruz, USA), BD Falcon (Franklin Lakes, USA), Pure Pak™ (Dallas, USA) or Sarstedt AG & Co. (Nümbrecht, Germany).

2.2.3 Chemicals

Tab. 4: Chemicals

Chemical	Supplier
2-Propanol	Roth, Karlsruhe, Germany
Acetic acid	Sigma-Aldrich, Steinheim, Germany
Agarose	Sigma-Aldrich, Steinheim, Germany
Alizarin Red S	Sigma-Aldrich, Steinheim, Germany
Ampicillin	USB, Cleveland, USA
Ascorbic-acid-2-phosphate	Sigma-Aldrich, St. Louis, USA
ATP	Sigma-Aldrich, Steinheim, Germany
β-Glycerophosphate	Sigma-Aldrich, St. Louis, USA

β-Mercaptoethanol	Merck KGaA, Darmstadt, Germany
Bromophenol Blue	Merck KGaA, Darmstadt, Germany
CaCl ₂	Sigma-Aldrich, Steinheim, Germany
Chloroform	Merck KGaA, Darmstadt, Germany
DAPI	Sigma-Aldrich, Steinheim, Germany
Dexamethasone	Sigma-Aldrich, Steinheim, Germany
D-Glucose	Sigma-Aldrich, Steinheim, Germany
DiL	(2Z)-2-[(E)-3-(3,3-dimethyl-1-octadecylindol-1-ium-2-yl)prop-2-enylidene]-3,3-dimethyl-1-octadecylindole; perchlorate
DMSO	Roth, Karlsruhe, Germany
Ethanol	Merck KGaA, Darmstadt, Germany
Ethidium Bromide	Sigma, Deisenhofen, Germany
EDTA Disodium Salt	VWR International Ltd, Luttersworth, UK
Formaldehyde	Calbiochem, San Diego, USA
Glycerol	Roth, Karlsruhe, Germany
Heparin	Biochrom AG, Berlin, Germany
HEPES	Sigma-Aldrich, Steinheim, Germany
Hydroxyurea	Sigma-Aldrich, Steinheim, Germany
Indomethacine	Sigma-Aldrich, Steinheim, Germany
Insulin	Sigma-Aldrich, Steinheim, Germany
HCl	Roth, Karlsruhe, Germany
KCl	Roth, Karlsruhe, Germany
KH ₂ PO ₄	Roth, Karlsruhe, Germany
DiI-labeled acetylated-low density lipoprotein (DiI-ac-LDL)	Biomedical Technologies Inc., Ward Hill, USA
Methanol	Roth, Karlsruhe, Germany
MgSO ₄	Sigma-Aldrich, Steinheim, Germany
MRS2365	Tocris Bioscience, Bristol, UK
MRS2500	Tocris Bioscience, Bristol, UK
NaCl	Roth, Karlsruhe, Germany
NH ₄ Cl	Roth, Karlsruhe, Germany
NaHCO ₃	Sigma-Aldrich, Steinheim, Germany
Na ₂ HPO ₄	Roth, Karlsruhe, Germany
Na ₂ S ₂ O ₃	Roth, Karlsruhe, Germany
N,N-Methylene Bisacrylamide	Sigma-Aldrich, Steinheim, Germany
Platelet derived growth factor (PDGF-ββ)	PromoCell GmbH, Heidelberg, Germany
Penicillin / Streptomycin (1000 µg/ml)	Biochrom AG, Berlin, Germany

SDS	Amersham Biosciences, Bucks, UK
TEMED	Sigma-Aldrich, Steinheim, Germany
Transforming growth factor- β 1 (TGF- β 1)	PromoCell GmbH, Heidelberg, Germany
TRIS	IBI-Shelton SCIENTIFIC, Peosta, USA
Triton-X	Sigma-Aldrich, Steinheim, Germany
Trypan Blue	Biochrom AG, Berlin, Germany
Trypsin / EDTA (0.5% / 0.2%)	Biochrom AG, Berlin, Germany
Tryptone	Roth, Karlsruhe, Germany
Tween 20	Roth, Karlsruhe, Germany

2.2.4 Oligonucleotides

Tab. 5: Oligonucleotides

Primer	Sequence	Product Size [bp]	Ta [°C]
eNOS Forward	ATCTTCAGCCCCAAACGGAG	527	55
eNOS Reverse	ACGGATGGAAACTGCTCCAG		
KDR Forward	GAG AGG TGC TGC TYM GAT TT	410	52
KDR Reverse	GGA AGG AAC TCT CAT TAG GA		
PECAM-1 Forward	GCCATTGTGCTTCAATGCTC	405	57
PECAM-1 Reverse	AATATCTGTTTTCCACTAAATCAGG		
β -Actin Forward	GCCATCCTGCGTCTGGACCTGG	565	55
β -Actin Reverse	TACTCCTGCTTGCTGATCCACA		
CD73 Forward	GTGGTGGGAGGACACWCCAACAC	314	56
CD73 Reverse	TGGGCACTCGACACTTGGTG		
CD90 Forward	TCCCGAGGGCAGAAGGTGACC	512	60
CD90 Reverse	GGCRTGGAGGAGGGAGAGGGA		
CD105 Forward	TTCCAGCTGGGCCTCTACCTCAG	462	67
CD105 Reverse	GAGGAGGCCGGGGCAGCCAC		
LPL Forward	GAGATTTCTCTGTATGGCACC	275	51
LPL Reverse	CTGCAAATGAGACACTTTCTC		
PPAR γ Forward	GCTGTTATGGGTGAAACTCTG	350	53
PPAR γ Reverse	ATAAGGTGGAGATGCAGGTTC		

2.2.5 Antibodies

Tab. 6: Antibodies

Antibody	Cat. No.	Host	Supplier
CD9	sc-13118	mouse	Santa Cruz, Heidelberg, Germany
CD63	sc-365604	mouse	Santa Cruz, Heidelberg, Germany
CD81	sc-70803	mouse	Santa Cruz, Heidelberg, Germany
eNOS	sc-376751	mouse	Santa Cruz, Heidelberg, Germany
KDR	sc-6251	mouse	Santa Cruz, Heidelberg, Germany
CD31	sc-376764	mouse	Santa Cruz, Heidelberg, Germany
VE-Cadherin	sc-9989	mouse	Santa Cruz, Heidelberg, Germany
Alexa 488	A32723	mouse	ThermoFischer Scientific, Martinsried, Germany
Alexa 594	A-11005	mouse	ThermoFischer Scientific, Martinsried, Germany
Adiponectin	sc-136131	mouse	Santa Cruz, Heidelberg, Germany
Leptin	sc-8391	mouse	Santa Cruz, Heidelberg, Germany
PPAR γ	sc-7273	mouse	Santa Cruz, Heidelberg, Germany
CD44-FITC	M1219U	mouse	Beckman Coulter, Krefeld, Germany
CD90-PC5	IM3793	mouse	Beckman Coulter, Krefeld, Germany
CD105-PE	A07414	mouse	Beckman Coulter, Krefeld, Germany
CD14-PC5	A07765	mouse	Beckman Coulter, Krefeld, Germany
CD34-PC7	A21691	mouse	Beckman Coulter, Krefeld, Germany
CD45-ECD	A07784	mouse	Beckman Coulter, Krefeld, Germany
IgG1-FITC	A07795	mouse	Beckman Coulter, Krefeld, Germany
IgG1-PE	A07796	mouse	Beckman Coulter, Krefeld, Germany
IgG1-ECD	A07797	mouse	Beckman Coulter, Krefeld, Germany
IgG1-PC5	A07798	mouse	Beckman Coulter, Krefeld, Germany
IgG1-PC7	737662	mouse	Beckman Coulter, Krefeld, Germany
HRP 2 nd	401253	mouse	Merck (Darmstadt, DE)

2.2.6 Buffers, Solutions and Medium

Tab. 7: Buffers, Solutions and Medium

Name	Reagents
Acidified Water	5 mL acetic acid to 1 L of distilled water
Agarose Gel (1-2%)	1-2 g agarose in 100 mL 1x TBE
Adipogenic Differentiation Medium	Stem cell culture medium with 1 μ M dexamethasone, 1 μ M insulin, and 200 μ M indomethacin
Alizarin Red S, pH 4.25	40 μ M Alizarin Red S, pH adjusted with HCl and NaOH
Alcian Blue 1%, pH 2.5	1% (v/w) Alcian Blue in 3% acetic acid solution, pH adjusted with acetic acid
Cell Line Culture Medium	DMEM with Phenol Red and stable 2 mM L-analyl-L-glutamine; 10% (v/v) FCS; 100 U penicillin; 100 μ g/mL streptomycin
Chondrogenic Differentiation Medium	DMEM-HG (w Phenol Red), 20 mM HEPES, 1 % Pen/Strep, 1 mM Na-pyruvate, 100 nM dexamethasone, 1 % ITS, 170 μ M ascorbic acid 2-phosphate, 350 μ M proline, 10 ng/mL TGF- β_3
DMEM Medium	DMEM with Phenol Red and stable 2 mM L-analyl-L-glutamine; 10% (v/v) FCS; 100 U penicillin; 100 μ g mL/1 streptomycin
DNA Gel Loading Buffer, 10x	0.21% (w/v) Bromophenol Blue, 0.21% (w/v) Xylene Cyanol FF, 0.2 M EDTA, pH 8.0, and 50% Glycerol
Erythrolysis Buffer “Winter”	1 x 100 mM EDTA (pH 8.0), 0.154 M NH_4Cl , 1 x 0,01 M KHCO_3
FACS Incubation Buffer	1x PBS + 0,5% BSA
FBS- (extracellular vesicle reduced)	FBS ultra-centrifuged (100.000 g for 20 h) supernatant
Freezing Medium	DMEM, 20% (v/v) FCS, 10% (v/v) DMSO
Laemmli Buffer	0.0625 mM TRIS-HCl (pH 6.8), 25% (v/v) Glycerol, 2% (w/v) SDS, 0.01% (w/v) Bromophenol blue, 0.71 M β -mercaptoethanol

Loading Dye (6x)	25% (v/v) Ficoll, 1% (w/v) Orange G, 0,5% (w/v) Bromphenol blue and 0.5% (w/v) Xylen Cyanol FF
Oil Red O Solution	4.284 x 0,001 M C ₂₆ H ₂₄ N ₄ O, 0.1 M C ₃ H ₈ O
Osteogenic Differentiation Medium	Stem Cell culture medium, 0.1 µM dexamethasone, 10 mM β-glycerophosphate, 50 µM ascorbic acid
PBS, 10x	1.37 M NaCl, 0.015 M KH ₂ PO ₄ , 0.081 M Na ₂ HPO ₄ , 0.027 M KCl, pH 7.4
PBSB, 0.1%	1x PBS, 0.1% (w/v) BSA
Protein Lysis Buffer	0.05 M TRIS (hydroxymethyl) amino ethane, 0.28 M NaCl, 10% glycerol, 0,2% EDTA and 0.002 M EGTA 0.5% NP40
Running Buffer, 10x	0.25 M TRIS (hydroxymethyl) amino ethane, 1.92 M Glycine, 1% (w/v) SDS
Stem Cell Culture Medium	DMEM; 10% (v/v) FCS; 100 U penicillin; 100 µg/mL streptomycin; 1% (v/v) L-analyl-L-glutamine (2 mM)
TBE, 10x	1M TRIS (hydroxymethyl) amino ethane, 0.97 M boric acid, 0.02 M EDTA, pH 8.3
TBST, 10x	0.2 M TRIS (hydroxymethyl) amino ethanol, 0.137 M NaCl, 1% (v/v) Tween 20, 1 M HCl; pH 7.6
Transfer Buffer, 10x Stock	0.25 M TRIS (hydroxymethyl) amino ethane, 1.92 M Glycine
Transfer Buffer, 1x	10% (v/v) 10x Transfer buffer, 10% (v/v) Methanol
Western Stacking Gel (4%)	2.9 mL water, 0.5 mL acrylamide (40%), 0.26 mL bisacrylamide (2%), 1.25 mL TRIS-HCl (0.5 M, pH 6.8), 50 µL SDS (10%), 5 µL TEMED, 25 µL APS (10%)
Western Resolving Gel (12%)	2.83 mL water, 3 mL acrylamide (40%), 1.6 mL bisacrylamide (2%), 2.5 mL TRIS-HCl (1.5 M, pH 8.85), 100 µL SDS (10%), 5 µL TEMED, 50 µL APS (10%)

2.2.7 Cell Lines

Tab. 8: Cell Lines

Cell Line	Description
C2C12	ATCC number: CLR-1772 Origin: <i>Mus musculus</i> Source: Muscle myoblast
HeLa	DSMZ number: ACC57 Origin: <i>Homo sapiens</i> Source: Cervix carcinoma
HEK-293FT	ATCC number: CRL-1573 TM Origin: <i>Homo sapiens</i> Source: Kidney epithelium
HMEC-1	ATCC number: CRL-10636 TM Origin: <i>Homo sapiens</i> Source: Microvascular endothelium
NIH-3T3	DSMZ number: ACC 59 Origin: <i>Mus musculus</i> Source: Swiss mouse embryo
MDA-MB-231	ATCC number: HTB-26 Origin: <i>Homo sapiens</i> Source: Adenocacinoma
MG-63	ATCC number: CRL-1427 TM Origin: <i>Homo sapiens</i> Source: Osteosarcoma
SaOS-2	DSMZ number: ACC 243 Origin: <i>Homo sapiens</i> Source: Osteosarcoma
U-87 MG	ATCC number: HTB-14 Origin: <i>Homo sapiens</i> Source: Glioblastoma and astrocytoma

2.3 Methods

2.3.1 Isolation of Primary Cells

Primary cells were obtained from humans and pigs. The ethical agreements (section 2.4) for the isolation and the usage of the cells can be viewed within the lab in Rheinbach, Germany.

2.3.1.1 Primary Endothelial Cells

Primary endothelial were isolated from pigs. Fresh porcine aortas were bought from a slaughterhouse in Rheinbach, Germany. The aortas were kept in ice-cold 1x PBS during transportation. Residual and connective tissues were removed. The aorta was cut longitudinal into 5 cm sections and then divided again into rectangles. Cutted aortas were placed into 1x PBS to avoid drying. The isolation method of endothelial cells was performed as described (Schwartz *et al.*, 1978) with modifications. The cutted aorta was washed with 1x PBS and carefully transferred into cell culture dishes coated with type I collagenase (10 mg/mL in 1x PBS) the inner layer (endothelium) attached to the collagenase and incubated for 60 min at 37°C. The endothelial cells were carefully scraped with a cell scraper and put onto gelatine (in 1x PBS (1%v/v) coated culture plates. DMEM medium was added to the freshly scraped cells and incubated at 37°C, 5% CO₂ in humidified conditions. After 24 hours, medium was exchanged to remove cell debris.

2.3.1.2 Mesenchymal Stem Cells

Human mesenchymal stem cells were isolated from lipoaspirates of different aged female donors. The usage of cells for this study was approved by the ethics commission of the University of Bonn (see chapter 2.4). The isolation procedure was performed according to a previous publication our laboratory (Zippel *et al.*,

2012). Lipoaspirates were diluted in a ratio of 1:2 with PBS at RT for 30 min in order to separate a lower blood and an upper fat phase. The upper phase was transferred into a new sterile container and diluted with PBS in a ratio of 1:2. Type I collagenase (7.2 mg/100 mL fat tissue) was added and incubated for 60 min at 37°C with agitation to release cells from the surrounding tissue. The upper phase was afterwards centrifuged at 200 g for 10 min at RT and the cell pellet was collected carefully. The pellet was subsequently treated with erythrolysis buffer “Winter” (see table 3) for 10 min at RT to destroy remaining red blood cells. The cells were finally collected by centrifuging (Centrifuge, Z 383 K, Hermle, Gosheim, Germany) at 200 g for 10 min at RT, seeded at a density of 3.300 cells/cm² with stem cell culture medium (see table 3) and incubated in a humidified atmosphere at 37°C with 5% CO₂. The cells were washed with 1x PBS to remove non-adherent cells after 24 hours and then supplied with fresh stem cell culture medium.

2.3.2 Cell Culture

2.3.2.1 Cell Line Culture

The cell lines HMEC-1, SaOS-2, MG-63, HEK-293FT, HeLa, MDA-MB-231, U-87 MG and NIH-3T3 were used for the conducted RT-PCR or Western blot experiments as positive or negative controls. They were cultured in cell line culture medium (see table 3). The medium was exchanged twice a week.

2.3.2.2 Primary Cell Culture

Primary cells used in this study were mesenchymal stem cells derived from human liposuction material and endothelial cells isolated from porcine aorta.

2.3.2.2.1 Primary Endothelial Cell Culture

Primary endothelial cells derived from porcine aorta were cultured in DMEM culture medium (see table 3) in a humidified atmosphere with 5% CO₂ at 37°C. The medium was changed two times a week. Confluent ECs were washed with 1x PBS and detached by incubation with 1x 0.5% trypsin / 0.2% EDTA for up to 10 min. Cells were counted by applying trypan blue solution (0.1% w/v) and the use of a counting chamber (Neubauer Counting Chamber, LO-Laboroptik GmbH, Friedrichsdorf, Germany). ECs were seeded in a ratio from 1:3 to 1:5 or frozen at -150°C in freezing medium marked with date of isolation and passage number if needed later.

2.3.2.2.2 Mesenchymal Stem Cell Culture

To investigate the impact of secreted exosomes from MSCs and adipogenic-differentiated MSCs only, exosomes from the FBS had to be removed. The FBS was ultra-centrifuged (Ultra Centrifuge, Beckmann Coulter, Krefeld, Germany) at 100.000 g at 4°C for 20 hours. The supernatant was sterile filtrated (0,22 µm) and stored at -20°C before use in culture medium and labeled F-.

Human MSCs derived from liposuction materials were grown in culture medium in a humidified atmosphere with 5% CO₂ at 37°C. The medium for the MSCs was changed twice a week. Confluent MSCs were washed with PBS and detached by incubation with 1x 0.5% trypsin / 0.2% EDTA for up to 10 min. Cells were counted by applying trypan blue solution (0.1% w/v) and the use of a counting chamber (Neubauer Counting Chamber, LO-Laboroptik GmbH, Friedrichsdorf, Germany), and subsequently seeded in a ratio from 1:3 to 1:5. MSCs stocks were frozen at -150°C in freezing medium (see table 3) labelled with donor gender, age and passage number.

2.3.3 Mesenchymal Stem Cell Differentiation

To confirm the multilineage differentiation potential of isolated MSCs were initially induced towards osteoblasts, adipocytes and chondrocytes. To obtain differentiated human smooth muscle and endothelial cells, all differentiation experiments were performed with cell passage No. 2-6.

2.3.3.1 Adipogenic Differentiation

Human mesenchymal stem cells were differentiated towards the adipogenic lineage by culturing the cells in adipogenic induction medium for 4 weeks. Every 7 days the medium was collected and exchanged with fresh induction medium after washing 3 times with PBS. The differentiation was performed in 12-well plates for staining and in T75 flasks for the collection of secreted EVs and proteins. All 3 donors were differentiated under standard conditions and in extracellular vesicle reduced FBS medium.

MSCs were differentiated under standard conditions in medium containing 10% FBS (F+) or FBS-depleted medium (F-). For the adipogenic differentiation, MSCs were seeded in a density of 2.600 cells per cm² in DMEM stem cell medium (see table 3). After one day of culture, the medium was changed to adipogenic differentiation medium that contained 1 μ M dexamethasone, 1 μ M insulin and 200 μ M indomethacin. Cells were grown in adipogenic differentiation medium for 28 days at 37°C with 5% CO₂ under humidified conditions. Fresh adipogenic medium was added once a week. As negative control, cells were cultured in stem cell culture medium (see table 3) without any induction agents. The supernatant was collected after weekly medium change for exosomes isolation (see 2.3.4.) and preparation of conditioned medium (see 2.3.6.). Supernatants were stored at -80°C.

2.3.3.2 Osteogenic Differentiation

For osteogenic induction, MSCs were seeded in a density of 1.300 cells per cm² in culture medium. After one day of culture, medium was exchanged to osteogenic differentiation medium (see table 3). The differentiation medium contained 0.1 µM dexamethasone, 10 mM β-glycerophosphate and 50 µM ascorbic-acid-2-phosphate. The medium was changed twice a week. MSCs were grown in osteogenic medium for 28 days in a humidified atmosphere as described above. As negative control, cells were cultivated in stem cell culture medium without the induction agents.

2.3.3.3 Chondrogenic Differentiation

For chondrogenic differentiation, the MSCs were seeded in aggregates, at 200 000 - 250 000 cells per aggregate in U-bottom 96-well polypropylene plates. Ten to twelve aggregates were created from each donor. Briefly, the required number of cells were distributed into the wells of the 96-well plate in 200 µL of DMEM HG-based chondrogenic medium (recipe in 2.2.6). The plate was centrifuged (Centrifuge, Z 383 K, Hermle, Gosheim, Germany) at 300 g for 10 min. Early next day, cell aggregates were detached from the bottom by pipetting 100 µL of the medium up and down. The medium exchange was performed strictly every 2-3 days for 5 weeks. During each medium exchange, the aggregates were released from the bottom by pipetting the medium up and down. Lastly, the aggregates were subjected to histological treatment as described in section 2.3.5.3.

2.3.4 Extracellular Vesicle Preparations

Extracellular vesicle preparations (EVPs) were obtained from supernatants of cell culture medium. Briefly, supernatants were centrifuged (Centrifuge, Z 383 K, Hermle, Gosheim, Germany) at 300 g for 10 min to remove cell debris. A part of the resulting supernatant was frozen (-80°C) for migration and angiogenesis

experiments. The rest was centrifuged at 10.000 g for 30 min and the supernatant was transferred to sterile ultracentrifugation tubes (Beckman Coulter, Krefeld) and ultra-centrifuged (Ultra Centrifuge, Beckmann Coulter, Krefeld, Germany) at 100.000 g for 70 min at 4°C. The supernatant was stored at -80°C and labeled as ultra-centrifuged conditioned medium (100 k) for migration and angiogenesis experiments. The pellet was washed in 5 mL 1x PBS and ultra-centrifuged at 100.000 g for 70 min at 4°C again. The resulting pellet was resuspended in 1 mL 1x PBS and sterile filtrated (0,22 µm pore size) into 1,5 mL Eppendorf tubes. The EVs containing samples were stored at -80°C for further investigations.

2.3.4.1 Measurement of EVP Concentration

The concentration of the EVPs was determined via a commercial kit (BCA Kit, BioRad, Feldkirchen, Germany) and was performed according to manufacturer. Briefly, a standard row with a range between 0 and 2.000 ng/mL was diluted from BSA stock solutions. 25 µL of standards and samples were pipetted into 96-well plates (triplicates). 200 µL of reaction buffer was added and the plate was incubated for 30 min at 37°C. The OD was determined on a plate reader at 550 nm wavelength.

2.3.5 Cell Specific Staining

2.3.5.1 Oil Red O Lipid Staining

The differentiated adipocytes were visualized by lipid droplet formation *via* Oil Red O staining. To do so the medium was removed. After rinsing with PBS, cells were fixed with a 10% freshly made formaldehyde solution for 90 min at 37°C in a humidified atmosphere. Afterwards cells were washed with 1x PBS three times and then incubated with a 4.284 mM Oil Red O staining solution (see table 3) at 37°C. After 30 min incubation, the staining solution was carefully removed.

Brightfield microscopy (Observer D1, Carl Zeiss, Germany) pictures were taken immediately. MSCs cultured in parallel in standard culture medium were counter stained as a negative control. Representative images of stained cells were obtained with a microscope (Observer D1, Carl Zeiss, Germany).

2.3.5.2 Alizarin Red S Staining

The osteogenic differentiated cells were visualized by staining extracellular calcium deposits with Alizarin Red S staining. To do so the medium was removed and cells were fixed with a 4% freshly made formaldehyde solution for 5 min at 37°C. The cells were then washed with 1x PBS and incubated with 4 M Alizarin Red S solution (pH 4.2) (see table 3) for 15 min at 37°C. MSCs cultured in parallel in standard culture medium were counterstained as a negative control. The calcium deposits in the ECM were evident as red. Representative images of stained cells were obtained with a microscope (Observer D1, Carl Zeiss, Germany).

2.3.5.3 Aican Blue Staining

After 32 days in culture, the chondrogenic aggregates were washed with 300 µL of 1x PBS twice, fixed in 300 µL Bouin's fixative (recipe in 2.1.6.) for 24 hours, washed four times with 70% ethanol over a 24 h-period and placed into histological cassettes equipped with thin sponges. Dehydration and paraffinization were performed using the automated tissue processor (Leica TP1020, Leica, Wetzlar, Germany) according to a routine histological procedure. Paraffin-embedding, cutting, deparaffinization, hydration, staining with Aican Blue (30 min, RT, wash with ddH₂O) and Nuclear Fast Red (30 min, RT, wash with ddH₂O) and dehydration were performed manually. Processed 6 µm sections were clarified in xylol, mounted on slides and subjected to microscopic imaging.

2.3.5.4 Immunofluorescence Staining of Endothelial Cells

Approximately 90% confluent cells were washed twice with PBS and fixed with 4% freshly made formaldehyde solution for 15 min at RT. After rinsed with 1x PBS twice, cells were incubated with each of following primary antibodies: eNOS, KDR, PECAM-1 (CD31) and KDR (VEGFR-2) in a ratio of 1:100 in antibody dilution buffer at 4°C overnight. After washing three times with 1x PBS cells were incubated with Alexa Fluor 488 or AlexaFluor594 mouse antibodies respectively in a dilution of 1:100 in antibody dilution buffer for 1 hour in the dark. Cell nuclei were stained with DAPI (1: 5.000 in 1x PBS) for 5 min. After subsequent washing with 1x PBS, images were taken with a fluorescent microscopy (Zeiss, Germany).

2.3.5.5 Immunofluorescence Staining of Mesenchymal Stem Cells

Undifferentiated and adipogenic-induced cells were washed twice with PBS and fixed with 4% (v/v) freshly made formaldehyde solution for 15 min at RT. After rinsed with 1x PBS twice, cells were incubated with each of following primary antibodies: PPAR γ , adiponectin (Acrp-30) and leptin (Rb-O) in a ratio of 1:100 in antibody dilution buffer at 4°C overnight. After washing with 1x PBS trice times, cells were incubated with Alexa Fluor 488 mouse antibodies in a dilution of 1:100 in antibody dilution buffer for 1 hour in the dark. Cell nuclei were stained with DAPI (1: 5.000 in 1x PBS) for 5 min. After subsequent washing with 1x PBS, images were taken with a fluorescent microscopy (Zeiss Observer D1).

2.3.5.6 Immunofluorescence Staining of Exosomes

Exosomes for uptake experiments (2.3.10.) were stained with primary antibodies CD9, CD63 and CD81, or DiL. Briefly, antibodies were added to isolated exosomes at a final dilution of 1:1.000 and incubated over night at 4°C on the shaker. After incubation, the exosomes were ultra-centrifuged (Ultra Centrifuge,

Beckmann Coulter, Krefeld, Germany) at 100.000 g for 70 min at 4°C. Exosome pellet was washed with 1 mL 1x PBS and ultra-centrifuged (Ultra Centrifuge, Beckmann Coulter, Krefeld, Germany) at 100.000 g for 70 min at 4°C again. The exosomes were resuspended in 1 x PBS and stored at -80°C.

2.3.6 Flow Cytometric Analysis

2.3.6.1 Endothelial Cell Marker

The isolated PAECs were analyzed using indirect flow cytometry *via* the endothelial cell markers eNOS, KDR and PECAM-1. Cells were scraped, centrifuged (Centrifuge, Z 383 K, Hermle, Gosheim, Germany) at 200 g for 5 min and the cells were fixated in 4% freshly made formaldehyde solution for 10 min at 37°C and afterwards resuspended in FACS incubation buffer. 100 µL of the cell solution was incubated with primary antibodies (10 µL, mouse, 1:100) over night at 4°C in the dark. After incubation, cells were washed with FACS incubation buffer and centrifuged (Centrifuge, Z 383 K, Hermle, Gosheim, Germany) at 200 g for 10 min RT. The pellet was resuspended in FACS incubation buffer and a secondary antibody (Alexa Fluor 488, mouse, 1:100) was added, and the cells were incubated over night at 4°C. After staining, the cells were washed with FACS incubation buffer and centrifuged at 200 g for 10 min at 4°C. The pellet was resuspended in 500 µL FACS incubation buffer for measurement of endothelial cell markers with flow cytometer (FACS, CYTOMICSTM FC 500, Beckman Coulter, Krefeld, Germany).

2.3.6.2 Mesenchymal Stem Cell Marker

The isolated MSCs were characterized using FACS analysis with the MSC positive markers CD44, CD90 and CD105 and negative markers CD14, CD45 and CD34. MSCs were scraped and centrifuged at 200 x g for 5 min. Cells were fixated in 4% freshly made formaldehyde solution for 10 min at 37°C and

resuspended in FACS incubation buffer (see table 3). 100 μ L of the cell solution was incubated in the dark with either the isotype control (3 μ L IgG1-FITC and IgG1-PE and 5 μ L of the tandem conjugates IgG1-ECD, IgG1-PC5 and IgG1-PC7) or the antibodies CD44-FITC (3 μ L), CD105-PE (3 μ L) and CD90-PC5 (5 μ L) as positive mesenchymal cell markers and CD14-PC5 (5 μ L), CD34-PC7 (5 μ L) and CD45-ECD (5 μ L) as negative markers for 60 min. Cells were washed with FACS incubation buffer, centrifuged at 200 g for 5 min and resuspended in 500 μ L FACS incubation buffer. The cytometer settings and cell gates were adjusted to the isotype control, followed by measurement of the stem cell markers using the same conditions on flow cytometer (FACS, CYTOMICSTM FC 500, Beckman Coulter, Krefeld, Germany).

2.3.6.3 Adipogenic Marker

The isolated MSCs were characterized by using FACS analysis with the adipogenic markers adiponectin, PPAR γ and leptin. Cells were scraped, centrifuged (Centrifuge, Z 383 K, Hermle, Gosheim, Germany) at 200 x g for 5 min. The MSCs were fixated in 4% freshly made formaldehyde solution for 10 min at 37 °C and resuspended FACS incubation buffer. Cells 100 μ L of the cell solution was incubated with primary antibodies (10 μ L, mouse, 1:100) over night at 4°C in the dark. After incubation, MSCs were washed with FACS incubation buffer and centrifuged (Centrifuge, Z 383 K, Hermle, Gosheim, Germany) at 200 g for 10 min RT. The pellet was resuspended in FACS incubation buffer and a secondary antibody (Alexa Fluor 488, mouse, 1:100) was added. Afterwards the cells were incubated over night at 4°C. After staining, MSCs were washed with FACS incubation buffer and centrifuged (Centrifuge, Z 383 K, Hermle, Gosheim, Germany) at 200 g for 10 min at 4°C. The pellet was resuspended in 500 μ L FACS incubation buffer for flowcytometric measurement of endothelial cell markers on

a flow cytometer (FACS, CYTOMICSTM FC 500, Beckman Coulter, Krefeld, Germany).

2.3.6.4 Exosomal Marker

EVs were analyzed using CD81-labeled micro beads according to manufacture. Briefly, isolated exosomes were incubated with 50 μ L of micro beads solution (System Biosciences, Palo Alto, USA) and incubated overnight at 4°C. After centrifugation (Centrifuge, Z 383 K, Hermle, Gosheim, Germany) at 2.000 g for 15 min at RT, the pellet containing exosomes bound to micro beads were stained with exosomal antibodies (CD9, CD63 and CD81, 1:100) at 4°C overnight in 1x PBS. After centrifugation (Centrifuge, Z 383 K, Hermle, Gosheim, Germany) at 2.000 g, for 15 min at RT, the pellet were washed with 1x PBS and secondary antibody (Alexa Fluor 488, mouse, 1:100) was added. Afterwards the EVs were incubated over night at 4°C. After staining, the exosomes were washed with 1x PBS. The pellet was resuspended in 250 μ L 1x PBS for flowcytometric measurement of exosomal markers on a flow cytometer (FACS, CYTOMICSTM FC 500, Beckman Coulter, Krefeld, Germany).

2.3.7 Extracellular Vesicle Uptake Assays

The uptake of EVs was investigated using 8-well slides and 24-well plates. The exosomes pre-stained with CD9, CD63, and CD81 (see section 2.3.5.6) were added to the culture medium in defined concentrations and time-points. After each time-point cells were washed with 1x PBS (3 times) and fixed with 4% freshly made formaldehyde for 10 min at 37°C. A secondary antibody was added (1:200 in antibody dilution buffer) and incubated overnight at 4°C. The cells were washed (3 times in 1x PBS) and nuclei counterstain was done with DAPI (5 min, RT).

2.3.7.1 Quantification of EV Uptake Assays

To determine the speed of EV uptake into endothelial cells and fibroblasts, immunofluorescence pictures were taken with the same settings of illumination time (2.000 ms) and magnification (200x & 400x). The resulting images were saved as zvi-format and converted to merged images and single channel images *via* Zeiss Zen software (Carl Zeiss, Jena, Germany). The single channel (Alexa488) images were converted to 8-bit images. Integrated density of cytosolic signals was measured within 5 random squares of 50 x 50 pixels per image using Image J software (imagej.nih.gov).

2.3.8 Migration Assays

2.3.8.1 Scratch Assays

Migration assays were conducted in 24-well plates for screening experiments and in 6 cm cell culture dishes for scratch assays. PAECs were grown to 95% confluence. 24 h before the scratch, cells were starved in DMEM containing 2% FBS. The cells were scratched with 200 μ L tips in 24-well plates or with the own developed scratch machine (Grimmig & Babczyk, 2021) in 6 cm cell culture dishes to create straight scratch edges. Microscope pictures (Zeiss Observer D1, Zeiss, Jena) of 3 randomly selected scratch edges were taken and marked. PAECs were incubated in the medium overnight for migration as follows: DMEM and FBS-depleted DMEM served as controls, whereas conditioned medium (from adipogenic differentiation, non-induced and induced supernatant) in a 1:1 mixture and DMEM and FBS-depleted DMEM plus addition of exosomes in different concentrations served as samples. After overnight migration at 37°C, the marked spots were photographed again to evaluate the migration of PAECs. After imaging the cells were re-scratched to collect only migrated cells. The re-scratched cell suspension was collected in 50-ml Flacons and centrifuged (Centrifuge, Z 383 K,

Hermle, Gosheim, Germany) for 5 min at 300 g. The pellet was lysed in protein lysis buffer (see section 2.2.6) and stored in -80°C for further investigations. Non-migrated PAECs were collected by scraping cells from 6-cm cell culture dishes.

2.3.8.2 Scratch Assay Evaluation

The scratch assays performed in 24-well plates were evaluated using the following procedure: After creation of the scratch, the spot of the scratch was imaged with bright field channel (Zeiss Observer D.1, Carl Zeiss, Jena, Germany) and marked with a pen on the bottom of the plate. After migration, the cells were fixated with 4% freshly made formaldehyde and stained with DAPI. The marked spot was imaged again with brightfield channel and additionally with the corresponding fluorescent DAPI channel. The resulting images were compared in Image J software. First, the scratch area of the first picture was marked with a square, which was then copied to the DAPI image of the migrated cells. The regions outside the square were deleted and the software's counting function counted the nuclei of the migrated cells. Data was translated to graphs using MS Excel (Microsoft, Redmond, USA).

For the scratch assay in 6 cm cell culture dishes the procedure was the following: After creating the scratch, a random spot of a scratch edge was imaged with the brightfield channel as above marked on the bottom of the dish. After migration the same spot was imaged again with the brightfield channel. The images taken after the scratch and after migration were saved as tiff-images, and sent to Prof. Odefey (Technische Hochschule Ostwestfalen-Lippe, Campusallee 12, 32657 Lemgo, Germany) for software automatic counting. Data was translated to graphs using MS Excel.

2.3.8.3 Boyden Chamber Assays

Endothelial cells were starved in DMEM containing 2% FBS for 24 h. Afterwards, the bottom of Boyden chambers were filled with control medium (F+/F-) or conditioned medium. PAECs were counted and 5.000 cells were resuspended in medium or in medium with additional exosomes (see section 2.3.4). The cell suspension was pipetted onto the membrane in the top chamber and incubated overnight at 37°C for migration. After migration, the membranes were fixed in 4% freshly made formaldehyde solution and stained with DAPI to label the nuclei.

2.3.8.4 Boyden Chamber Assay Evaluation

After DAPI stain of the cells, membranes were imaged (Zeiss Observer D1, Zeiss, Jena, Germany) at 5 random spots to detect total cell number. After imaging, the top side of the membranes was swiped with cotton swabs (Ekos, Hamburg, Germany). Migrated cells stayed on the bottom side of the membranes. Membranes were imaged again on 5 random spots. The number of migrating cell number was calculated using Image J software particle analyzer (imagej.nih.gov) by measuring difference of total cell number and migrated cells.

2.3.9 Angiogenesis Assays

The angiogenic capacity of PAECs was investigated using Matrigel (BD Biosciences, CA, USA). 24-well and 12-well plates were pre-cooled (4°C) and loaded with a mixture of Matrigel and EGM-2 medium (2:1) according to the manufacturer (300 µl for 12 wells 150 µl for 24 wells). The Matrigel loaded plates were transferred to the incubator for hardening for 2h. Afterwards, the wells were loaded with PAECs suspended in medium for investigation (50.000 cells/well for 12-well plate and for 12-well plate 100.000 cells/well). After overnight incubation at 37°C, the wells were imaged under the microscope (Zeiss Observer D1, Zeiss,

Jena, Germany). For 24-well plates 3 random spots per well were imaged. For 12-well plates 9 connected images per well were taken and merged to one image.

2.3.9.1 Evaluation of Angiogenesis

To evaluate the angiogenesis of PAECs under different conditions (control EGM-2 and addition of exosomes in different concentrations) images were converted to 8-bit black and white pictures using ImageJ software. The parameters of network formation (size, length, junctions) AngioQuant software was used (<https://bio.tools/angioquant>).

2.3.10 RNA Isolation and Reverse Transcription

RNA was extracted with innuSOLV RNA Reagent (IST Innuscreen GmbH, Berlin, Germany). Afterwards, the quantity of total RNA was evaluated spectrophotometrically at 260 nm using a spectrophotometer (Cambridge, UK) and the RNA solution was either directly used for reversed transcription or frozen at -80°C. Complementary DNA was synthesized from 2.0 µg total RNA using reverse transcriptase, 2 mM dNTPs, RT-Buffer, RI (RNase inhibitor) and an oligo-dT primer for one hour at 42°C.

2.3.11 Polymerase Chain Reaction

Semi-quantitative RT-PCRs were performed with respective primer sequences (see table 2). A reaction mix contained 1x PCR reaction buffer, 0.5 µM of each primer, with primer-dependent magnesium chloride concentration (see table 2), 0.2 mM dNTPs and 1.25 U DreamTaq polymerase (ThermoFisher, Darmstadt, Germany), and template cDNA. The mixture was filled up with ultrapure water to a final volume of 25 µL. Amplification conditions for each specific product were conducted as followed: the initial denaturation was performed at 94°C for 3 min and cyclic denaturation was run for 30 seconds. The cyclic annealing step

was performed for 30 seconds with the respective annealing temperatures (see table 2). The target gene was elongated at 72°C for 45 seconds. A final extension of 3 min at 72°C was set with subsequent cooling down to 4°C. GAPDH and β -actin served as the controls for experiments, respectively. The RT-PCR product was evaluated using a agarose gel (1 - 2%) electrophoresis at 12V/cm for 30 min in 1x TBE buffer. Agarose gels included 4 μ L ethidium bromide solution (10 mg/mL) per 100 mL. A 100 bp DNA ladder was utilized to confirm the product size.

2.3.12 Protein Isolation

Proteins were isolated from cell lines (as controls), adipogenic differentiated MSCs, PAECs from scratch assays (6-cm cell culture dishes), PAECs from Matrigel assays, and exosomes.

2.3.12.1 Protein Isolation from Cells in Culture

Cells mentioned above (see section 2.3.12) were washed with ice cold 1x PBS and scraped in 1 mL PBS with a cell scraper. Afterwards, the cell pellet was collected by centrifuging (Centrifuge, Z 233 MK-2, Hermle, Gosheim, Germany) for 5 min at 500 g. Protein lysate was obtained by treating cells in protein lysis buffer (see table 3) for 30 min on ice. The protein samples were centrifuged at 22.000 g for 5 min at RT to remove cellular debris. The protein lysates were stored at -80°C in lysis buffer for later analysis.

2.3.12.2 Protein Isolation from Scratch Assays

The collection of migrated PAECs was mentioned in section 2.3.8.1. The frozen protein sample samples (see section 2.3.12.1) were centrifuged (Centrifuge, Z 233 MK-2, Hermle, Gosheim, Germany) at 22.000 g for 5 min at RT to remove cellular debris prior to Western blot analysis.

2.3.12.3 Protein Isolation from Matrigel Assays

The PAECs networks were flushed from the Matrigel by pipetting the medium 10 times with pressure within the wells. After centrifugation (Centrifuge, Z 383 K, Hermle, Gosheim, Germany) at 2.000 g for 5 min at 4°C, the pellet was resuspended in 1x PBS and centrifuged (Centrifuge, Z 233 MK-2, Hermle, Gosheim, Germany) at 22.000 g for 5 min at RT. The protein samples were washed in 1x PBS and centrifuged (Centrifuge, Z 233 MK-2, Hermle, Gosheim, Germany) again at 22.000 g for 5 min at 4°C and resuspended in 100 µL lysis buffer. The protein lysates were stored at -80°C.

2.3.12.4 Protein Isolation from Extracellular Vesicle Particles

EVP protein lysate was obtained by treating the exosomes solution protein lysis buffer (1:1 mix) for 30 min on ice and 3x 15 sec ultra-sonication using an ultrasonic Homogenizer (VTSYIQI, China). The protein samples were centrifuged (Centrifuge, Z 233 MK-2, Hermle, Gosheim, Germany) at 22.000 g for 15 min at 4°C to remove debris. The protein lysates were stored at -80°C.

2.3.12.5 Measurement of Protein Concentration

The protein concentration of lysates was determined using Ready-to-Use Bradford Reagent (BioRad) according to manufacturer's protocol. Briefly, 5 µL of sample was mixed with 250 µL of Bradford reagent in a 96-well. After 15 min incubation at RT the OD was measured at 595 nm next to a standard curve (range 0 -2 mg/mL BSA) in triplicates.

2.3.13 Western blotting

The protein lysates were diluted 1:4 in 4x Laemmli buffer (Bio-Rad), boiled for 5 min at 95°C and separated by electrophoresis with a SDS-polyacrylamide gel. A pre-stained marker (Cell Signaling Technology) and a biotin-labeled marker (Cell Signaling Technology) were used to evaluate the protein bands. A stacking gel (4%) and resolving gel (12%) were prepared (see table 4) and protein (about 25 µg protein samples were added for each lane) separation was run at 30 mA for approximately 1.5 hours.

The protein was semi-dry transferred to a PVDF membrane (Amersham Biosciences, Bucks, UK) for 2 hours at a maximum of 25 V. The membrane was blocked in 1x TBST containing 5% nonfat dry milk for 60 min. Afterwards, the membrane was incubated with antibodies in 1x TBST containing 5% (w/v) nonfat dry milk overnight at 4°C. After washing 10 min. with 1x TBST for three times, the membranes were incubated with the secondary antibodies (see section 2.2.5) against mouse for 60 min. in 1x TBST containing 5% (w/v) nonfat dry milk at RT in a ratio of 1:1.000. The membrane was washed in 1x TBST three times for 10 min. Immunofluorescence pictures were taken (Zeiss Observer D1, Zeiss, Jena, Germany) after 3 min of incubation with the Immobilon Western Chemiluminescent HRP Substrate (Merck Millipore, Billerica, USA). The density of the bands was analyzed by using Image J software (imagej.nih.gov). As positive controls, protein extracts from different cell lines (see section 2.2.7) were used as described in each experiment in details.

2.3.14 Phosphorylation Kinase Array

The different PAEC signaling pathways were evaluated measuring the phosphorylated levels of kinases with a human phosphor-kinase array (R&D Systems). The experiment and measurement was performed according to the

protocol from the company. The Western HRP substrate was used to detect the fluorescent signals with a Bio-Rad Chemidoc (Bio-Rad, Richmond, USA) imaging machine. The relative levels of phosphorylation of the kinase phosphorylation sites were analyzed using Image J software (imagej.nih.gov).

2.3.15 Cytokine Array

The different conditioned medium conditions were evaluated measuring different membrane spotted cytokines (R&D Systems). The experiment and measurement was performed according to the protocol from the company. The Western HRP substrate was used to detect the fluorescent signals with a Bio-Rad Chemidoc imaging machine. The relative levels of phosphorylation of the kinase phosphorylation sites were analyzed using ImageJ software.

2.3.16 Adipokine Array

The different conditioned medium conditions were evaluated measuring different membrane spotted adipokines (R&D System). The experiment and measurement was performed according to the protocol from the company. The Western HRP substrate was used to detect the fluorescent signals with a Bio-Rad Chemidoc imaging machine. The relative levels of phosphorylation of the kinase phosphorylation sites were analyzed using ImageJ software.

2.3.17 Statistical Analysis

Statistical analysis was applied for the experiments using the Microsoft Office program Excel 2010. Data are shown as means $\pm$ standard deviation. Experiments were repeated at least three times for three different donors. The probability (p)

value was calculated using the LSH test to assess differences between two groups. Levels of significance were labeled as follows (* $p \leq 0.05$), (** $p \leq 0.01$) and (***) $p \leq 0.005$). Significance was given with the appropriate number of asterisks or in numbers.

2.4 Ethics Agreement

The ethics committee of the University of Bonn approved the use of human tissue material for the isolation, differentiation and characterization of adult mesenchymal stem cells as well as on scaffolds (209/04, title: “Gewinnung humaner mesenchymaler Stammzellen aus Fettgewebe von Liposuktionspatienten und deren Charakterisierung und Differenzierung zu unterschiedlichen Zelltypen auf unterschiedlichen Trägermaterialien”, date of approval: 05.11.2004). All donors signed an informed consent.

3. RESULTS

3.1 Isolation and Characterization of Porcine Aortic Endothelial Cells

Porcine aortic endothelial cells (PAECs) were obtained from aortas of pigs, which have been slaughtered only a few hours prior to isolation according to a modified protocol of Schwartz (Schwartz *et al.*, 1978). The cultured PAECs were grown in standard culture medium until use in following experiments.

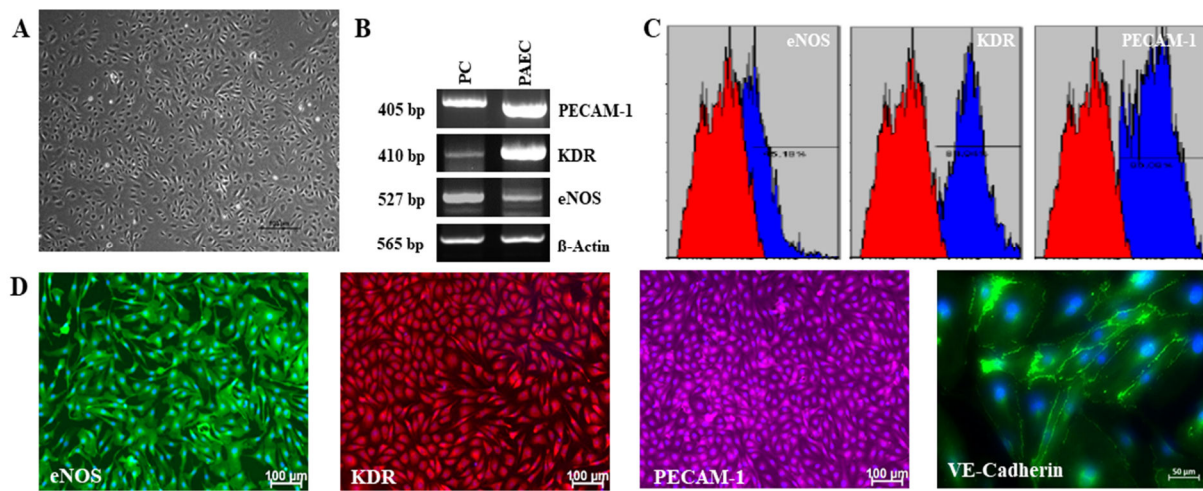


Fig. 9: Isolation and Characterization of PAECs. (A) PAEC form a cobblestone pattern in cell culture dishes (passage 0). Endothelial specific marker expression was confirmed *via* (B) PCR, (C) FACS and (D) IF stain. PCR positive control: HMEC-1.

Isolated PAECs formed a cobblestone-patterned monolayer in cell culture dishes (Fig. 9A). To confirm the isolation of endothelial cells, the expression of specific the endothelial markers eNOS, KDR and PECAM-1 was shown on gene level (Fig. 9B) and on protein level via flow cytometry (Fig. 9C) and immunofluorescence staining. Additionally, VE-Cadherin expression was shown for immunofluorescence staining (Fig. 9D).

3.2 Isolation and Characterization of Mesenchymal Stem Cells

Mesenchymal stem cells (MSCs) were isolated from liposuction material from female donors aged between 23 and 46 years. The liposuction bags usually contained between 1 L and 3 L of material, depending on the side of surgery (Fig. 10A). For the experiments, a mixture of liposuction material from different body regions was used (thigh, belly and hips). The fatty phase (top) containing MSCs was separated from the liquid phase and the cells could be isolated via enzymatic digestion and centrifugation steps. MSCs were cultured in standard medium or exosomes depleted medium and used until passage 7.

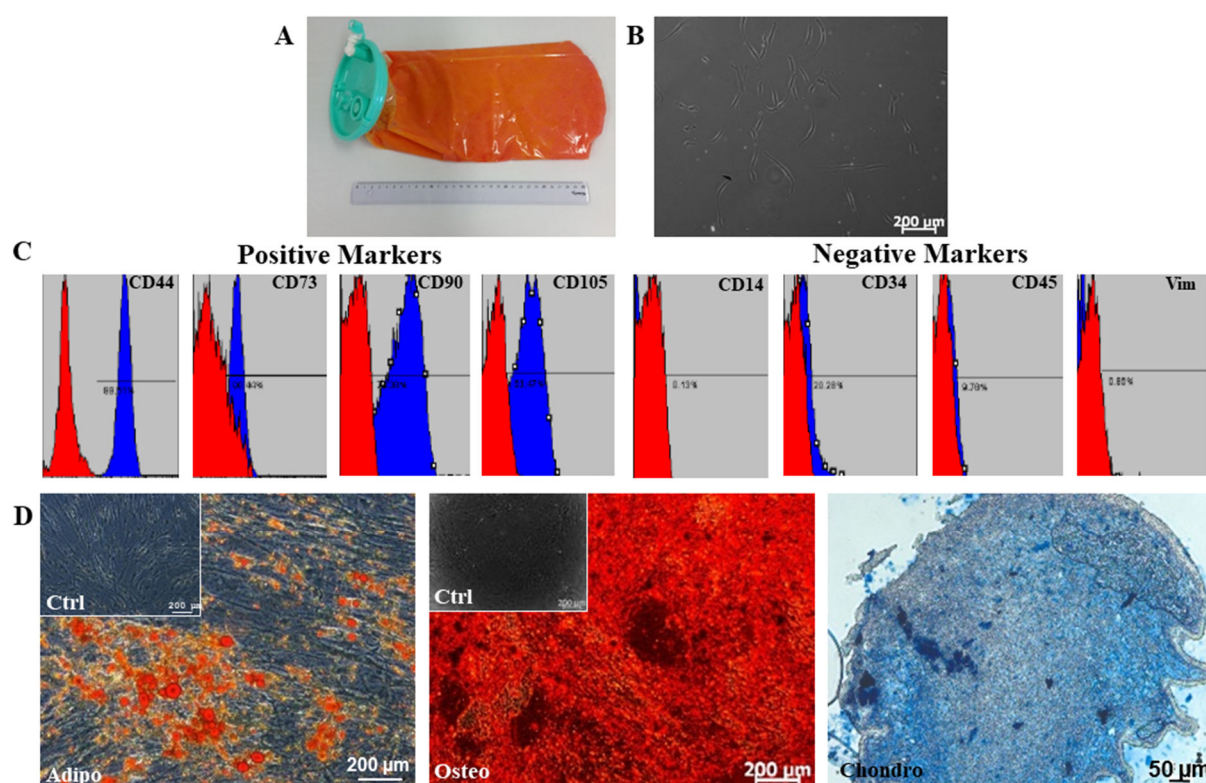


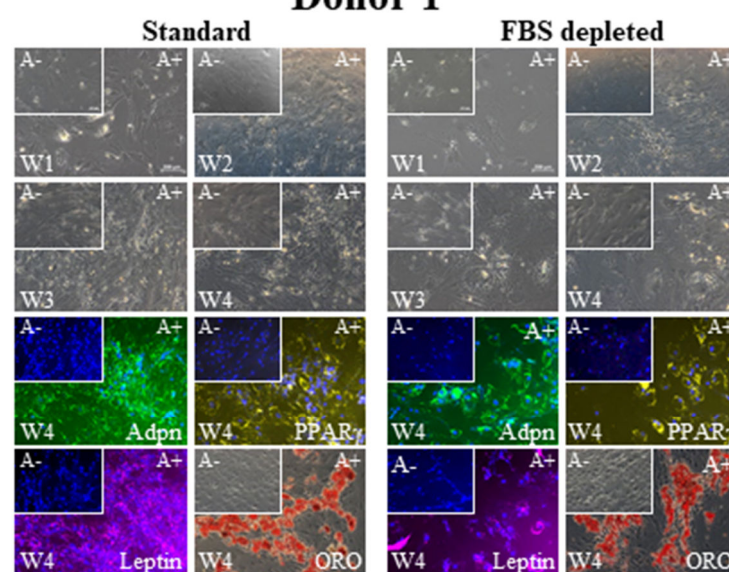
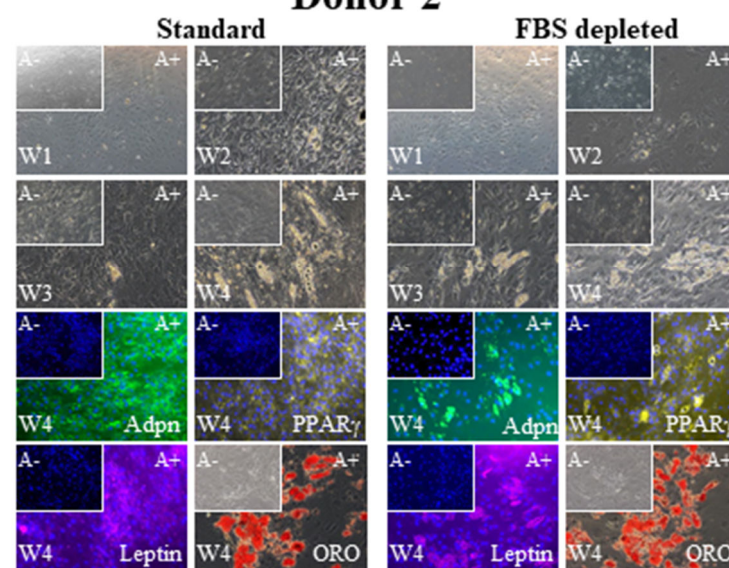
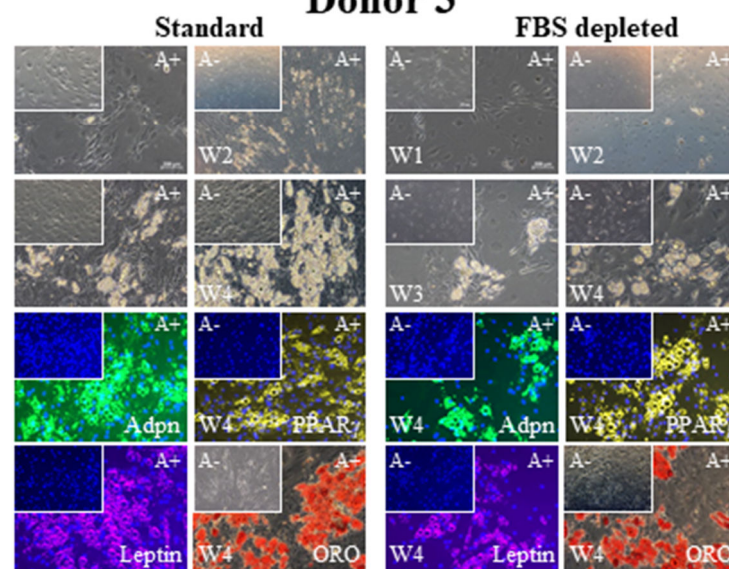
Fig. 10: Isolation and Characterization of MSCs. (A) Liposuction bags containing up to 3 L of liposuction material was mixed 1:1 with 1x PBS. (B) Isolated MSCs showed adherence to plastic and a fibroblast-like morphology. (C) Flow cytometric analysis showed the expression of the positive markers CD44, CD73, CD90, CD105 and the lack of expression for the negative markers CD14, CD34 and CD45. The lack of vimentin expression ensured a MSC culture without fibroblast cross-contamination. (D) Oil Red O Stain, Alizarin Red S Stain and Alcian Blue stain confirmed adipogenic, osteoblast and chondrogenic differentiation. (The data shown are representative for the tested donors).

MSCs showed adherence to plastic culture dishes and a fibroblast-like morphology in passage 0 (Fig. 10B). According to the International Society for Cellular Therapy (ISCT) MSCs additionally have to express CD44, CD73, CD90 and CD105 (all > 95 % positive), and lack the expression of CD14, CD34, and CD45, which was confirmed via flow cytometry (Fig. 10C). The lack of vimentin expression showed that there was no cross-contamination with fibroblasts. The differentiation capacity of the isolated MSCs towards adipocytes, osteoblasts and chondrocytes was confirmed *via* specific staining after *in vitro* differentiation of the cells (Fig. 10D).

3.3 Adipogenic Differentiation of MSCs

3.3.1 Adipogenic Differentiation of hAT-MSCs in FBS Medium

Human mesenchymal stem cells were differentiated towards the adipogenic lineage by culturing the cells in adipogenic induction medium for 4 weeks.

A**Donor 1****Donor 2****Donor 3**

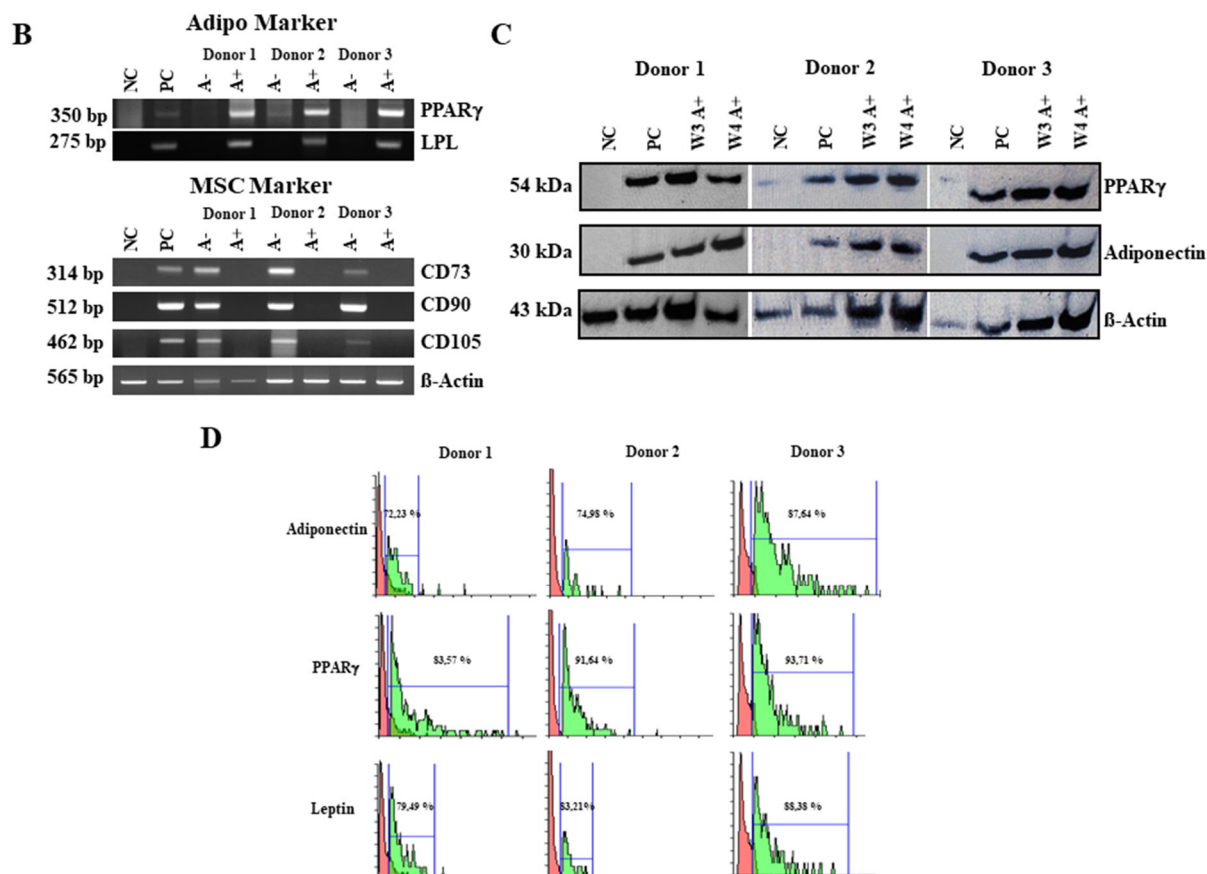


Fig. 11: Adipogenic Differentiation of hAT-MSCs. (A) Three donors were differentiated towards the adipogenic lineage under standard conditions and FBS depleted conditions. After day 4, 14, 21 and 28 cells were photographed to protocol the differentiation status by the development of lipid droplets. After 4 weeks immunofluorescence staining was performed for adipogenic markers (Adiponectin, Adpn; PPARγ; Leptin) and Oil Red O staining was performed to show lipid droplets (red). (B) PCR for adipogenic markers (PPARγ & Lipoprotein lipase, LPL) and MSC markers after week 3 and week 4 of adipogenic differentiation. Beta-Actin served as housekeeping control. (C) Western blot for adipogenic markers (PPARγ & Adiponectin) after week 3 and week 4 of adipogenic differentiation. Beta-actin served as housekeeping control. (D) Flow cytometric analysis of adipogenic differentiated MSCs after week 4 for adipogenic markers (Adiponectin, PPARγ and Leptin). Red histograms show isotype control, green histograms show positive cells (proportion of positive cells are given in %). PCR, Western blot & FACS negative control: HMEC-1; PCR, Western blot & FACS positive control (Adipo): Characterized MSC-derived adipocytes; PCR, Western blot & FACS positive control (MSC): Characterized MSCs.

All 3 donors showed lipid droplets after 2 weeks of differentiation under standard conditions and FBS-depleted conditions. After week 3 and week 4 of differentiation, the highest number of lipid droplets was seen (Fig. 11A). Immunofluorescence staining for adipogenic markers adiponectin, PPARγ and

leptin confirmed the adipogenic differentiation of MSCs after 4 weeks for all 3 donors under standard conditions and FBS-depleted conditions. Oil Red O staining indicated the formation of lipid droplets after 4 weeks of adipogenic differentiation (Fig. 11A). On gene level the adipogenic differentiated cells showed expression for PPAR γ and LPL after 4 weeks of differentiation. While only the controls (not differentiated cells, A-) showed expression of the MSC markers CD73, CD90 and CD105 (Fig. 11B). On protein level adipogenic differentiated cells of week 3 and week 4 were positive for the adipogenic markers adiponectin and PPAR γ (Fig. 11C). Flow cytometric analysis showed that the majority of the cells were positive for the adipogenic markers adiponectin, PPAR γ and leptin after week 4 of the adipogenic differentiation (Fig. 11D).

3.3.2 Adipogenic Differentiation of hAT-MSCs in Human Plasma Lysate Medium

MSCs were additionally differentiated towards adipocytes for 4 weeks in 3% hPL supplemented medium. Immunofluorescence staining of specific adipogenic markers and Oil Red O staining showed that the differentiation was efficient (Fig. 12). The supernatants from week 3 and week 4 of differentiation were collected and EVPs were isolated for further migrations investigations.

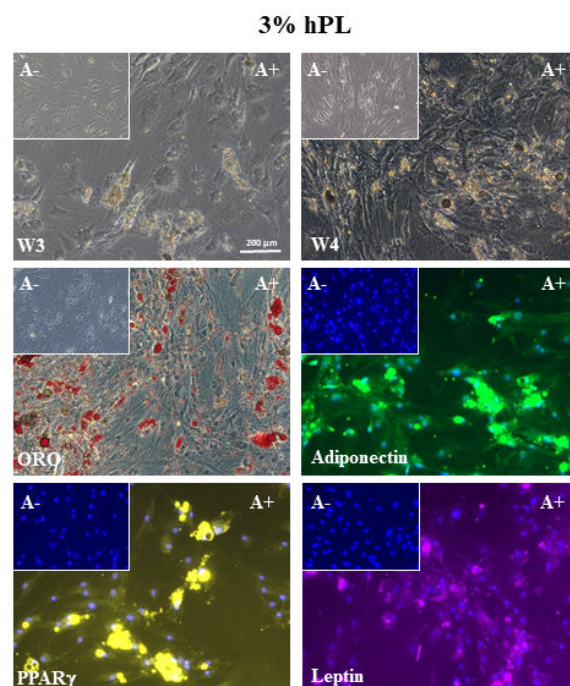


Fig. 12: Adipogenic Differentiation of hAT-MSCs in Platelet Lysate. MSCs were differentiated towards the adipogenic lineage in DMEM supplemented with 3% hPL. After day 21 and day 28 (MSCs) cells were images to protocol the differentiation status by the development of lipid droplets. After differentiation, immunofluorescence staining was performed for the adipogenic markers (Adiponectin, Adpn; PPAR γ ; Leptin) and Oil Red O staining was performed to visualize the lipid droplets (red).

3.4 Extracellular Vesicle Preparation

3.4.1 Preparation of EVs from MSC Cultures

Medium of MSC cultures and adipogenic differentiation of week 4 was collected and used for extracellular vesicle preparation (EVP). The EVs were photographed using an electron microscope (Fig. 13A). Spheroid structures with a diameter of 50 – 150 nm can be seen. EVs secreting MSCs were labeled with antibodies against the exosome markers CD9, CD63, CD81, and the membrane staining dye DiL for flow cytometry (Fig. 13B). MSCs grown in standard culture medium expressed 69% CD9-positive, 87% CD63-positive cells, and 95% CD81-positive cells (Fig. 13B). The EVs were characterized by Western blot for the exosome markers CD9, CD63 and CD81 (Fig. 13C). Additionally, EVs from MSCs

cultured under standard conditions and FBS-depleted conditions were bound to CD81-labeled capture beads and stained with the above-mentioned exosomal markers for flow cytometry. MSCs grown in standard culture medium secreted EVs expressing 97% CD9-positive, 98% CD63-positive, and 72% CD81-positive EVs. In contrast, MSCs cultured in depleted medium secreted EVs expressing almost 100% CD9-positive, CD63-positive, and CD81-positive EVs (Fig. 13D). Further, EVs were detected via immunofluorescence staining of CD9 (Fig. 13E, top). The uptake into PAECs was determined via DiL pre-labeled EVs after overnight incubation. PAEC exosomes can be seen in green while the MSC EVs were taken up by the cells after the incubation (Fig. 13E, red, bottom).

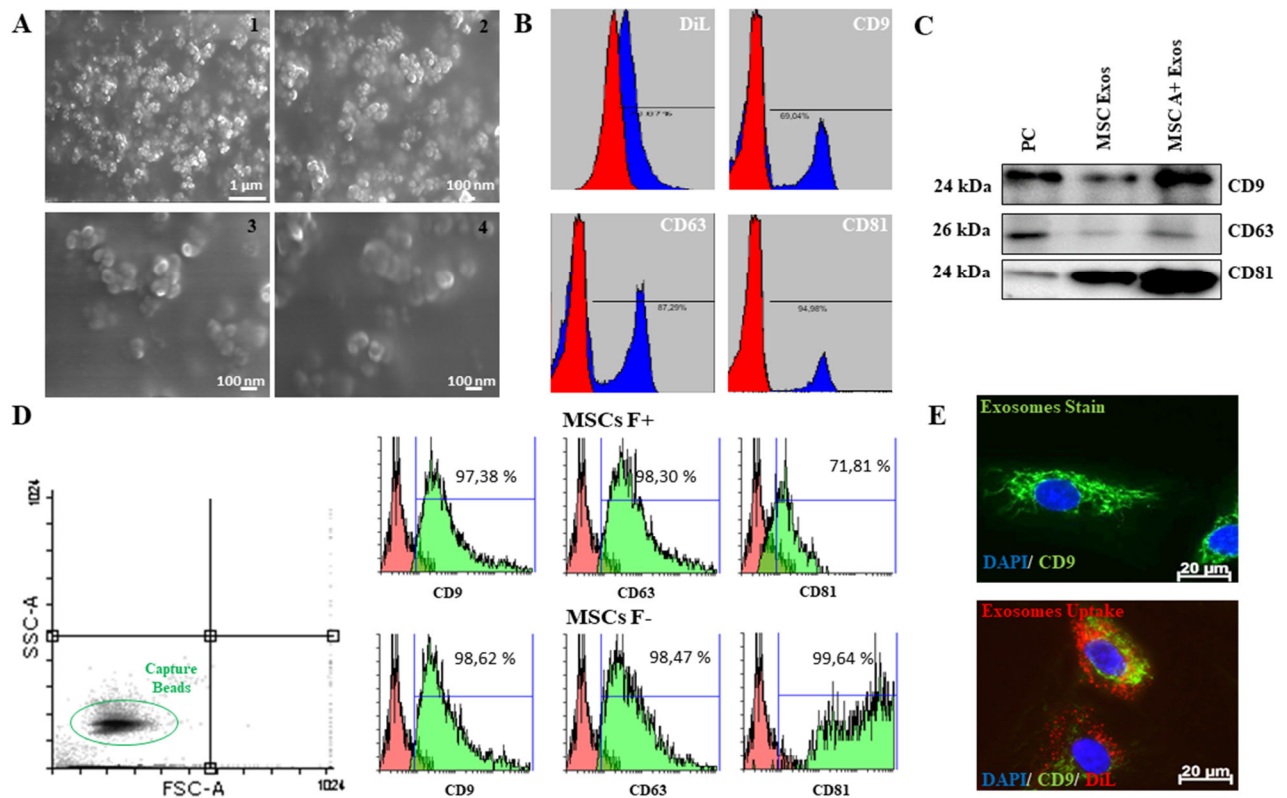


Fig. 13: EVPs from MSC Cultures and their Characterization. (A) Electron microscope images of isolated EVs (1 & 2: 10.0000x; 3: 32.000x; 4: 48.000x). (B) Flow cytometry of MSC secreting EVs. (C) Western blot of EVPs. (D) CD81-labeled capture beads flow cytometry. (E) Exosomes stain and EV uptake into PAEC. Positive Controls: MSCs.

3.4.2 Preparation of EVs from Adipogenic Differentiated MSCs

During the adipogenic differentiation of MSCs from the three donors (Section 3.3.1), medium was collected after every medium change and at the end of the differentiation (week 1 – 4). The EVs were isolated after standard differentiation with 10 % FBS (F+) and after FBS-depleted differentiation (EV reduced FBS: F-).

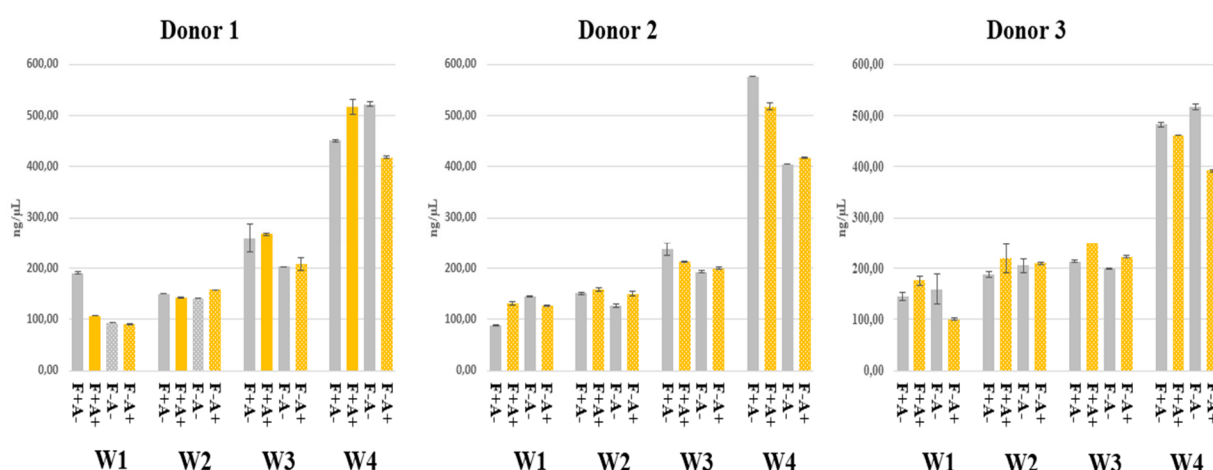


Fig. 14: Concentration of EVs during Adipogenic Differentiation. EVs were collected out of approximately 50 mL of medium supernatant each. The concentration is given in ng/ μ L. Grey: non-induced exosomes; yellow: adipogenic induced exosomes.

All three donors showed the highest concentration of EVs at the end of differentiation at week 4. There was no difference between standard conditions (F+) and FBS-depleted conditions (F-). Additionally, the concentrations did not differ between all three donors. Until week three of adipogenic differentiation the concentrations ranged between 100 – 200 ng/ μ L. At the end of differentiation the concentration of EVs more than doubled (400 – 500 g/ μ L) for both, induced and non-induced cells.

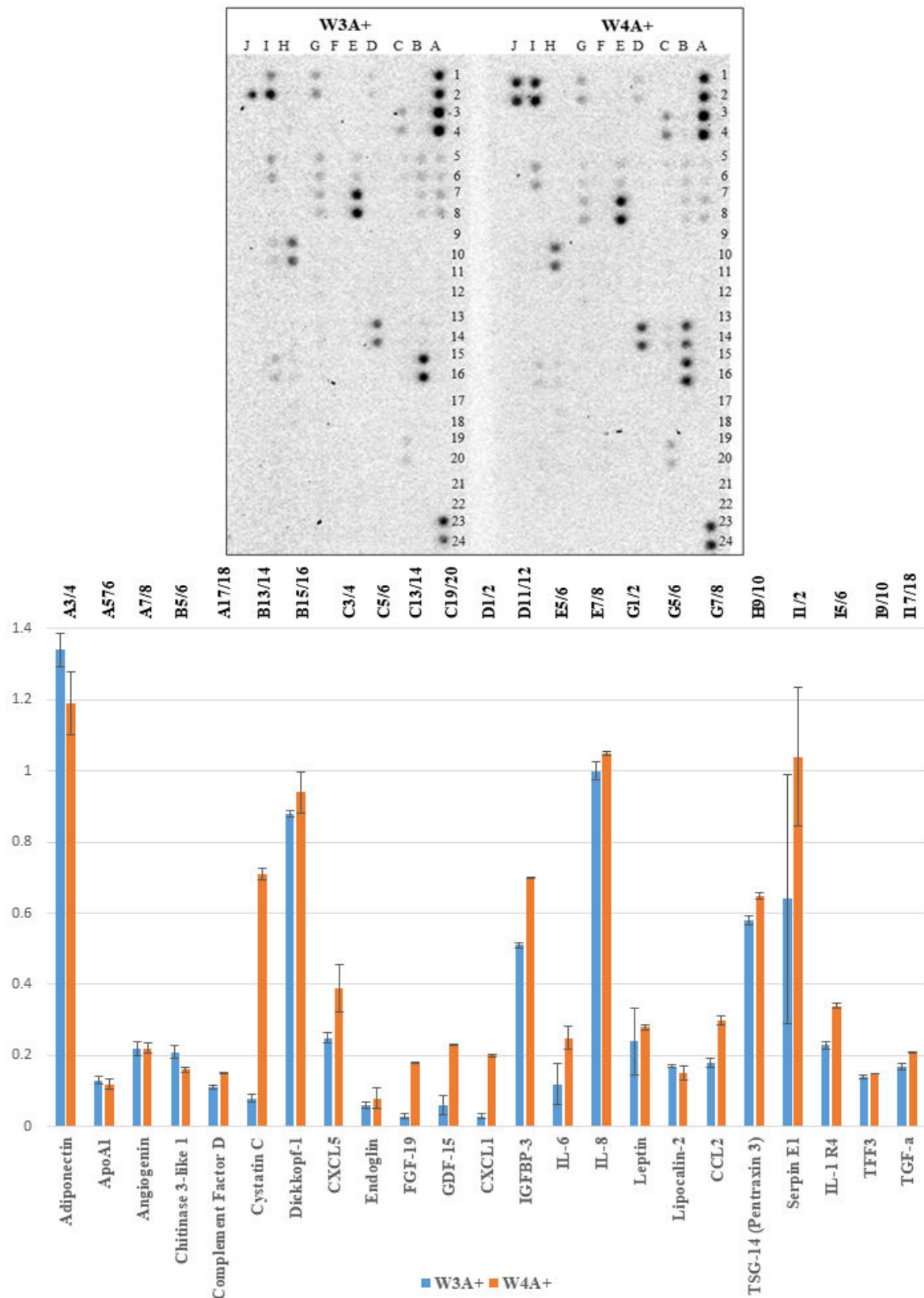


Fig. 15: Cytokine Array for Adipogenic Conditioned Medium. Medium from week 3 and week 4 of adipogenic-induced MSCs was collected and used in cytokine arrays. Top: incubated membranes; bottom: quantification of dots.

The quantitative analysis of the cytokine arrays (R&D systems) for adipogenic-induced medium from week 3 and week 4 showed no differences in the secretion of cytokines. Only the level of secreted cystatin C was significantly higher in week 4. The cytokines FGF-19, GDF-15, CXCL1, IL-6 and IL-R4 were elevated in week 4. To investigate if EVs preparation (EVP) was successful, the EVP of week 3 from donor 3 was investigated with a commercial exosomes detection kit (SBI System Biosciences). EVs were purified and put on membranes for overnight incubation before detection according to manufacturer's instruction.

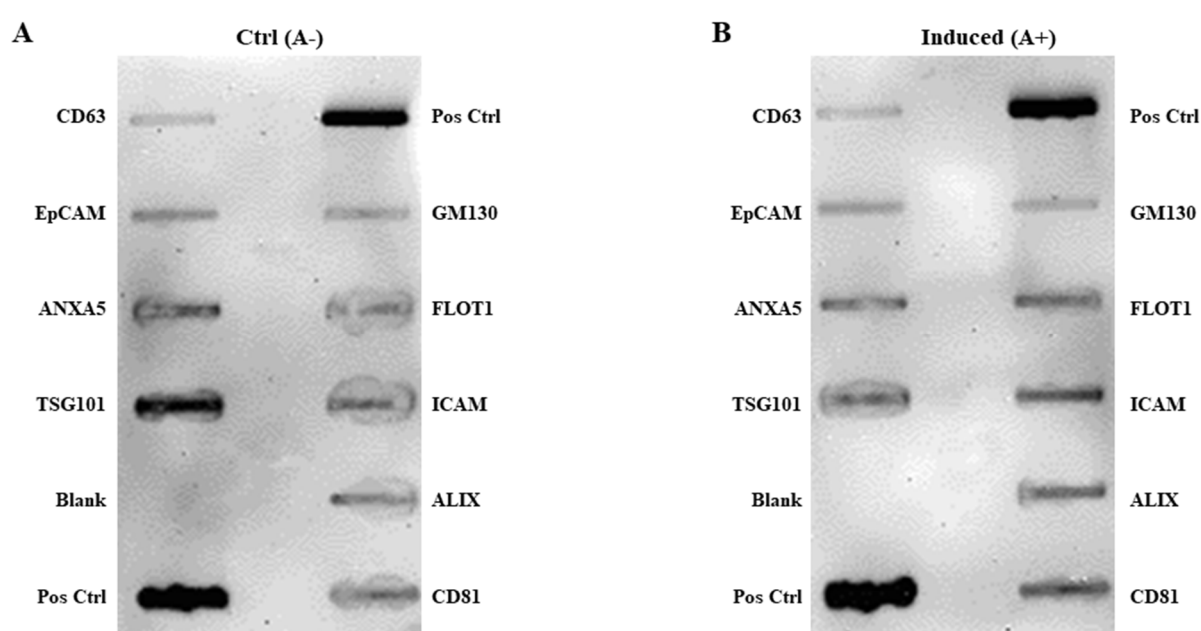


Fig. 16: Exosome Antibody Array. (A) EVs from non-induced MSCs (F-) from donor 3 after 3 weeks of culturing. (B) EVs from adipogenic-induced MSCs (F-) from donor 3 after 3 weeks of culturing. 25 µg of EVs were used for each membrane.

EVs from both, MSCs fractions (non-induced and adipogenic-induced) from donor 3 showed all exosomal markers (CD63; EpCAM, ANXA5; TSG101; GM130; FLOT1; ICAM; ALIX; CD81). Most markers displayed a similar strength of bands. CD81 seemed to be more prominently expressed on adipogenic-induced EVs whereas TSG101 gave a stronger signal on non-induced MSC EVs.

3.5 Extracellular Vesicle Uptake Assays

3.5.1 Uptake of Pre-Stained EVPs from MSCs

The uptake of EVPs was investigated by pre-staining the isolated EVPs with antibodies for the specific exosomal markers CD9, CD63 and CD81, and the membrane staining dye DiL. To investigate if the pre-staining of EVPs was functional, PAECs were loaded with 1 μ g/mL of CD9, CD63 & CD81 pre-stained EVPs. Immunofluorescence images (Fig. 17A) showed that the pre-stained EVPs were taken up by the cells. To verify the staining and to investigate the time period needed for the uptake, EVPs were stained with DiL. After 0 h (no addition of EVs) only CD9 from the PAEC exosomes was visible. After 1 h of incubation, EVPs (DiL signal in red) could be observed additionally to the CD9 signal of PAEC exosomes. Over a time period of 4 hours both signals could be detected (Fig. 17B).

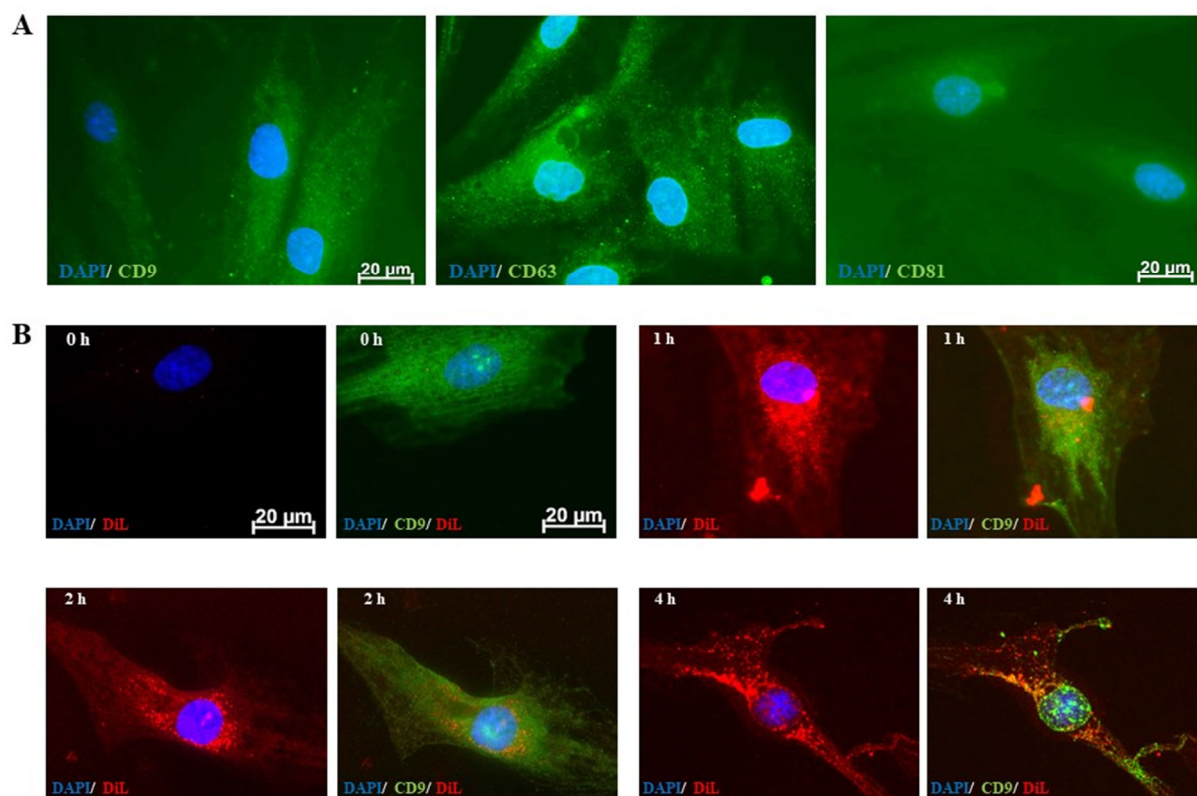


Fig. 17: Uptake of pre-stained EVPs into/onto PAECs. (A) EVPs were stained with the exosomal markers CD9, CD63 & CD81 and added to the medium (1 $\mu\text{g}/\text{mL}$) on PAECs for overnight incubation. PAECs were incubated with secondary fluorochrome-labeled antibody (Alexa488, green) and showed uptake of EVPs. (B) EVPs were stained with DiI (red) and added to the medium (1 $\mu\text{g}/\text{mL}$) on PAEC for time-point experiment. PAECs were stained for CD9 and fixed after 1, 2 and 4 hours and (green). Directly after EVP addition (0h), no prestained EVPs were visible. Uptaken EVs could be seen after 1, 2 6 4 h after addition (red). Counterstain: Nucleus (DAPI, blue).

3.5.2 Uptake of Pre-Stained EVPs from Adipogenic Differentiation

The uptake of EVPs from adipogenic-differentiated MSCs was quantified by immunofluorescence staining. The EVPs were stained with antibodies against the exosomal markers (CD9, CD63, CD81) and added to the medium for 1- 4 hours. Visualization was performed for each time point with the same image settings for quantification of signal strength.

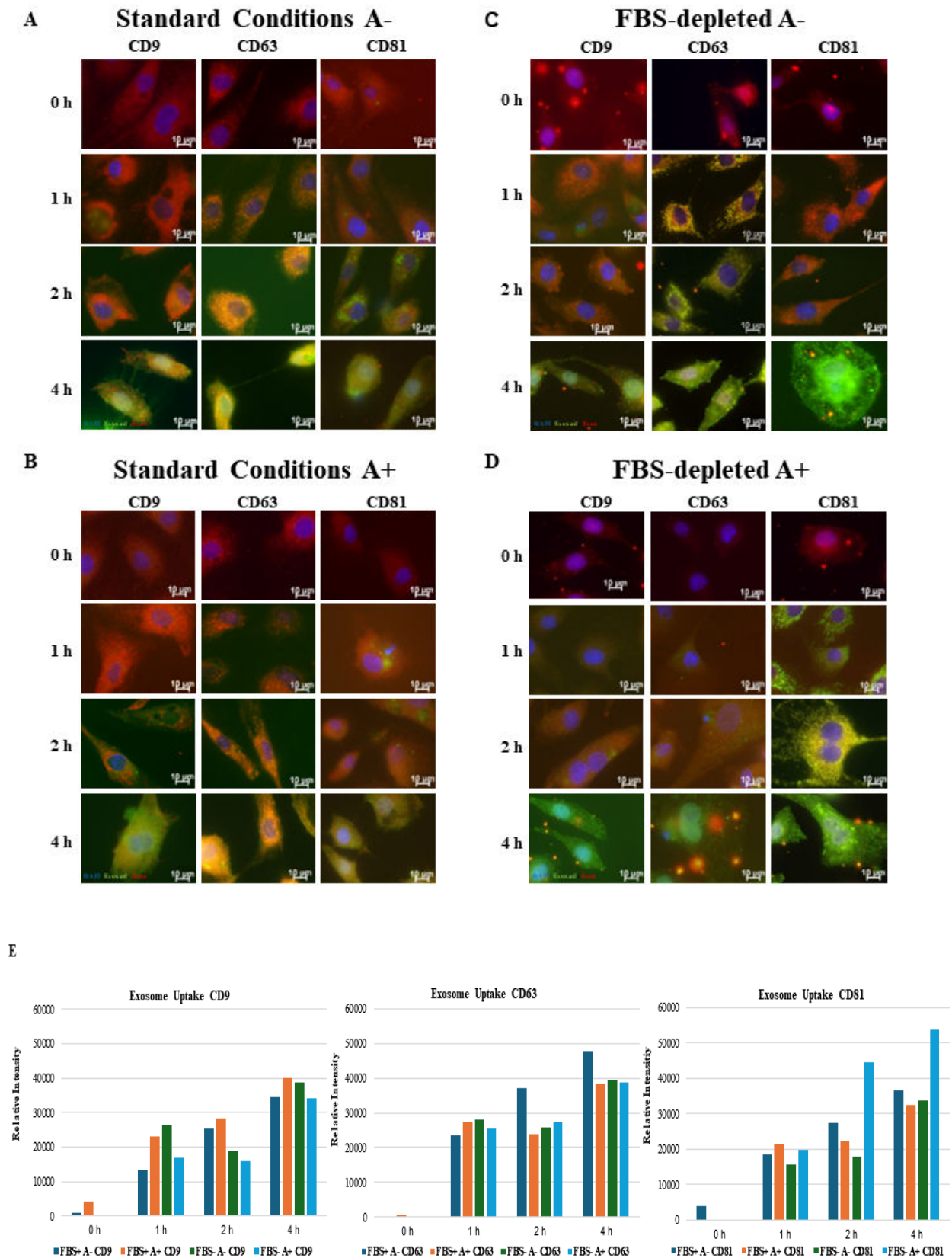


Fig. 18: Uptake of Pre-stained Adipogenic EVPs into/onto PAECs. EVPs were stained with the exosomal markers CD9, CD63 & CD81 and added to the medium (1 µg/ mL) on PAEC. PAECs were incubated with secondary fluorochrome-labeled antibody (Alexa488, green). Origin exosomes of PAEC were stained with the exosomal markers CD9, CD63 & CD81. PAECs were incubated with the secondary fluorochrome-labeled antibody (Alexa594, red). Counter stain: DAPI (nucleus, blue). **(A)** Standard conditions (F+A-): EVPs from non-differentiated MSCs. **(B)** Standard conditions (F+A+): EVPs from adipogenic-differentiated MSCs. **(C)** FBS-depleted (F-A-): EVPs from non-differentiated MSCs. **(D)** FBS-depleted (F-A+): EVPs from adipogenic-differentiated MSCs.

The images and the quantitative analysis showed that the controls (0 h) without additional EVs gave no corresponding green signal from pre-stained EVPs. Only PEAC exosomes could be detected (red). The first signals for all 3 markers appeared after 1 h of incubation and increased within the next 3 hours. This was observed for pre-stained EVPs gained under standard conditions (Fig. 18 A, B) as well as for pre-stained EVPs gained from EV reduced FBS medium (Fig. 18 C, D). Additionally, there was no difference between EVPs from non-induced MSCs and adipogenic-induced MSCs.

To exclude any cross-talk of fluorochromes, the uptake experiments were repeated by only imaging pre-stained EVPs and not PAEC exosomes. The results above could be confirmed by fluorescence imaging and quantification. In the controls without EVPs (0 h) no signals could be detected. EVPs gained from standard conditions and EVPs from FBS-depleted conditions started to show signals for all 3 exosomal markers after 1 h of incubation which increased during the next 3 hours. CD81 was higher expressed after 2 h and after 4 h incubation with adipogenic EVPs from FBS-depleted conditions.

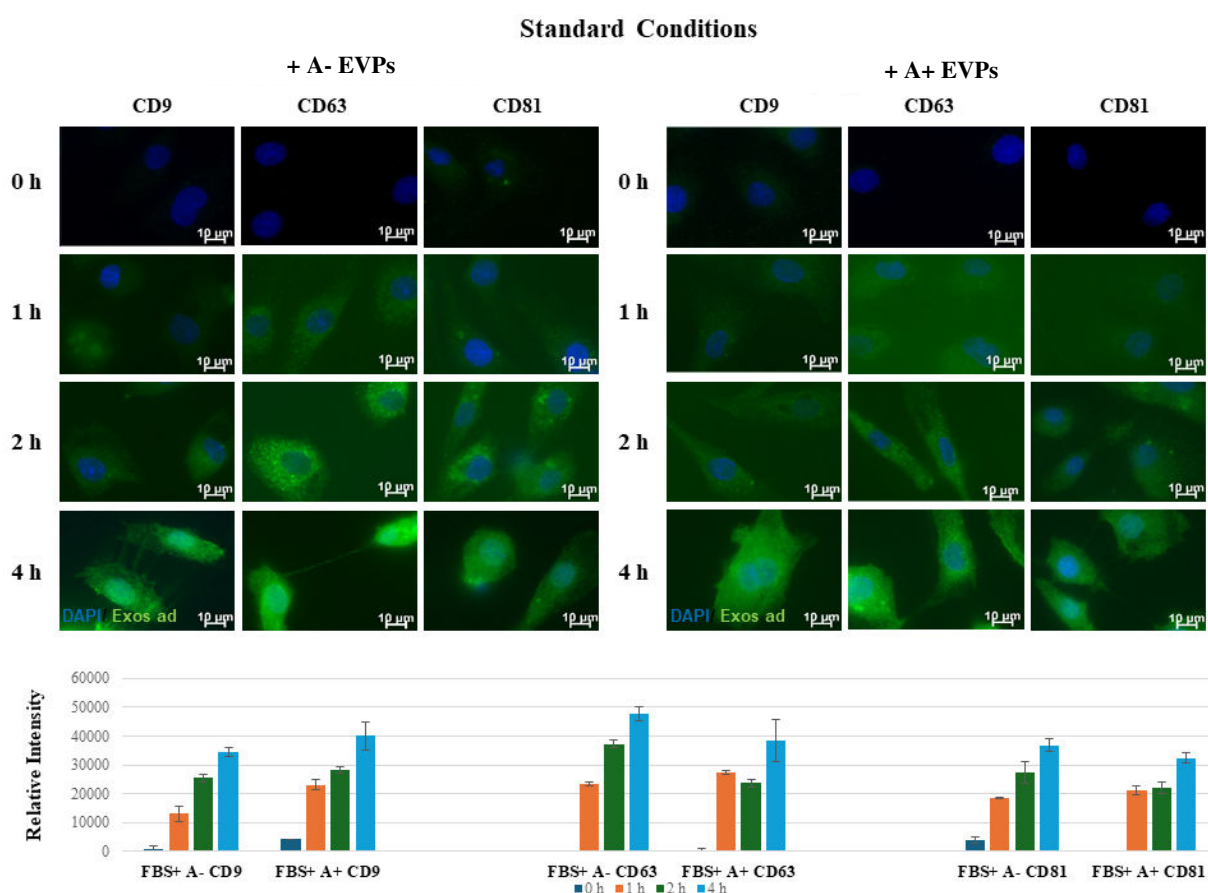


Fig. 19: Uptake of Exosomal Marker Pre-stained F+ EVPs into/onto PAECs. F+ EVPs were stained with the exosomal markers CD9, CD63 & CD81 and added to the medium (1 $\mu\text{g}/\text{mL}$) on PAECs for overnight incubation. PAECs were incubated with secondary fluorochrome-labeled antibody (Exos ad, green). Counter stain DAPI (nucleus, blue). The uptake of both, non-induced and adipogenic-induced EVPs is visible after 1 h and increases during the next 3 hours for all 3 pre-stained EVPs.

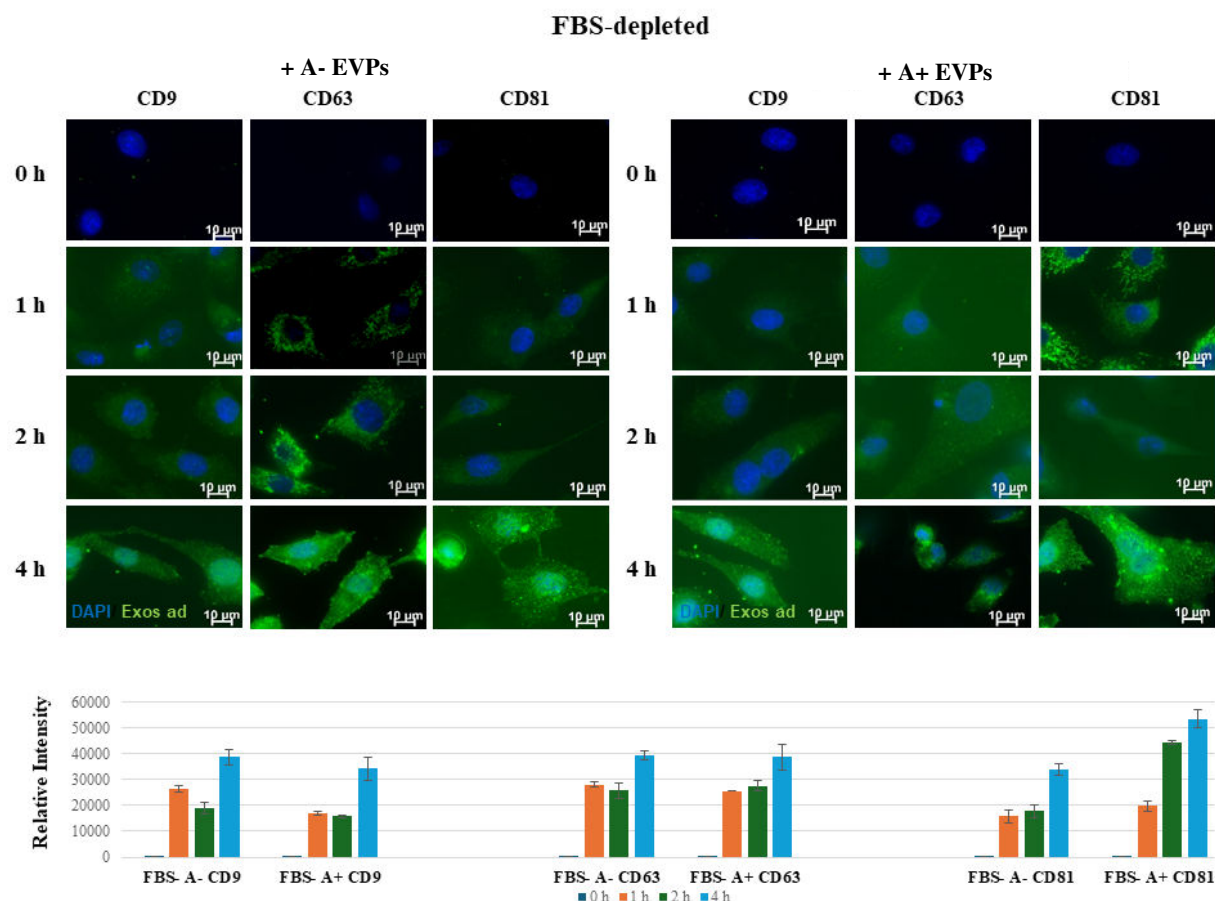


Fig. 20: Uptake of Exosomal Marker Pre-stained F- EVPs into/onto PAECs. F- EVPs were stained with the exosomal markers CD9, CD63 & CD81 and added to the medium (1 µg/ mL) on PAECs for overnight incubation. PAECs were incubated with secondary fluorochrome-labeled antibody (Exos ad, green). Counter stain DAPI (nucleus, blue). The uptake of both, non-induced and adipogenic-induced EVPs is visible after 1 h and increases during the next 3 hours for all 3 pre-stained EVPs.

As the uptake of EVPs was very similar for EVPs obtained under standard conditions and obtained under FBS-depleted conditions, the question if there is a difference in the uptake of EVPs in other endothelial cells was addressed. Therefore, human umbilical vein endothelial cells were incubated with pre-stained EVPs and imaged for quantification.

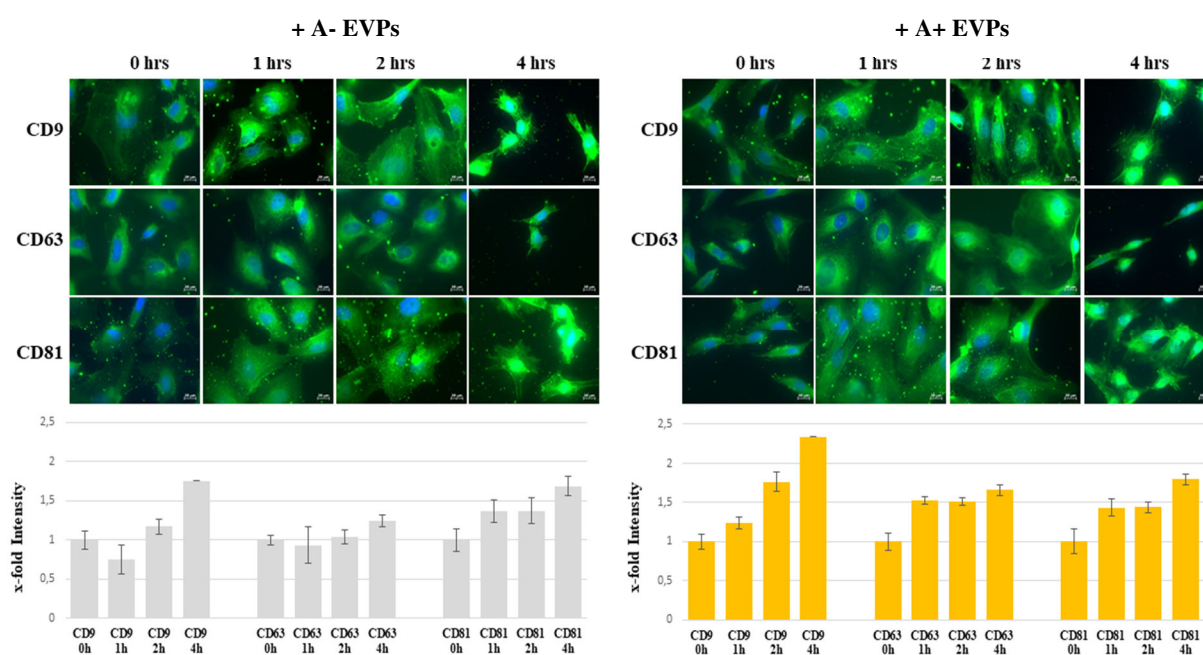


Fig. 21: Uptake of EVPs into/onto HUVECs. HUVEC were incubated with non-induced and adipogenic-induced EVPs for 1, 2 and 4 hours and stained for the exosomal markers CD9, CD63 and CD81. After overnight incubation, HUVEC were stained with the fluorochrome-labeled secondary antibody (Alexa488, green). Counter stain: DAPI (nucleus, blue).

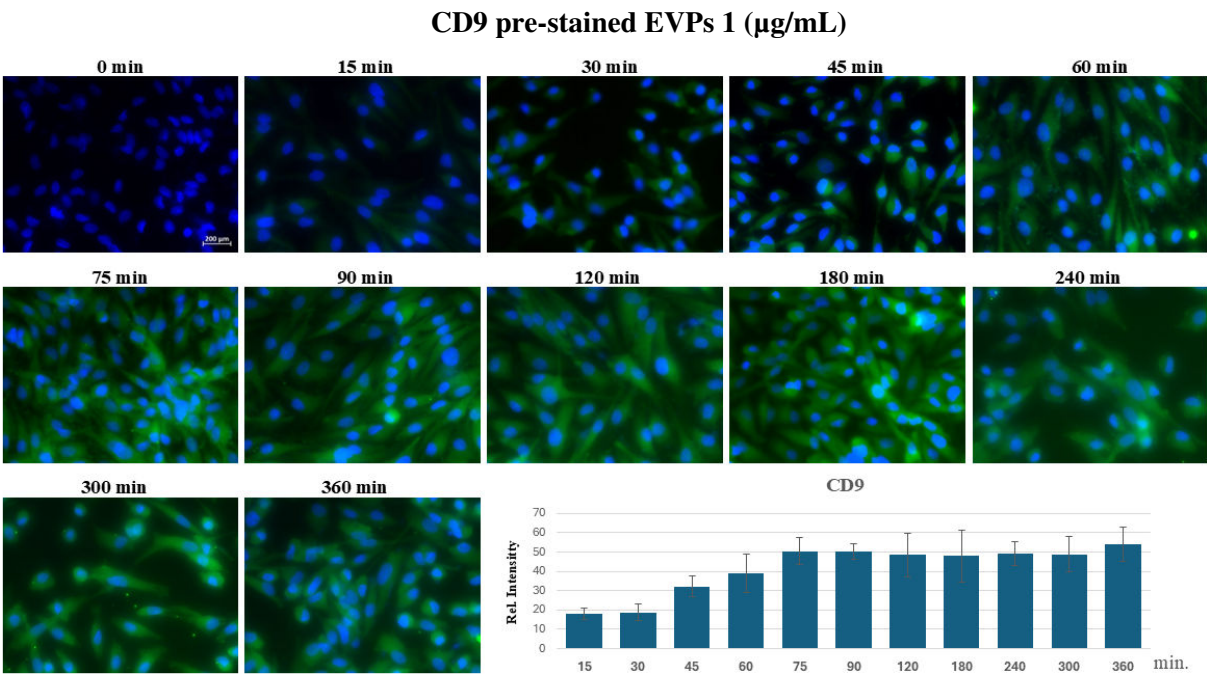
Because of lacking pre-stained EVPs, human umbilical vein endothelial cells were incubated with EVPs and stained. The control cells directly showed signals for all 3 exosomal markers. The signals increased during the 4 hours of incubation for both, non-induced and adipogenic-induced EVPs.

3.5.3 Determination of the Uptake Peak in Different Cell Types

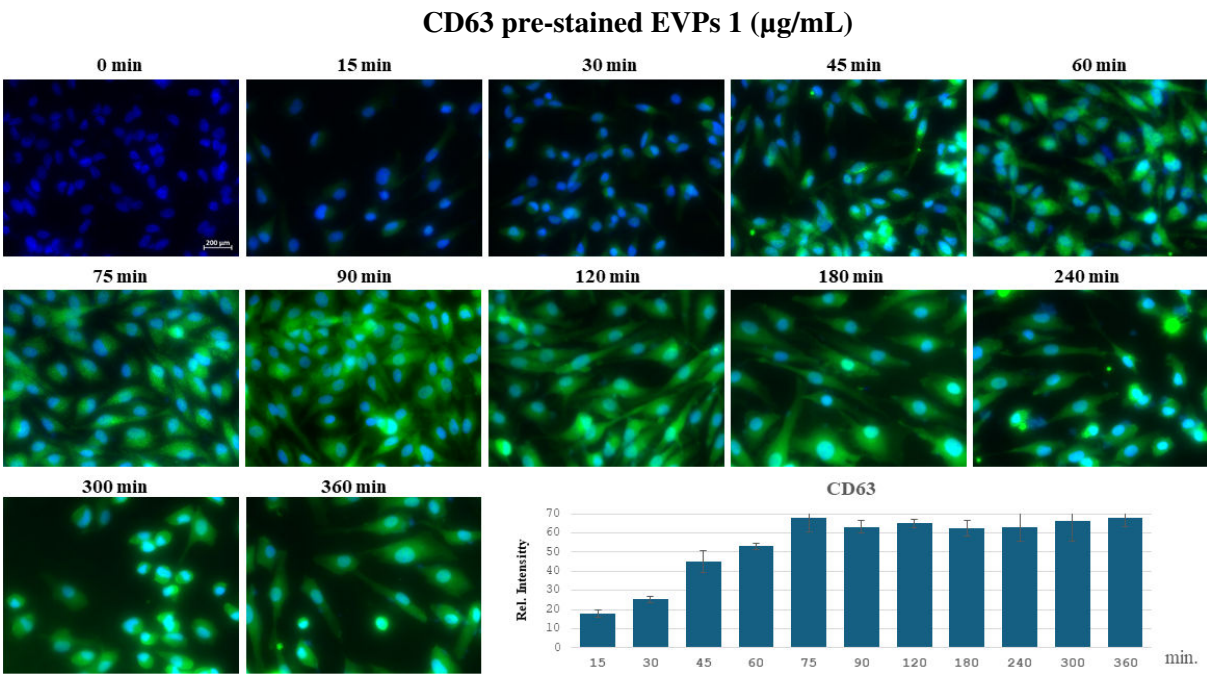
The results from sections 3.5.1 and 3.5.2 revealed that the uptake of EVPs took place in a time-frame between 0 h and 1 h and reached it's a maximum after 4 hours. To determine the starting time-point and the maximum uptake time-period PAECs and porcine smooth muscles cells (PSMCs) were incubated with pre-stained EVPs (CD9, CD63 6 CD81) for up to 6 h. The incubation was stopped after every 15 min within the first 2 hours and every hour (3 – 6 h) afterwards. For the uptake in PAECs it could be observed that within the first 30 min of incubation the signals for all 3 markers were very low. Increasing signals could

be detected after 45 min and reached a plateau after 75 min of incubation (Fig. 22).

A



B



C

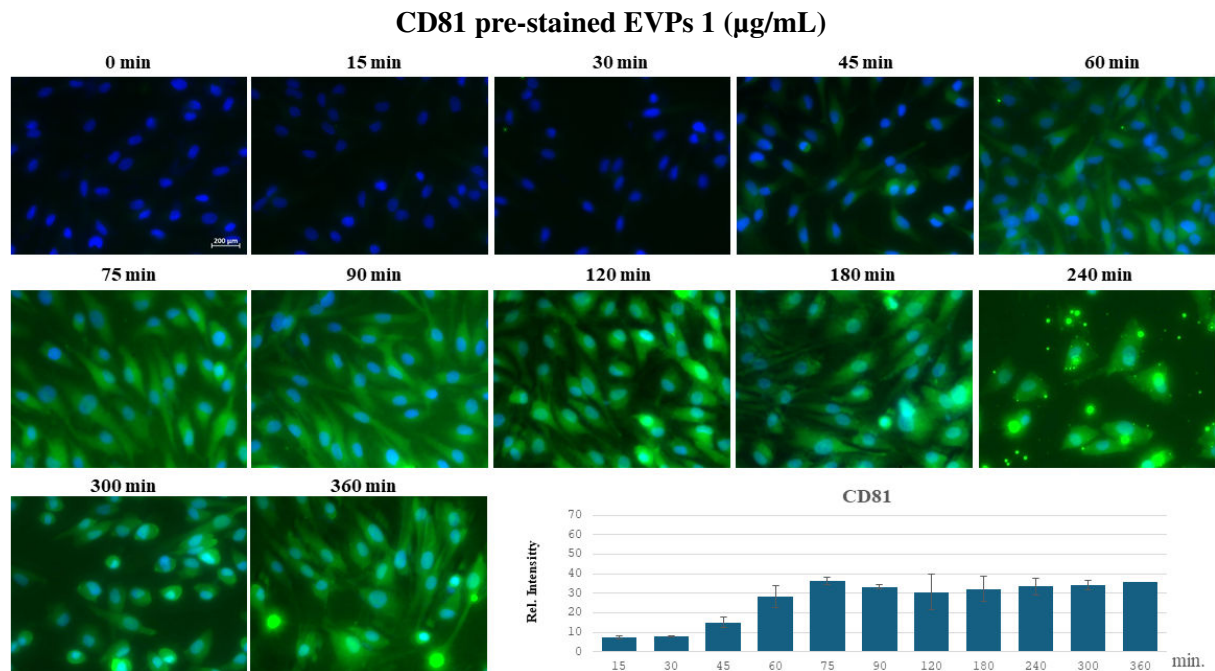
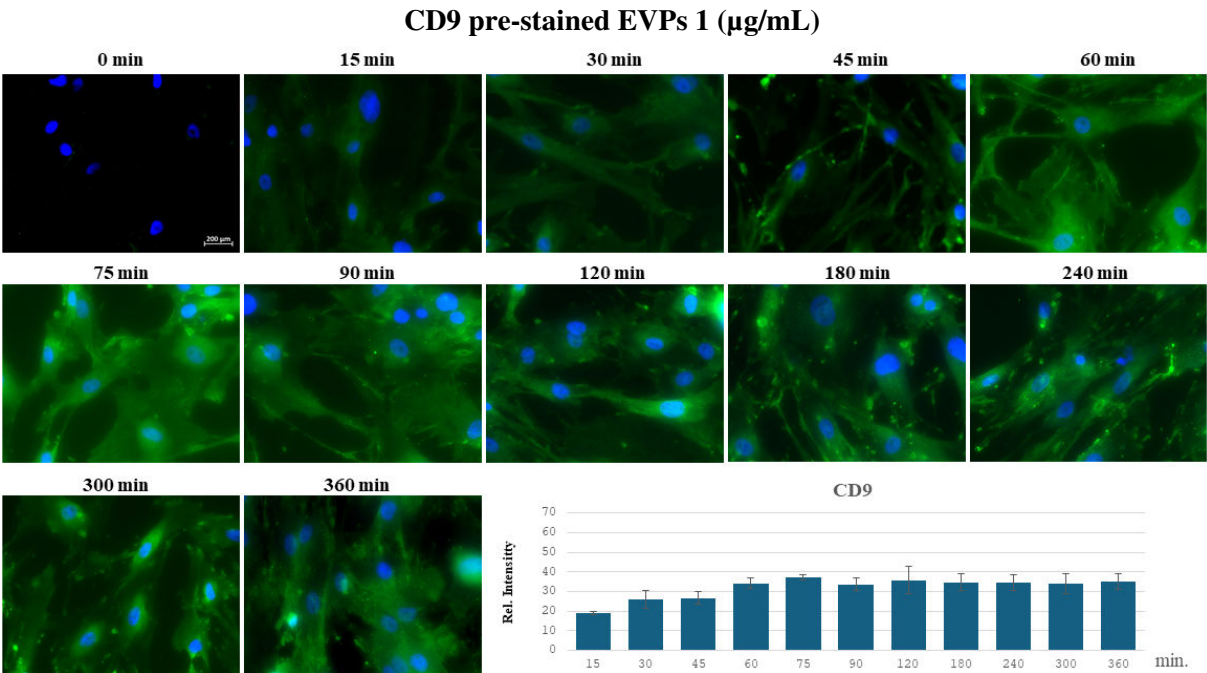


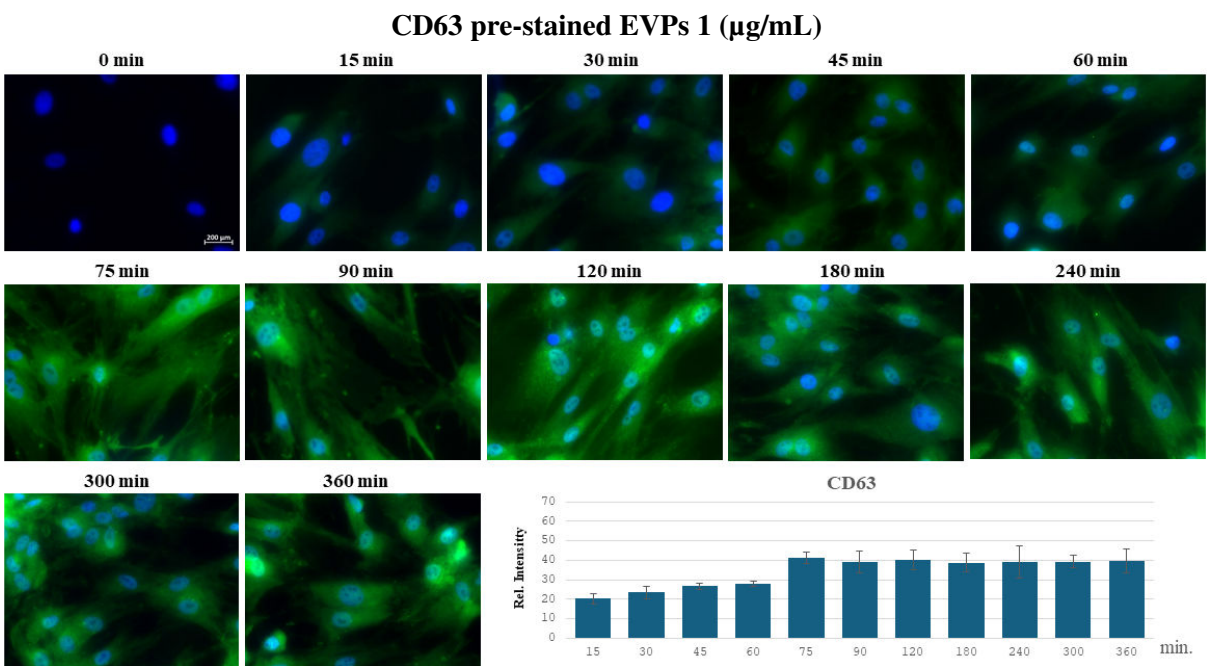
Fig. 22: Time-dependent Uptake of Pre-stained EVPs into/onto PAECs. PAECs were incubated with pre-stained (CD9, CD63 and CD81) EVPs from MSCs. After overnight incubation PAECs were washed with 1 x PBS and stained with fluorochrome-labeled secondary antibody (Alexa488, green). (A) CD9 (B) CD63 (C) CD81. Counter stain: DAPI (nucleus, blue).

For the uptake in PSMCs it could be observed that until 15 min of incubation the signals for all 3 markers were very low. Increasing signals were detected after 30 min and reached a plateau after 75 min of incubation (Fig. 23). The uptake into smooth muscle cells was 15 min faster than into endothelial cells.

A



B



C

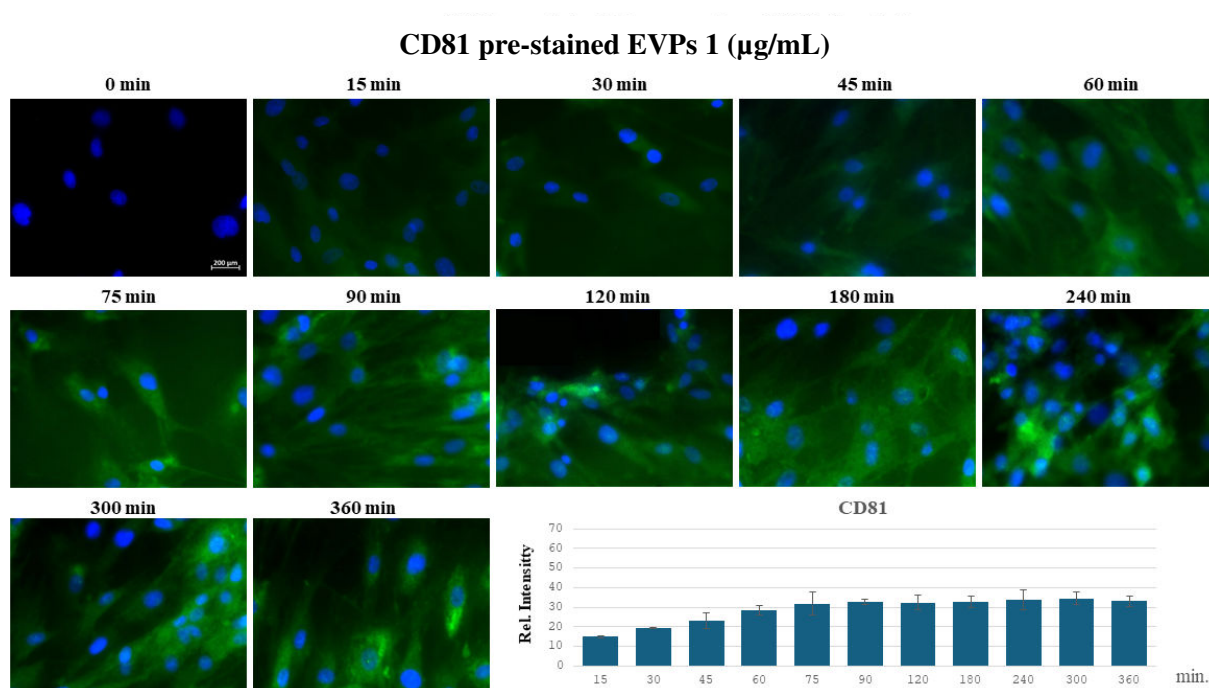


Fig. 23: Time-dependent Uptake of Pre-stained EVPs into/onto PSMCs. PSMCs were incubated with pre-stained (CD9, CD63 and CD81) EVPs from MSCs. After overnight incubation PSMCs were stained with the fluorochrome-labeled secondary antibody (Alexa488, green). (A) CD9 (B) CD63 (C) CD81. Counter stain: DAPI (nucleus, blue).

The immunofluorescence staining of exosome uptake experiments showed that all 3 exosomal markers could be detected on both, endothelial cell types (PAECs & HUVECs) and on smooth muscle cells (PSMCs) within 4 hours. The pre-stained exosomes could be detected on endothelial cells after 1 hour. The signals for all 3 markers increased during a time-frame of 4 h, but the CD81 signal was significantly higher when adipogenic-derived exosomes from FBS-depleted differentiations were applied to the cells. Experiments with PAEC and PSMC showed that the uptake of exosomes starts within 45 min on PAEC and within 30 min on PSMCs. The signal plateau was reached after 75 min of incubation for all 3 markers for both cell types.

3.6 Migration Assays

Migration Assays were performed in 24-well plates (handmade scratches with tips), 6 cm cell culture dishes (scratch machine), and Boyden chambers. The migration assays served as potency assays to investigate the impact of conditioned medium and EVPs on endothelial cell migration as a parameter for EC activation.

3.6.1 Scratch Assays with PAECs in Conditioned Medium

Supernatant from adipogenic differentiation of 3 donors was collected over a time period of 4 weeks. The supernatant was applied in a 1:1 mixture with fresh DMEM to confluent grown PAECs. Hydroxyurea (HU) was added to the medium to exclude proliferation effects. After overnight incubation and migration, the number of cells migrating over the scratch were counted.

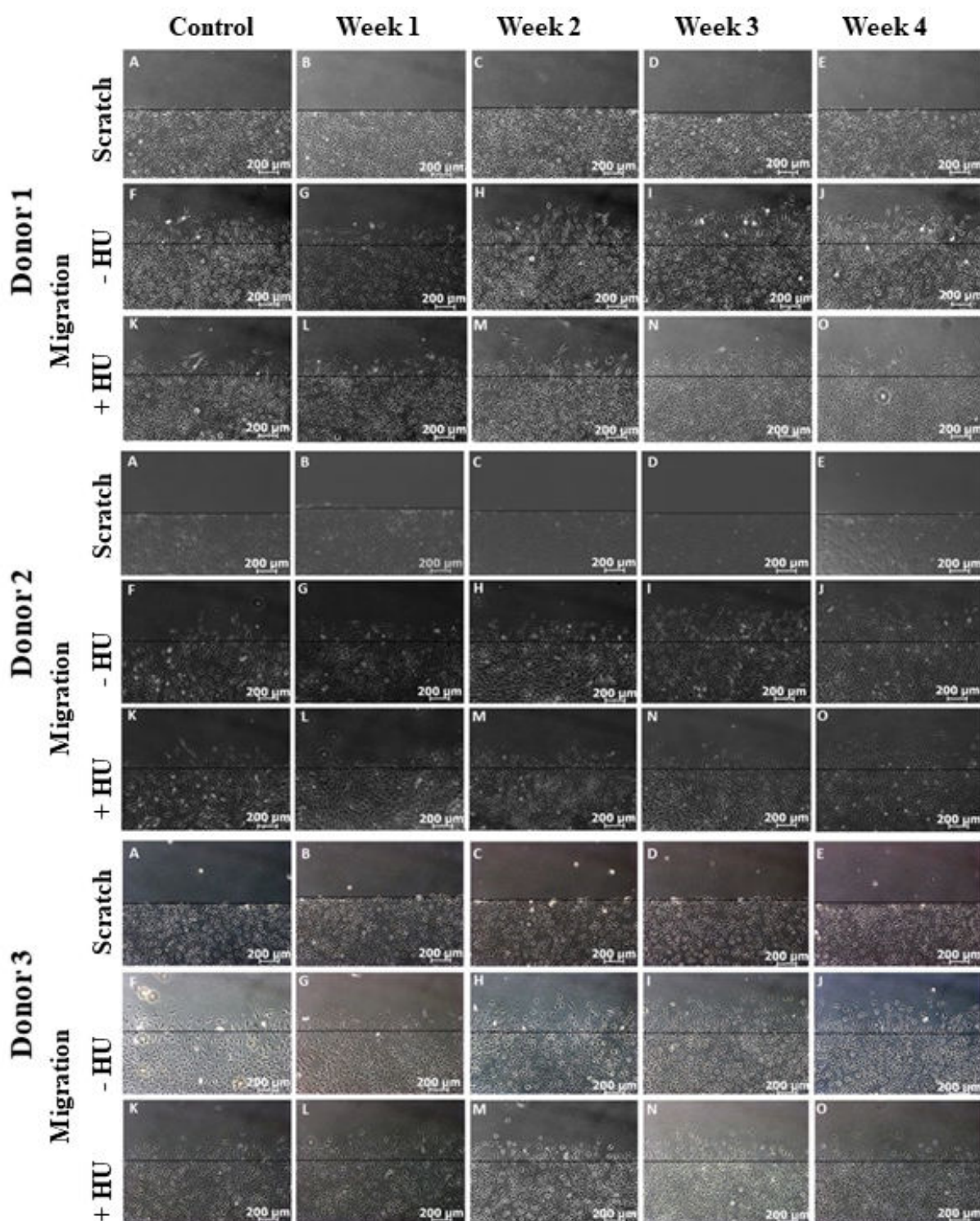


Fig. 24: Scratch Assays with PAECs in Conditioned Medium. Confluent grown were scratched and supernatant from adipogenic differentiation of 3 MSC donors was added. After 24 hours migrated cells were counted. Black line marks scratch. Migration of PAECs was highest in conditioned medium of week 3 and week 4. HU: hydroxyurea.

Microscope images showed that PAECs migrated in all 4 conditioned media from the 3 MSC donors. The addition of HU lowered the number of migrated cells for all conditions.

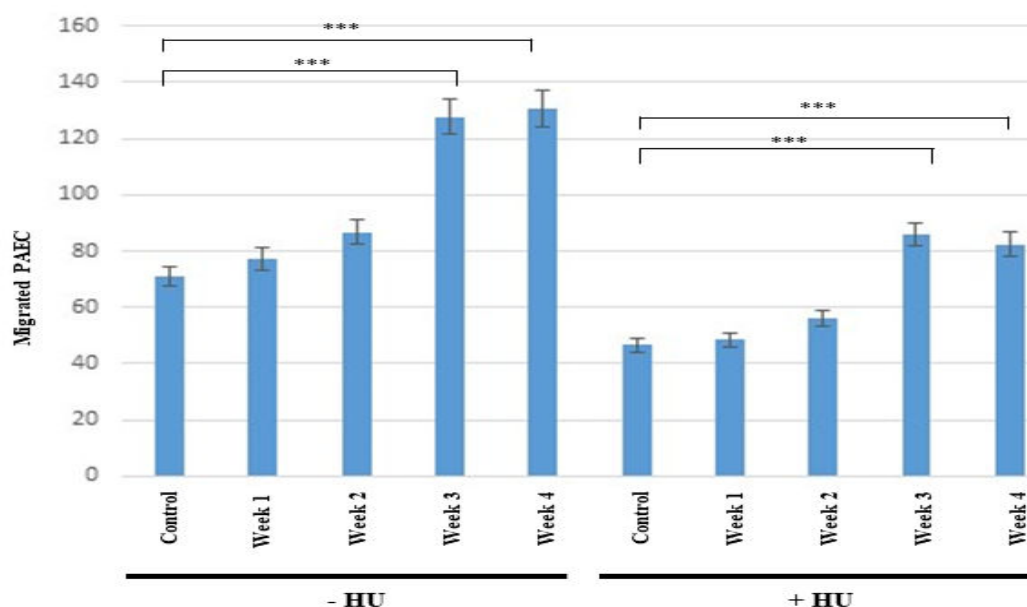


Fig. 25: Quantification of Scratch Assays with PAECs in Conditioned Medium. Migrated PAEC in conditioned medium for 4 weeks of adipogenic differentiation were counted. Proliferation was excluded by using hydroxyurea. Conditioned medium of week 3 and week 4 lead to the highest migration in both, conditioned medium with and without HU. HU: hydroxyurea.

The quantification of PAEC migration in conditioned medium showed that in week 3 and week 4 the highest migration can be seen with and without HU. These results confirm previous data of our lab (Lange, 2013). The number of migrated cells lowers when HU was added. To define the best tool for later collection of migrated cells for further investigations the data were compared with Boyden chamber assays.

3.6.2 Comparison Scratch Assay versus Boyden Assay

In section 3.6.1 the high migration rate of PAECs with conditioned medium was shown for week 3. Therefore, this medium was used for comparison of scratch

assays with Boyden chamber migration assays. Conditioned medium from week 3 of the adipogenic differentiation (W3) was compared with week 3 medium from non-induced MSCs (W3C) and culture medium in scratch assays and Boyden chambers.

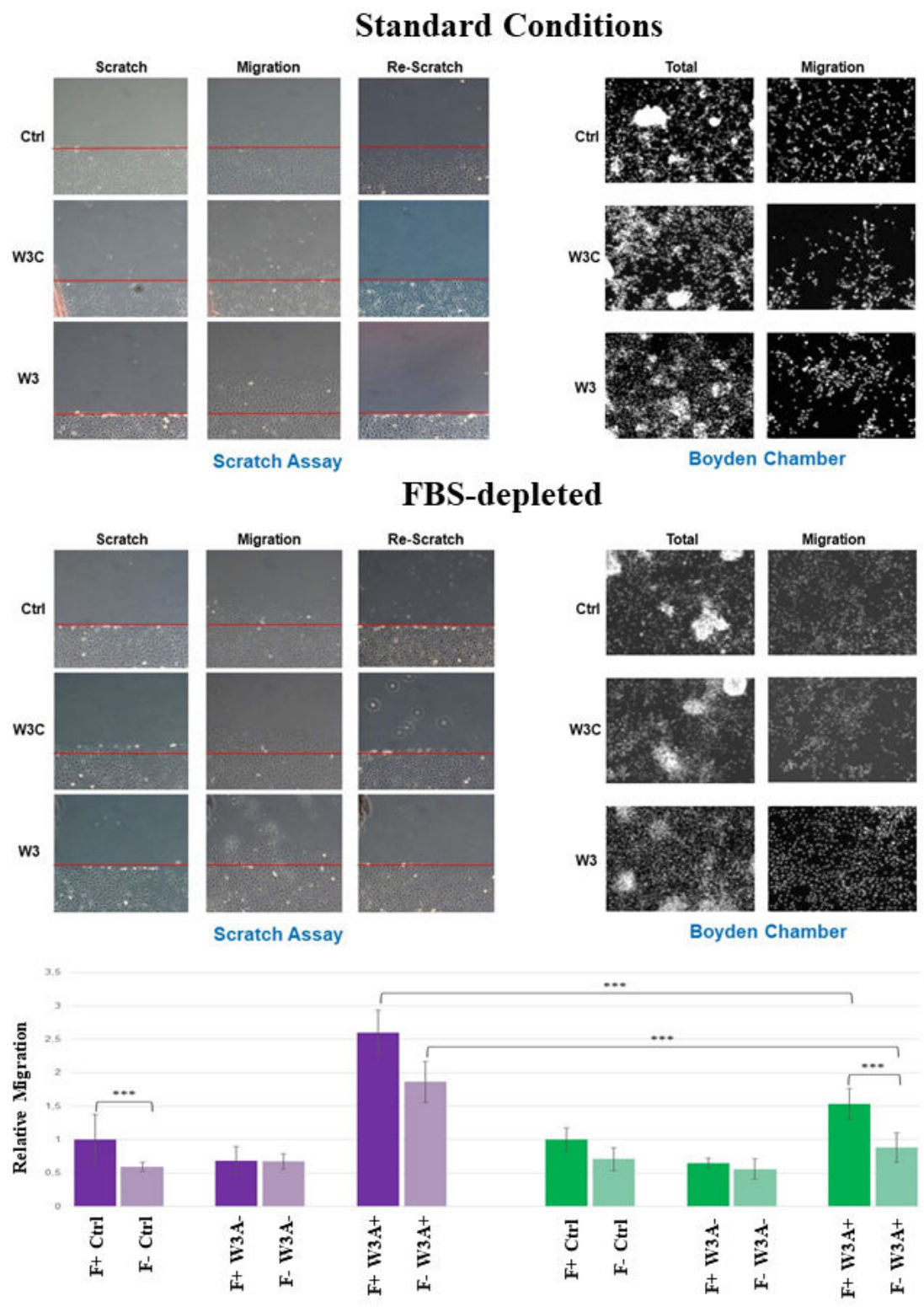


Fig. 26: Comparison of Scratch Assays versus Boyden Chamber Assays. Confluent grown PAECs were scratched and supernatant from week 3 of adipogenic differentiation of the donor (from standard conditions and from FBS-depleted conditions) was added. After 24 hours migrated cells were counted. In Boyden chambers conditioned medium was added to the bottom chamber as chemoattractant. Images for Boyden chamber assays were taken before swiping (total) and after swiping (migration) the DAPI stained membrane.

In both migration assays conditioned medium of week 3 from adipogenic induced MSCs had the highest impact on PAEC migration. EV reduced FBS medium decreased the migration of ECs in all conditions. The relative PAEC migration was higher in scratch assays compared to the migration in Boyden chambers.

3.6.3 Boyden Assay with Extracellular Vesicle Preparations Addition

The impact of EVPs on endothelial cell migration was initially investigated in Boyden chambers. MSC-derived EVPs were added either to the lower chamber or to the PAECs in the top chamber. The medium in both chambers was always EV-reduced FBS DMEM to exclude a bovine exosome impact. The addition of EVPs into the top chamber lead to higher migration rates than adding EVPs into the bottom chamber. With increasing EVPs concentration clumping of PAECs occurred.

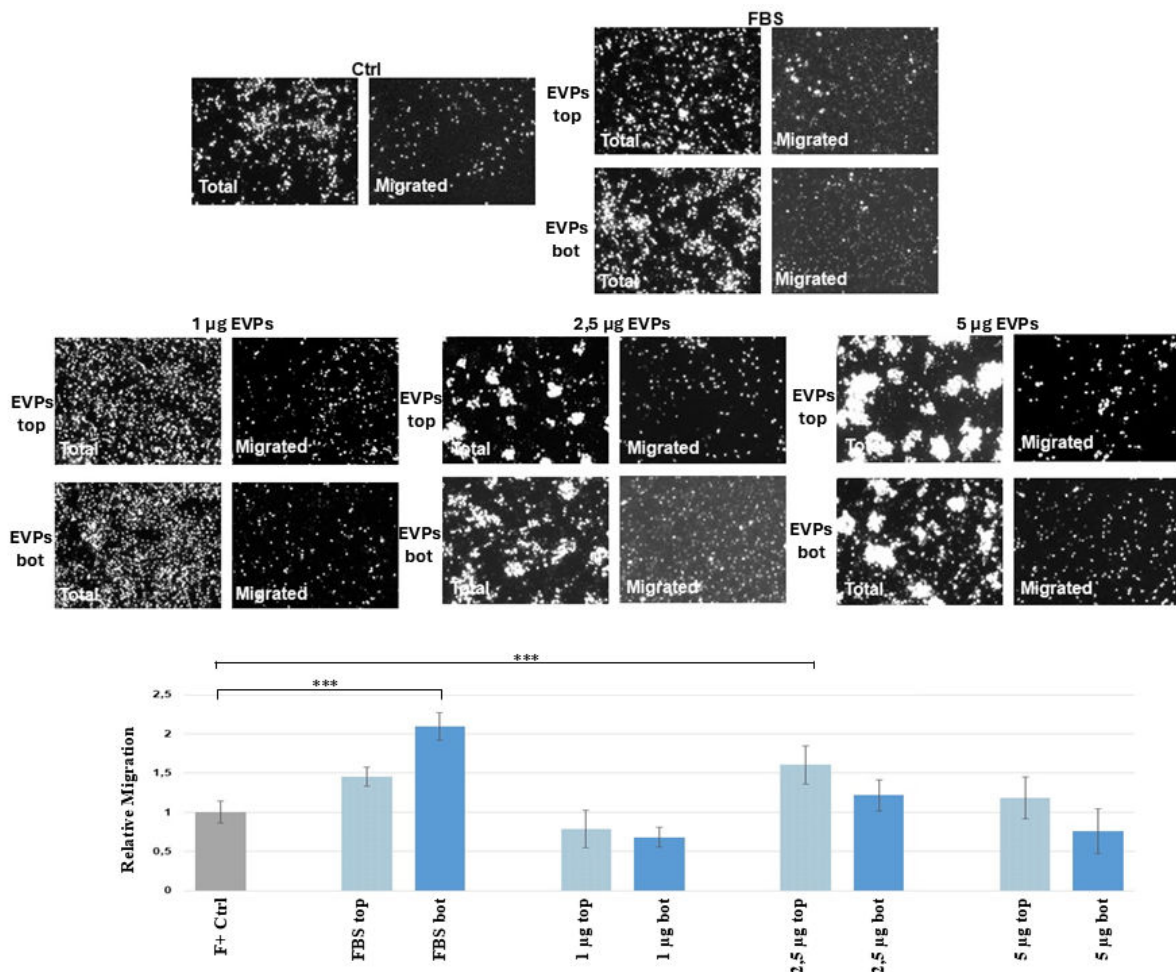


Fig. 27: Boyden Chamber Assay with PAECs and Addition of EVPs. EVPs in different concentrations were added either to the medium in the bottom chamber (bot) or to the cell suspension in the top chamber (top). After migration, membranes were stained with DAPI and migrated cells were counted. Images for Boyden chamber assays were taken before swiping (total) and after swiping (migration) the DAPI stained membrane. Exo top: addition of EVPs in top chamber; Exo bot: addition of EVPs in bottom chamber.

The addition of 1 µg/mL EVPs showed no clumping of the cells. The addition of 2,5 µg/mL and 5 µg/mL lead to increased clumping of PAECs. FBS within the bottom chamber as chemoattractant showed the highest migration rate of PAECs. Due to the clumping effect, the following scratch assays were performed in 24 well plates for screening assays and in 6 cm culture dishes for collection of migrated cells.

3.6.4 Scratch Assay in 24-Well Plates

Due to the high number of conditions (see table 9) and the low volume of medium and EVPs (500 μ L – 1000 μ L), the screening scratch assays were performed in 24 well plates. For each donor the medium and EVPs (1 μ g/mL & 2 μ g/mL) from both, standard conditions and FBS-depleted conditions, was applied to PAEC scratch assays in triplicates. Images of the wells were taken directly after the scratch and after incubation. The total number of migrated cells into the scratch area of a whole image was counted by using NIH Image J software.

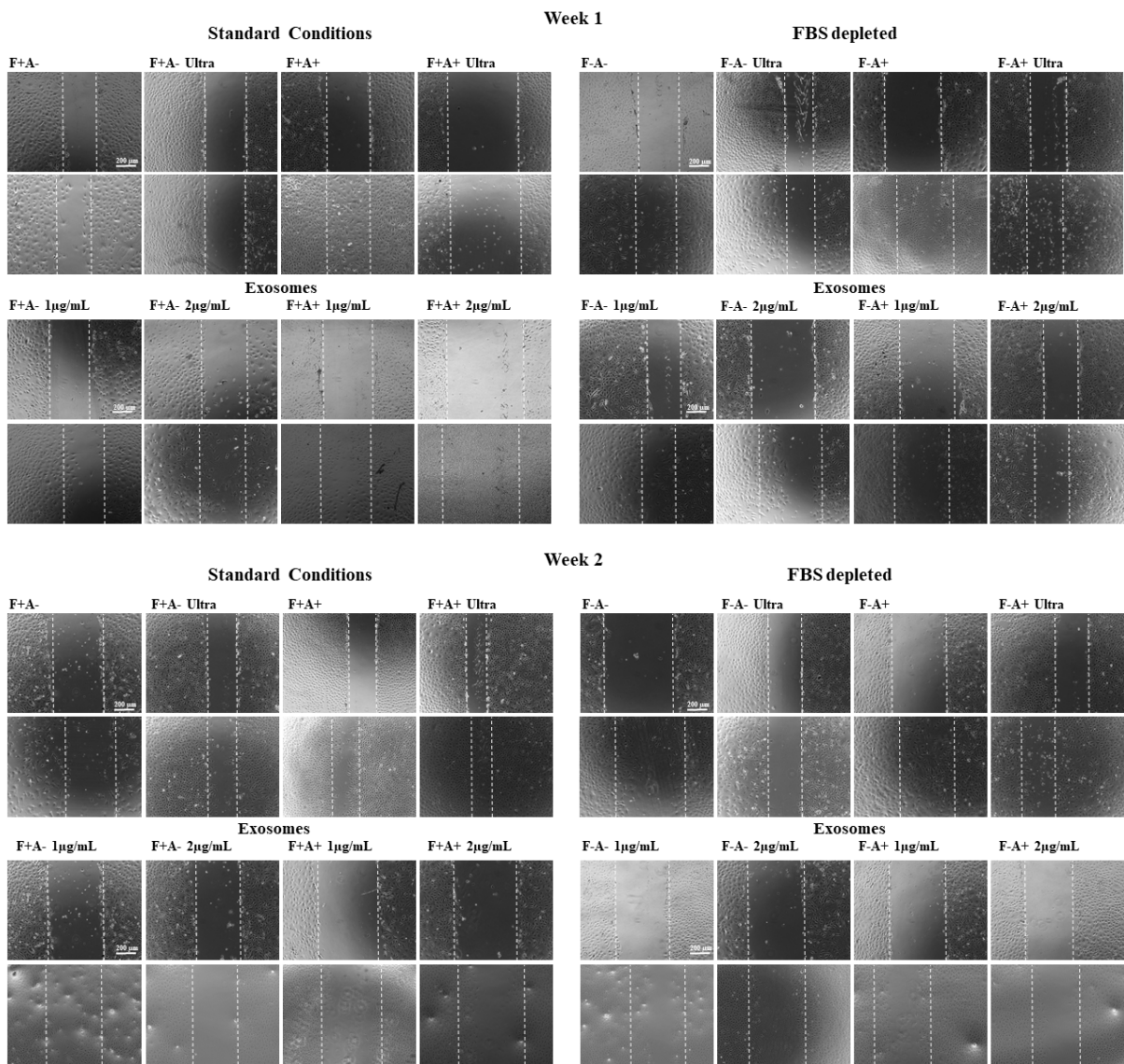
Tab. 9: Calculated Number of Wells for Scratch Conditions in 24-Well Plates

Donor 1	Standard Conditions								FBS-depleted								
Week	F+A-	F+A- Ultra	F+A+	F+A+ Ultra	F+A- EVPs 1 μ g/ml	F+A- EVPs 2 μ g/ml	F+A+ EVPs 1 μ g/ml	F+A+ EVPs 2 μ g/ml	F+A-	F+A- Ultra	F+A+	F+A+ Ultra	F+A- EVPs 1 μ g/ml	F+A- EVPs 2 μ g/ml	F+A+ EVPs 1 μ g/ml	F+A+ EVPs 2 μ g/ml	
1	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	
2	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	
3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	
4	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	
Total Wells:								96	Total Wells:								96
Donor 2	Standard Conditions								FBS-depleted								
Week	F+A-	F+A- Ultra	F+A+	F+A+ Ultra	F+A- EVPs 1 μ g/ml	F+A- EVPs 2 μ g/ml	F+A+ EVPs 1 μ g/ml	F+A+ EVPs 2 μ g/ml	F+A-	F+A- Ultra	F+A+	F+A+ Ultra	F+A- EVPs 1 μ g/ml	F+A- EVPs 2 μ g/ml	F+A+ EVPs 1 μ g/ml	F+A+ EVPs 2 μ g/ml	
1	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	
2	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	
3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	
4	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	
Total Wells:								96	Total Wells:								96
Donor 3	Standard Conditions								FBS-depleted								
Week	F+A-	F+A- Ultra	F+A+	F+A+ Ultra	F+A- EVPs 1 μ g/ml	F+A- EVPs 2 μ g/ml	F+A+ EVPs 1 μ g/ml	F+A+ EVPs 2 μ g/ml	F+A-	F+A- Ultra	F+A+	F+A+ Ultra	F+A- EVPs 1 μ g/ml	F+A- EVPs 2 μ g/ml	F+A+ EVPs 1 μ g/ml	F+A+ EVPs 2 μ g/ml	
1	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	
2	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	
3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	
4	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	
Total Wells:								96	Total Wells:								96

The pictures below show scratch (upper rows) and migration (lower rows) of PAEC after addition of conditioned medium and EVPs from donor 1. Scratch assays showed that ultra-centrifuged medium from each week resulted in a lower migration rate of PAEC.

During the first two weeks under standard conditions, the migration rate of PAECs dropped significantly following ultra-centrifugation. The reduction was more pronounced in the adipogenic-induced medium, where migration rates decreased by up to 61%, compared to a 51% drop in non-induced medium. This initial reduction highlights the impact of ultra-centrifugation on the cellular

environment, possibly due to the removal of certain factors that support cell migration.



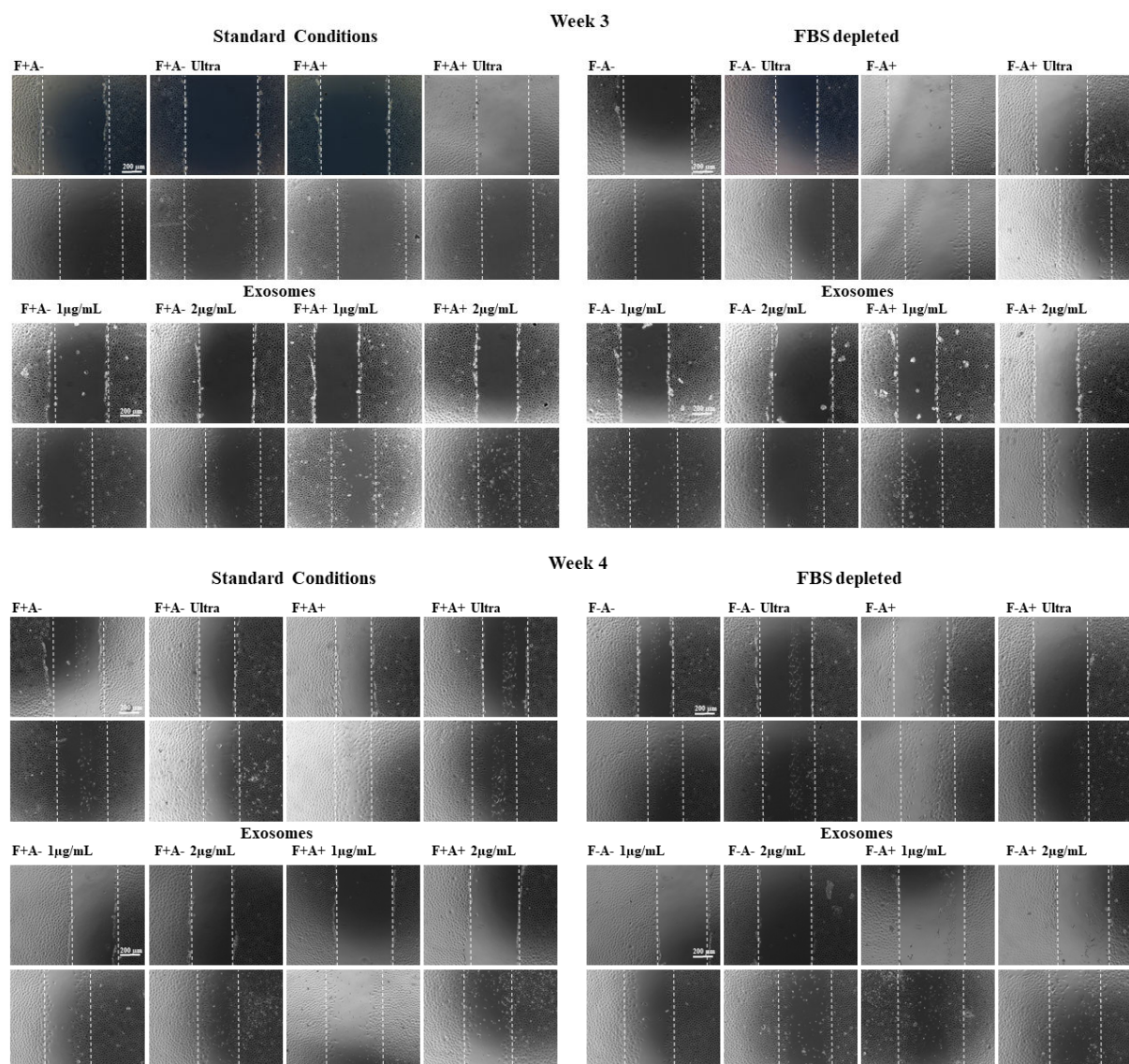


Fig. 28: 24-Well Scratch Assay with PAECs in Conditioned Medium and Addition of EVPs. Confluent grown PAECs in 24 wells were scratched and supernatant or EVPs from each week of adipogenic differentiation of donor 1 (mixture 1:1 with fresh DMEM) was added. After 24 hours migrated cells were counted. Scratches were performed with medium from standard conditions and FBS-depleted conditions. Ultra: ultra-centrifuged supernatant (without EVs). Dashed lines indicated original scratch. Top row: after scratch; bottom row: after migration. (The data shown are representative for the tested donors).

By the third week, the difference in migration rates between centrifuged and non-centrifuged media diminished to 12% compared to the control, indicating a partial recovery or adaptation of the cells to the altered medium. However, by the fourth week, conditioned media, whether centrifuged or not, universally reduced PAEC migration rates. This suggests that over time, the medium's ability to support

migration wanes, potentially due to the depletion of essential migratory cues or the accumulation of inhibitory factors.

The addition of adipogenic EVPs from weeks 3 and 4 had a significant impact on PAEC migration. When 1 $\mu\text{g/mL}$ of EVPs was added, PAEC migration increased by up to 57% in week 3 and 51% in week 4. This effect was even more pronounced with a higher concentration (2 $\mu\text{g/mL}$), which resulted in an 82% increase in migration by week 4. These findings suggest that EVPs, particularly those from adipogenic-induced MSCs, contain factors that robustly stimulate PAEC migration.

Interestingly, EVPs from undifferentiated MSCs from donor 1 also promoted PAEC migration in weeks 3 and 4, indicating that not only adipogenic but also undifferentiated MSCs release EVPs capable of enhancing migration. This points to a potentially broad application of MSC-derived EVPs in promoting endothelial cell migration, a key process in tissue repair and regeneration.

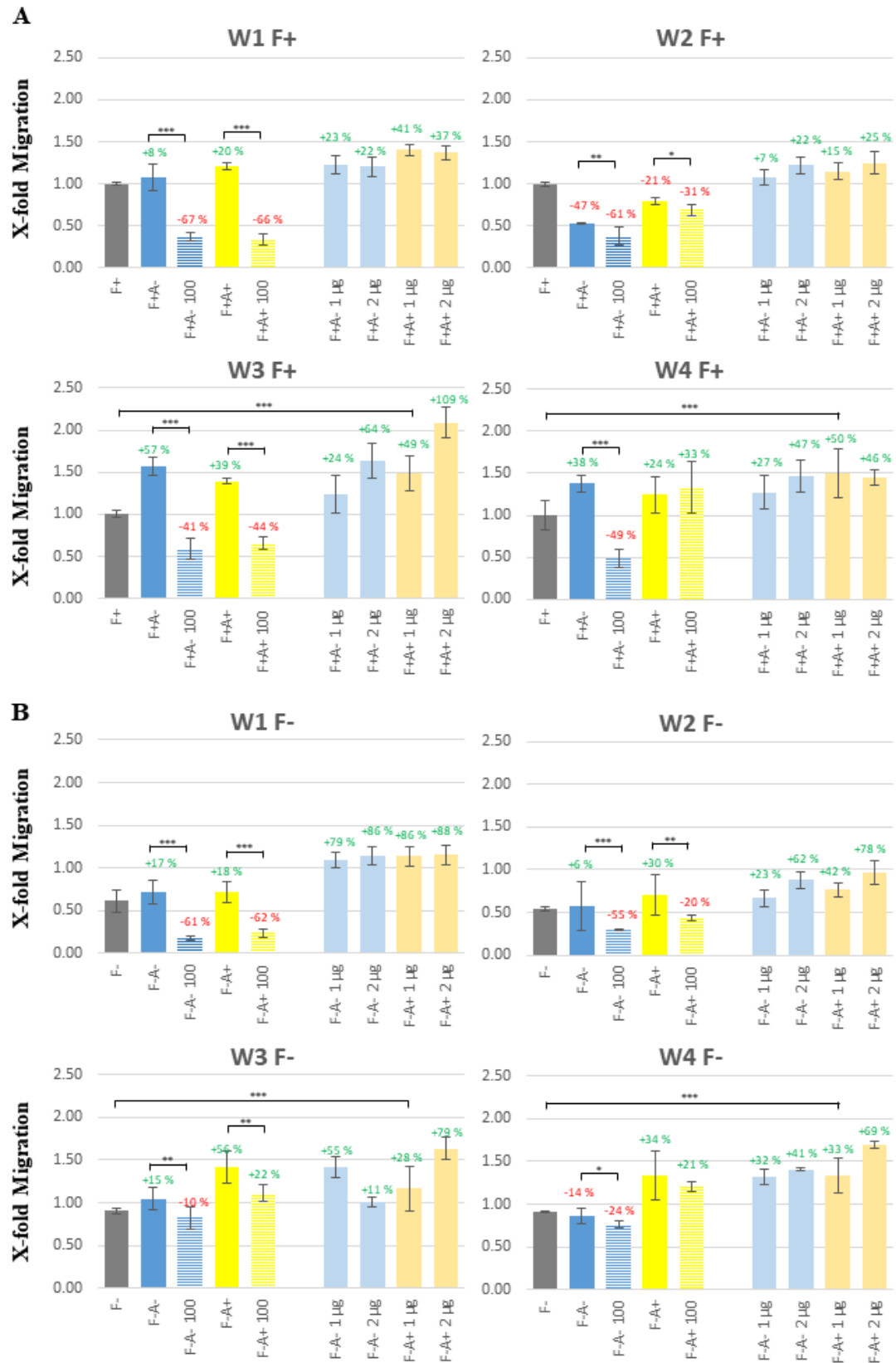


Fig. 29: Quantitative Analysis of Scratch Assays of MSC Donor 1 in 24-Well Plates. Migrated cells under different conditions were counted and compared to the controls under (A) standard conditions and (B) FBS-depleted conditions. Numbers give increase/decrease of migration in %. Significance: * < 0,05; ** < 0,01, *** < 0,005.

Under FBS-depleted conditions, PAEC migration exhibited a similar pattern to that observed under standard conditions, though with some variations in magnitude. During the first two weeks, the migration rate decreased by up to 40% in non-induced medium and 29% in adipogenic-induced medium after ultra-centrifugation. As in the standard conditions, the difference between centrifuged and non-centrifuged media decreased by week 3.

In the fourth week, conditioned media again reduced migration across all conditions. However, the addition of adipogenic EVPs continued to have a stimulatory effect on migration, though the increases were slightly lower than under standard conditions. Specifically, 1 µg/mL of EVPs raised migration by 30% in week 3 and 44% in week 4, while 2 µg/mL increased it by 62% and 82%, respectively. These results reinforce the idea that EVPs can counteract some of the negative effects of ultra-centrifugation and support PAEC migration even under nutrient-depleted conditions.

Again, EVPs from undifferentiated MSCs from donor 1 were effective in promoting migration during weeks 3 and 4, underscoring the consistency of this effect across different MSC sources.

To further validate these findings, scratch assays were performed, which visually confirmed that ultra-centrifuged media resulted in lower PAEC migration rates compared to non-centrifuged media. The addition of conditioned media and EVPs from different donors (donors 2 and 3) consistently increased migration, although the specific percentages varied depending on the donor and the week.

For instance, under standard conditions, migration rates during the first three weeks dropped by up to 67% in non-induced medium and 66% in adipogenic-induced medium after ultra-centrifugation. By week 3, the difference between centrifuged and non-centrifuged media reached its peak, but the addition of adipogenic-conditioned medium from week 4 significantly increased PAEC migration.

The EVPs from donor 2, when added at 1 $\mu\text{g/mL}$, increased PAEC migration by 57% and 51% in weeks 3 and 4, respectively, while 2 $\mu\text{g/mL}$ led to increases of 39% and 82%. Similarly, EVPs from undifferentiated MSCs from donor 2 also promoted migration in weeks 3 and 4.

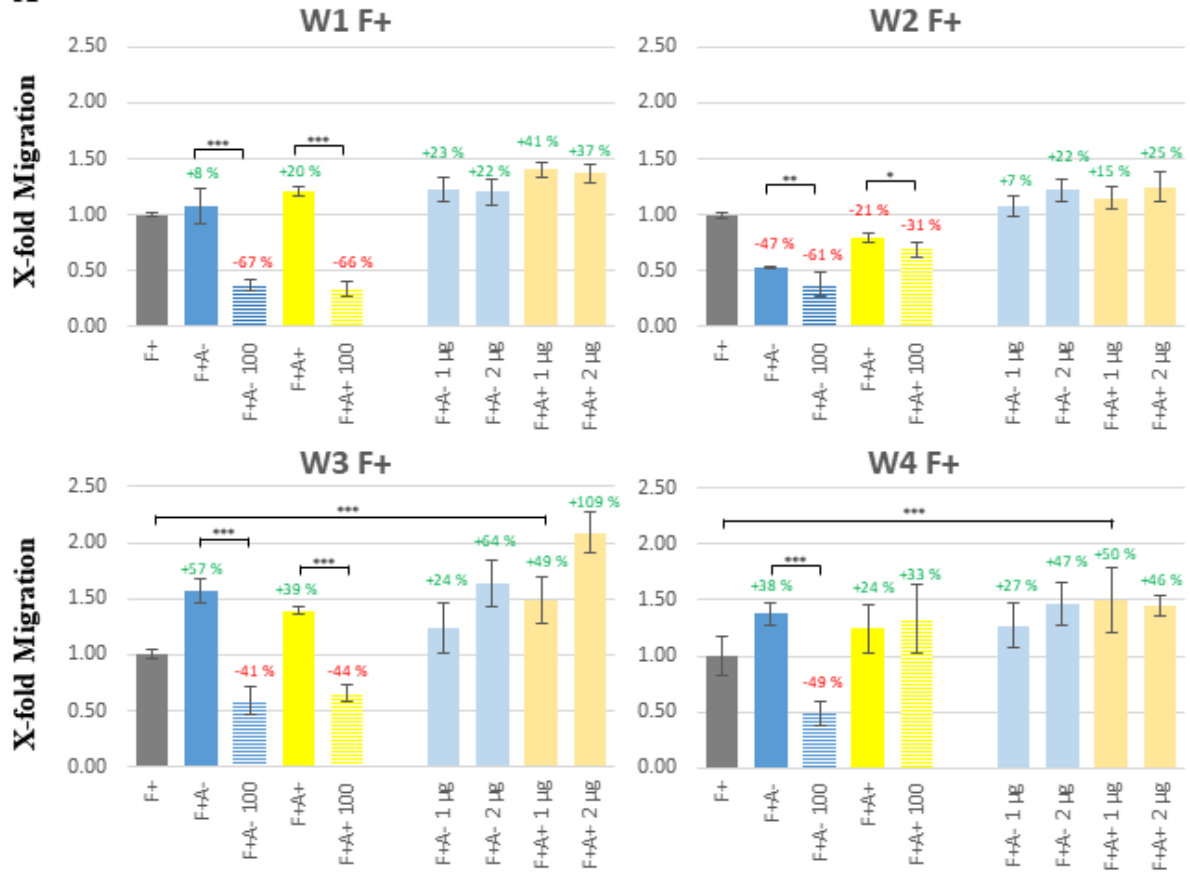
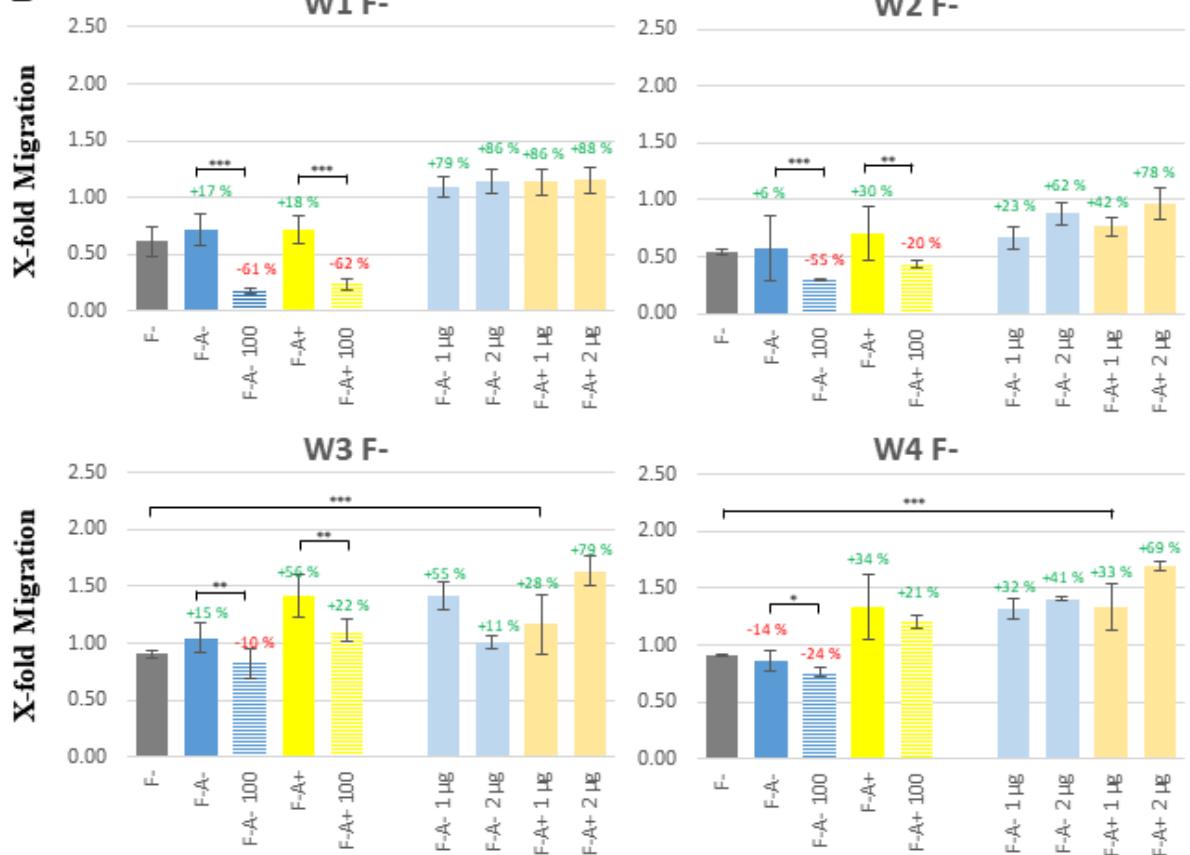
A**B**

Fig. 30: Quantitative Analysis of Scratch Assays of MSC Donor 2 in 24-Well Plates. Migrated cells under different conditions were counted and compared to the controls under (A) standard conditions and (B) FBS-depleted conditions. Numbers give increase/decrease of migration in %. Significance: * < 0,05; ** < 0,01, *** < 0,005.

Under FBS-depleted conditions, PAEC migration showed a slightly different pattern. While the first week's migration was increased by all conditioned media, only non-centrifuged medium led to a significant increase in week 2, with migration rates rising by up to 56% in non-induced and 67% in adipogenic-induced medium. The third week again saw an increase in migration across all conditions, though in the fourth week, only the non-centrifuged medium enhanced migration.

EVPs from donor 3 also demonstrated effectiveness in promoting PAEC migration under both standard and FBS-depleted conditions. The addition of 1 µg/mL adipogenic EVPs increased migration by up to 63% in week 3 and 9% in week 4 under standard conditions, with a more modest increase of 16% and 40% under FBS-depleted conditions. Higher concentrations (2 µg/mL) resulted in increases of up to 31% in week 3 and 35% in week 4 under standard conditions, and 28% and 54% under FBS-depleted conditions.

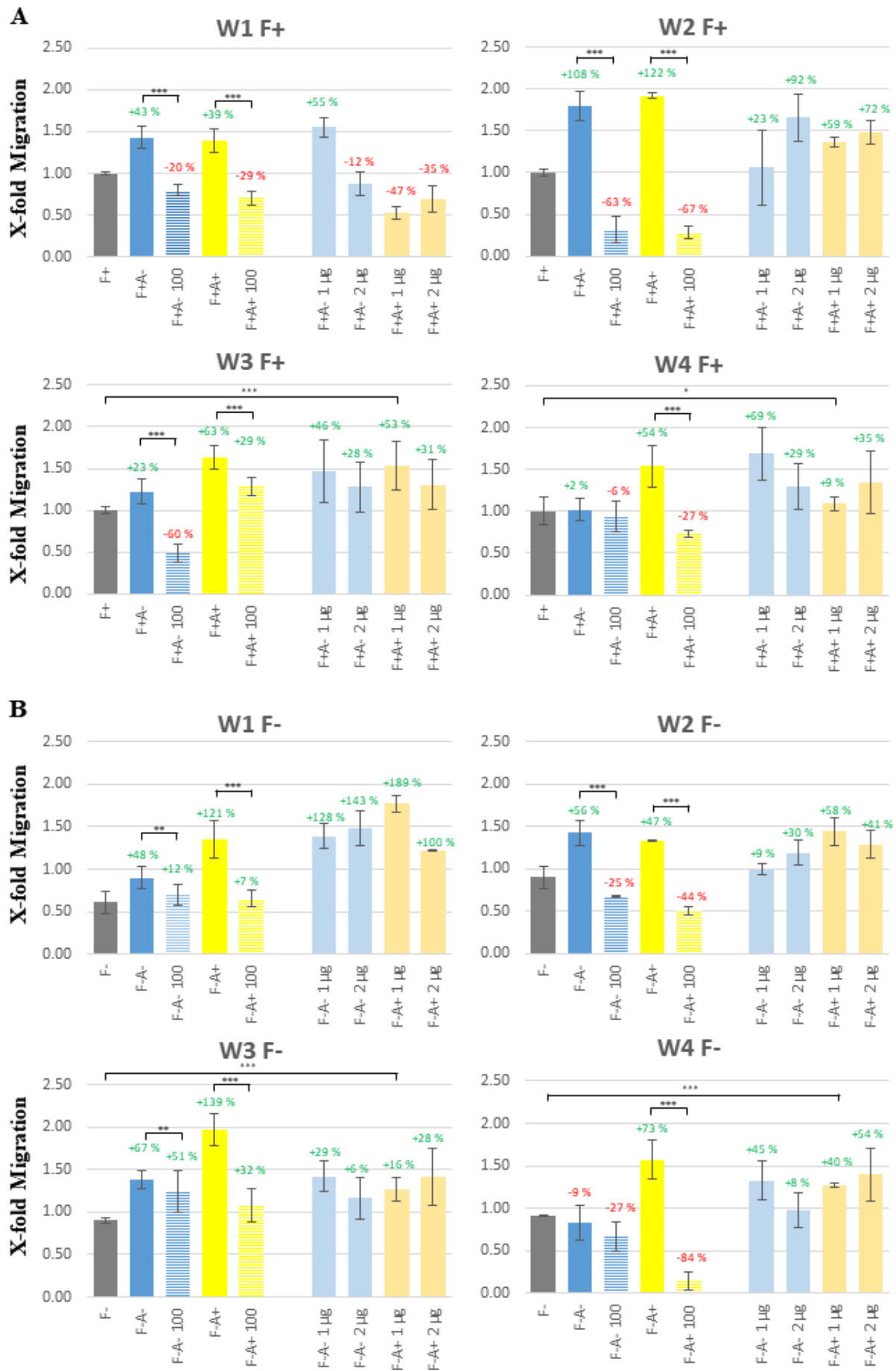


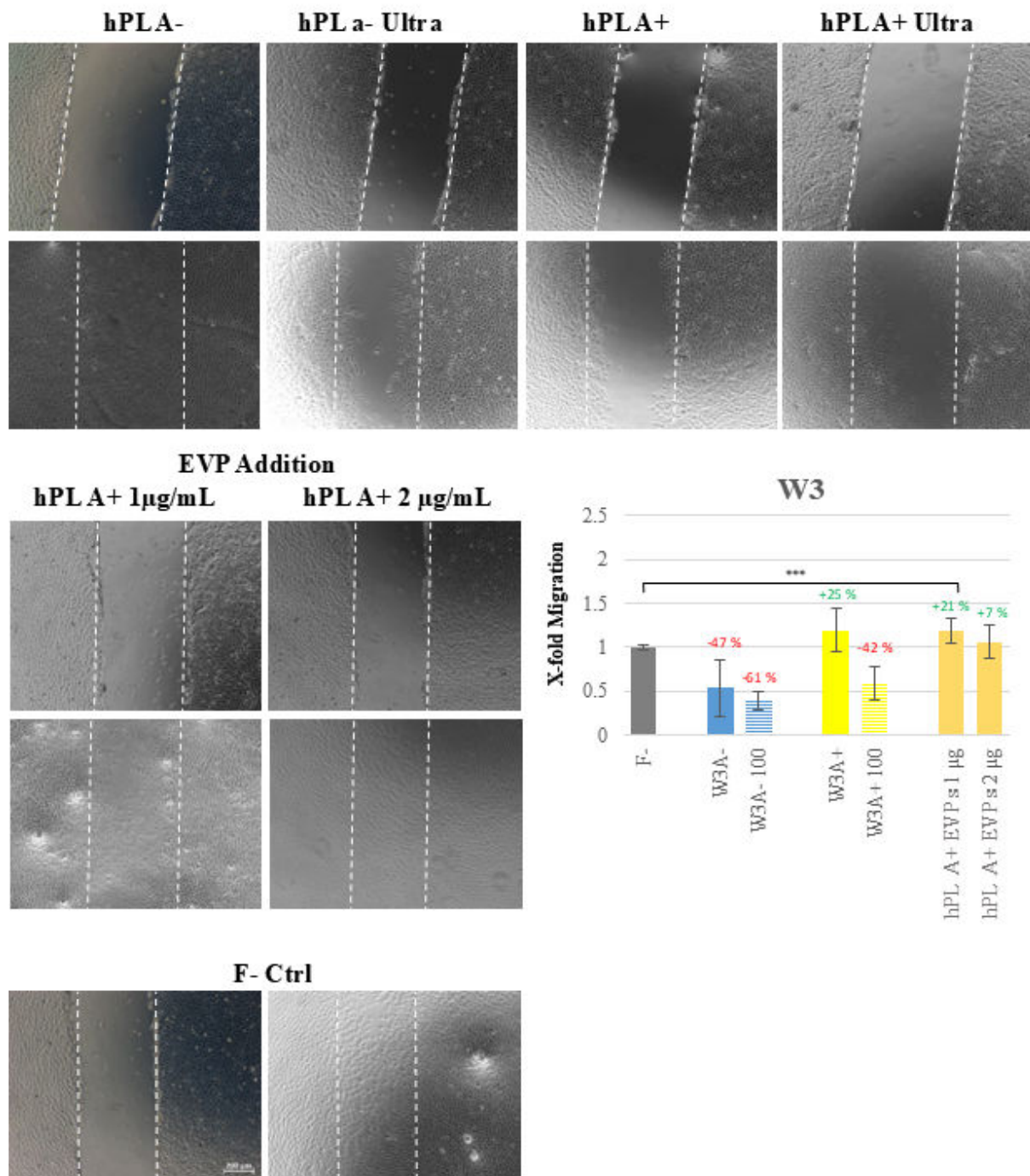
Fig. 31: Quantitative Analysis of Scratch Assays of MSC Donor 3 in 24-Well Plates. Migrated cells under different conditions were counted and compared to the controls under standard conditions (A) and FBS-depleted conditions (B). Numbers give increase/decrease of migration in %. Significance: * < 0,05; ** < 0,01, *** < 0,005.

In summary, the addition of adipogenic EVPs from week 3 and week 4 increased EC migration significantly, while ultra-centrifuged conditioned medium lead to a significant decrease of migration. A correlation between the difference of EVP concentration was not observed.

3.6.5 Scratch Assay with Supernatant from Platelet Lysate Supplement

To confirm the impact of MSC-derived EVPs, adipogenic differentiations were also performed without FBS. Human platelet lysate was added to control medium and adipogenic differentiation medium (3% each) instead of FBS. The supernatant of week 3 and week 4 was used in scratch assays in 24-well plates.

A



B

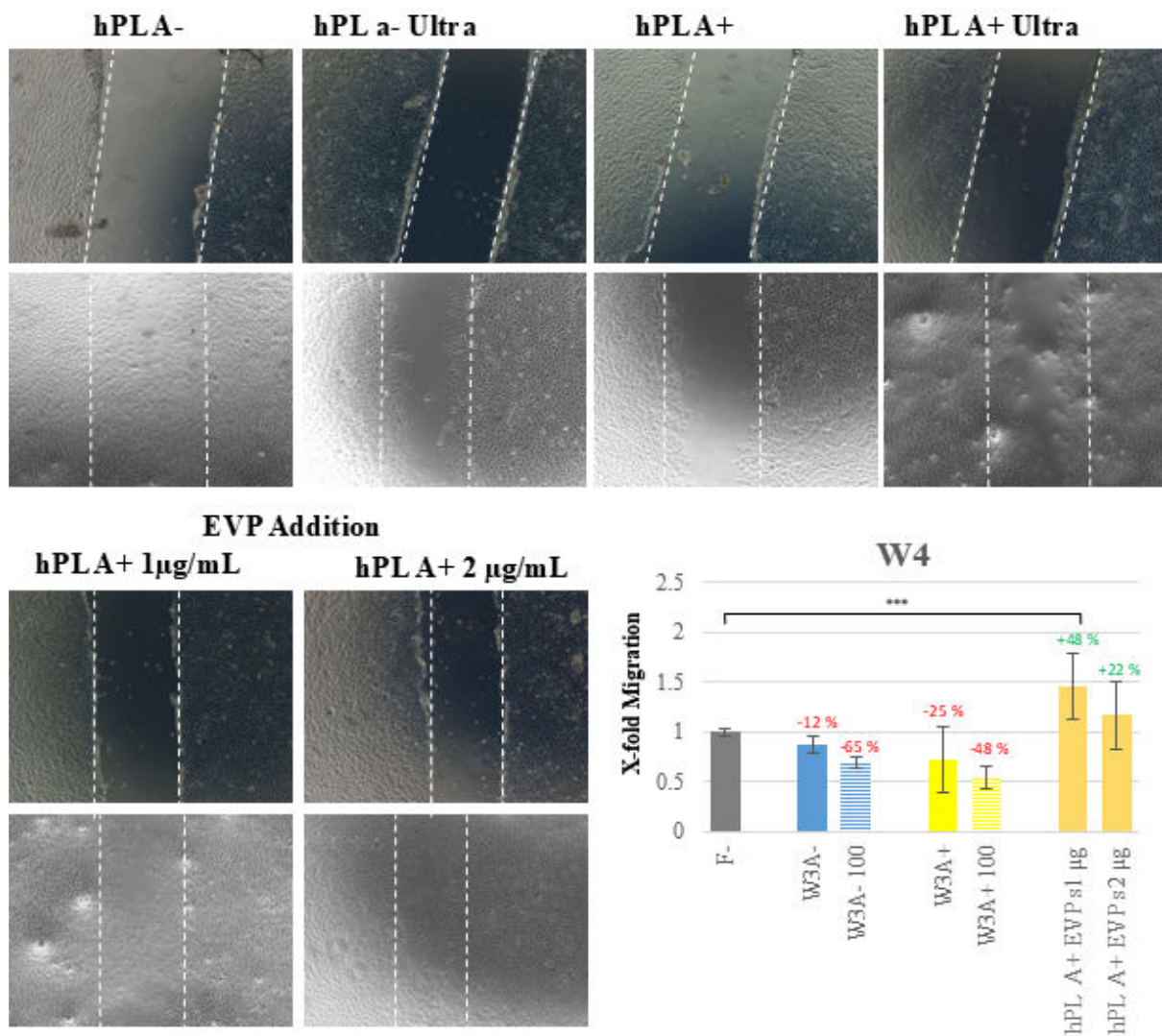
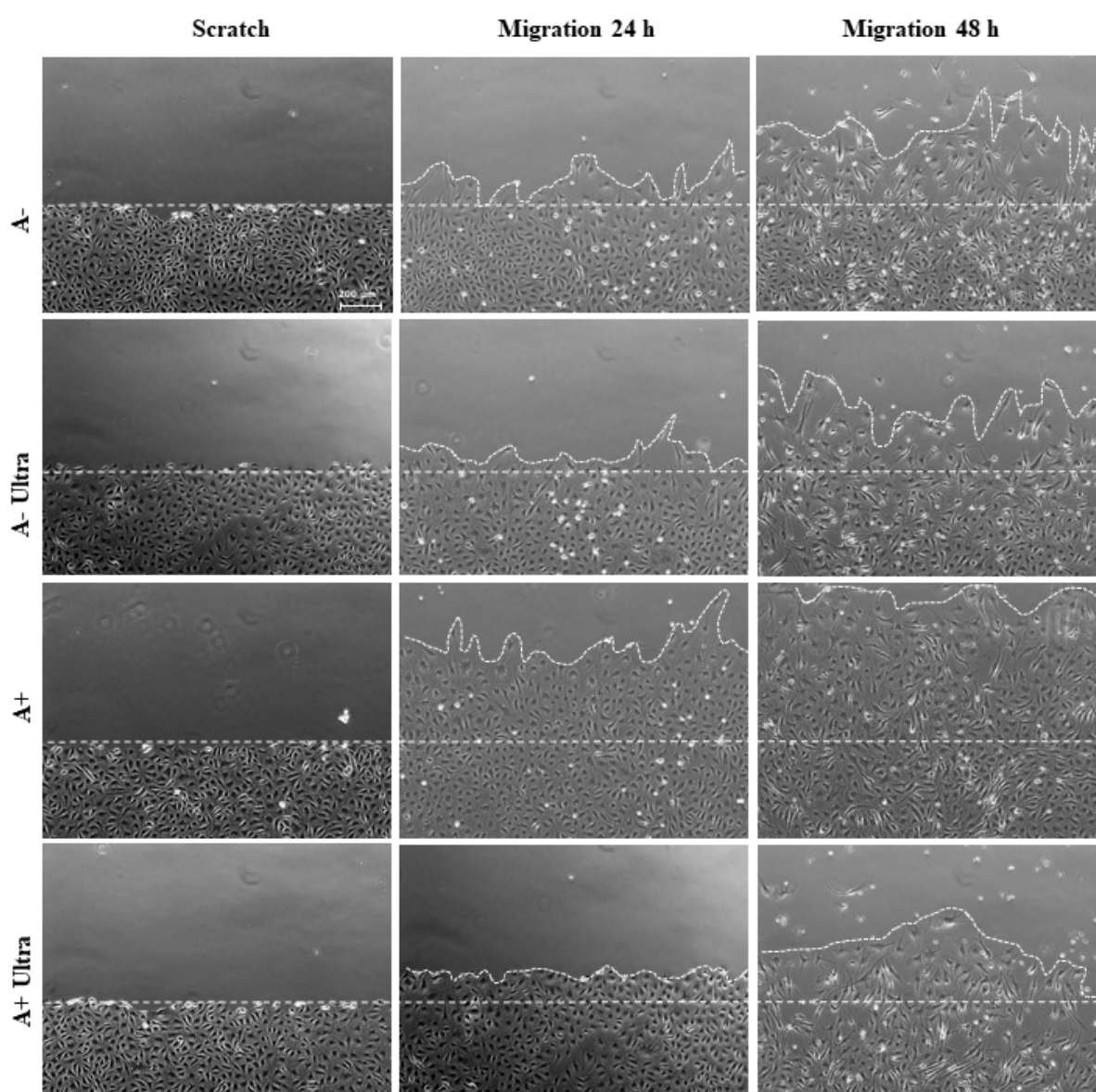


Fig. 32: Scratch Assay with PAECs in Conditioned Medium and Addition of EVPs from Platelet Lysate. Confluent grown PAECs in 24 wells were scratched and supernatant or EVPs from week 3 (A) and week 4 (B) of adipogenic differentiation in hPL supplemented medium (mixture 1:1 with fresh DMEM) was added. After 24 hours migrated cells were counted. Scratches were performed with medium from standard conditions and FBS-depleted conditions. Ultra: ultra-centrifuged supernatant (without EVs). Dashed lines indicated original scratch. Top row: after scratch; bottom row: after migration. Numbers give increase/decrease of migration in %. Significance: *** < 0,005.

Ultra-centrifuged medium decreased PAEC migration in week 3 and in week 4. The adipogenic-induced medium of week 3 increased migration significantly. The addition of adipogenic EVPs from week 3 and week 4 increased migration at concentrations of 1 µg/mL and 2 µg/mL.

3.6.6 Scratch Assay with Scratch Machine

After scratch assays showed that week 3 medium had the highest impact on PAEC migration, conditioned medium of week 3 from all 3 donors was used for scratch assays with the scratch machine to re-collect migrated cells for further investigations. To exclude impact of FBS EVs only EV reduced FBS medium (FBS-depleted) and EVPs were applied on the assays.



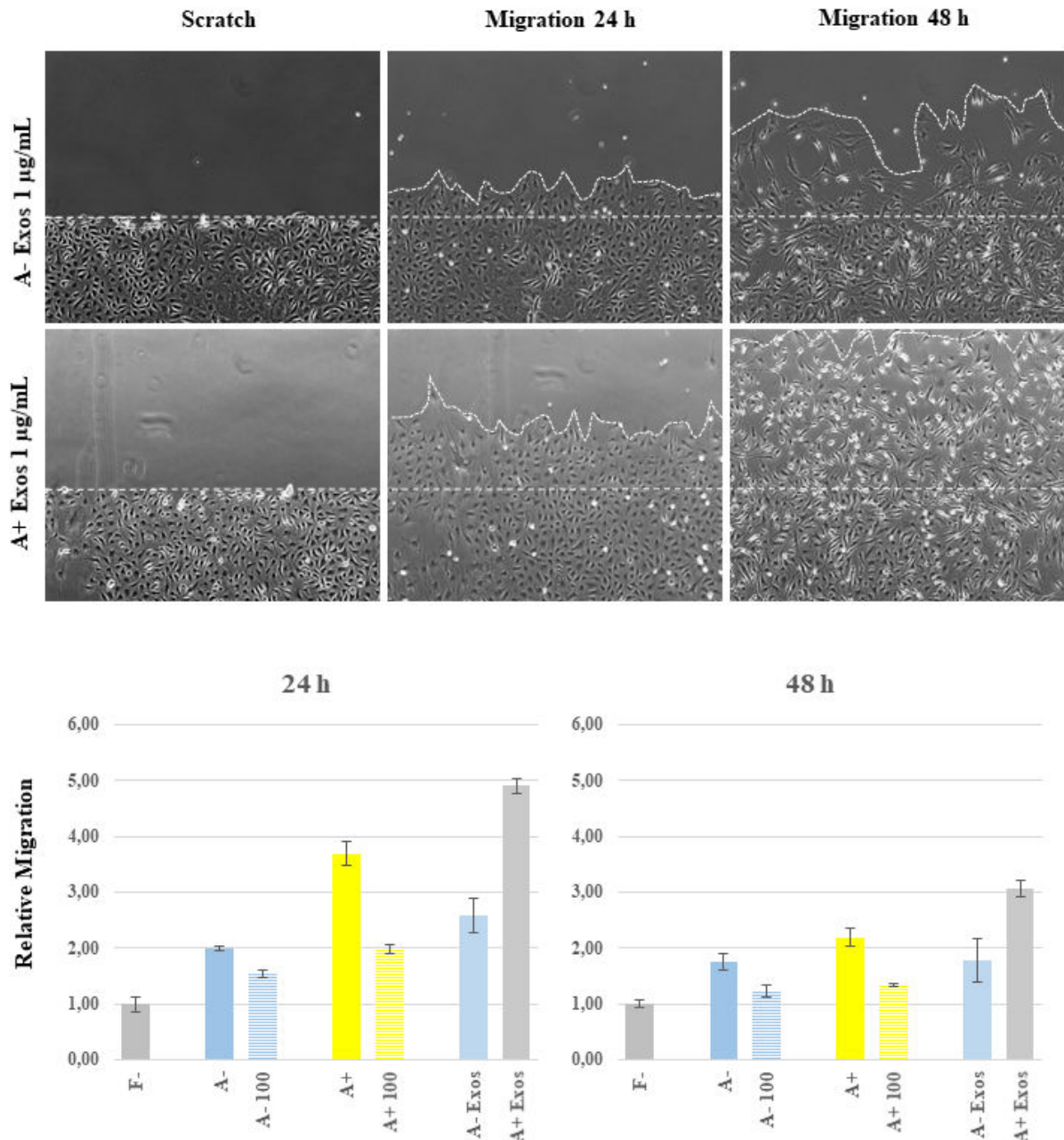


Fig. 33: Scratch Assay with PAECs in Conditioned Medium and Addition of EVPs. Confluent grown PAECs in 6 cm dishes were scratched and supernatant or EVPs from week 3 of adipogenic differentiation (mixture 1:1 with fresh DMEM) was added. After 24 and 48 hours migrated cells were counted. Scratches were performed with medium from standard conditions and FBS-depleted conditions. Ultra: ultra-centrifuged supernatant (without EVs). Dashed horizontal lines indicated original scratch. Zigzag lines indicate migration front. Significance: *** < 0,005 (The data shown are representative for the tested donors).

PAEC migration was elevated in non-induced medium and in adipogenic-induced medium of donor 1 after 24 and 48 hours whereas the ultra-centrifuged medium

decreased the migration. The addition of adipogenic-induced EVPs from week 3 resulted in a significantly higher migration after 24 (3x higher) and 48 hours (5x higher) compared to the control (F-). Compared to conditioned medium from adipogenic differentiation, migration of ECs was increased after adipogenic EVP addition (1µg/mL). Non-induced EVPs had no significant impact after 24 h but increased EC migration after 48 h.

For the second donor PAEC migration was more elevated after 24 than after 48 hours compared to donor 1. The ultra-centrifuged medium also decreased the migration, but the adipogenic-induced medium could also increase migration. The addition of adipogenic EVPs from week 3 resulted in a significantly higher migration after 24 hours (5x higher) and 48 hours (3x higher) compared to the control (F-). Compared to conditioned medium from adipogenic differentiation the migration of ECs was increased after adipogenic EVP addition (1µg/mL). Non-induced EVPs also increased EC migration after both 24h and 48 h.

The results of donor 3 medium and addition of EVPs were similar to donor 2. PAEC migration was more elevated after 24 than after 48 hours. The ultra-centrifuged medium also decreased the migration compared to non-centrifuged supernatant, but the adipogenic-induced medium could also increase migration. The addition of adipogenic EVPs from week 3 resulted in a significantly higher migration after 24 (4x higher) and 48 hours (2x higher) compared to the control (F-). Compared to conditioned medium from adipogenic differentiation, migration of ECs was increased after adipogenic EVP addition (1µg/mL). Non-induced EVPs also increased EC migration after both 24h and 48 h.

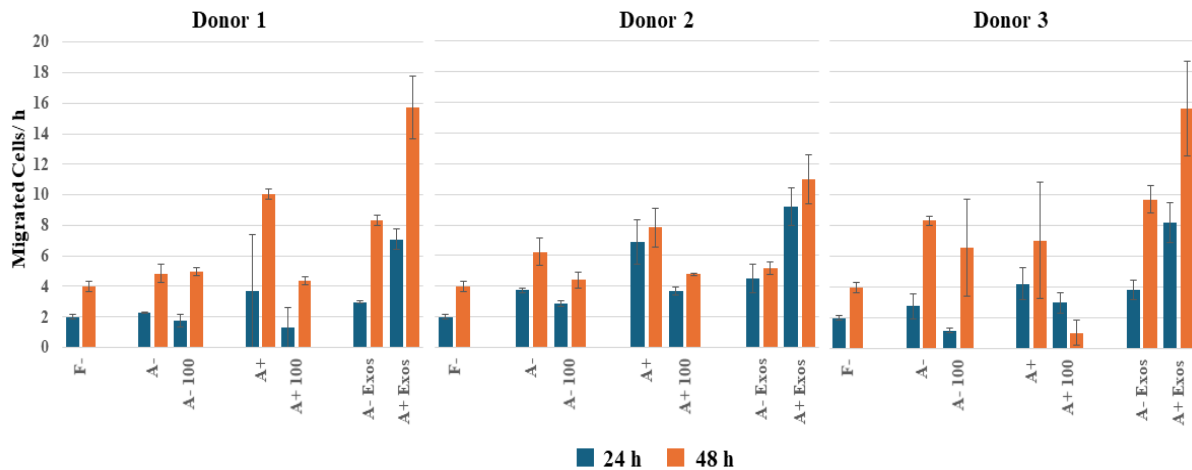


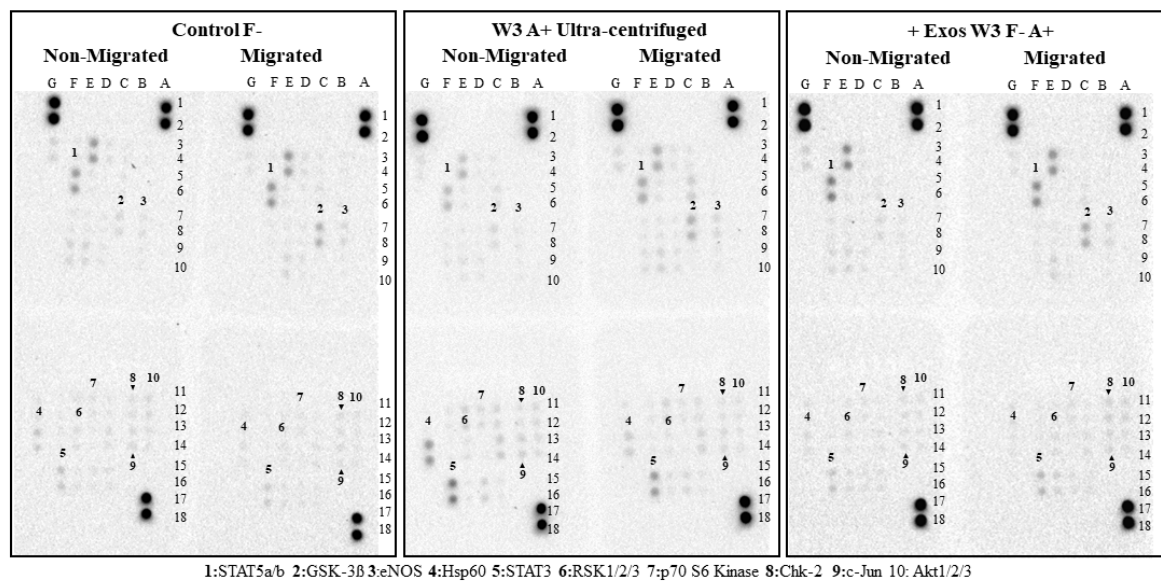
Fig 34: PAEC Migration Rate Depending on Time. The counting data from the scratch assays above were translated to migrated cells per hour.

When translating the data of migrated cells to traveled cells per hour, it can be seen that the numbers for 48 hours are higher than for 24 hours. Adipogenic-induced medium and adipogenic EVPs increased the number of travelled cells per hour for all 3 donors. Since 24 hours migration already resulted in a significantly increased PAEC migration, the cells were collected after 24 hours for further investigations.

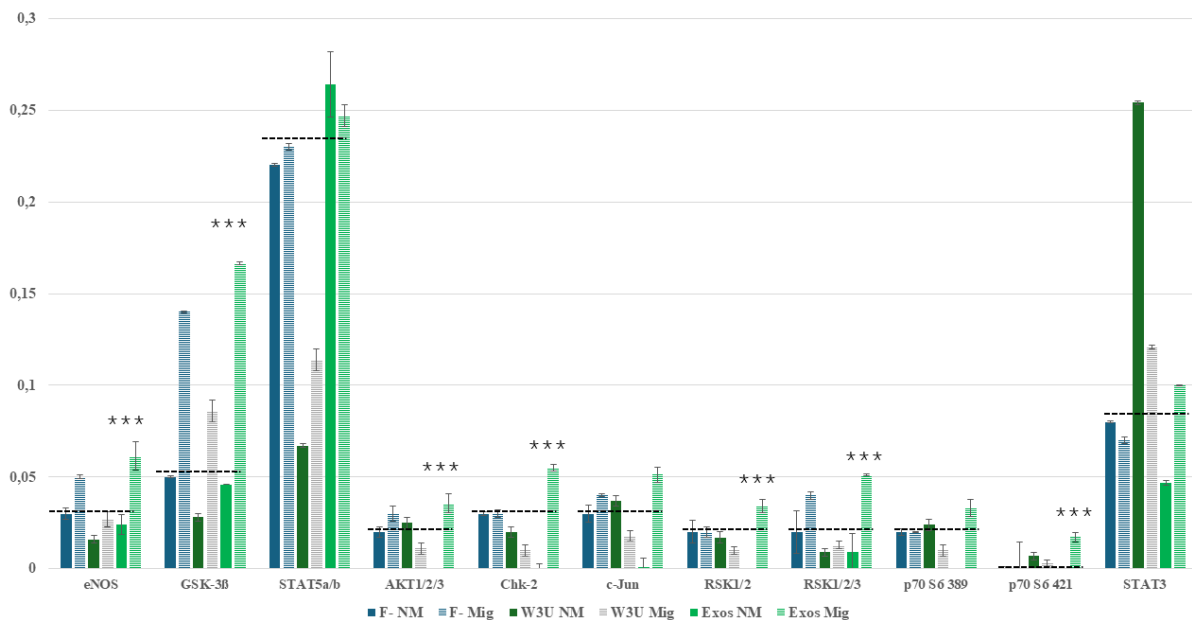
3.6.7 Signal Transduction in Migrated Endothelial Cells

The lysates of non-migrated cells and of migrated cells was applied to phosphokinase arrays in equal concentrations. Three conditions were investigated: 1.) control medium (EV reduced FBS medium) 2.) addition of 1 mg/mL adipogenic-derived EVPs in control medium and 3.) ultra-centrifuged conditioned medium from adipogenic-induced MSCs (1:1 in control medium). The latter was used to investigate a possible difference between secreted soluble factors within the medium and the adipogenic EVPs.

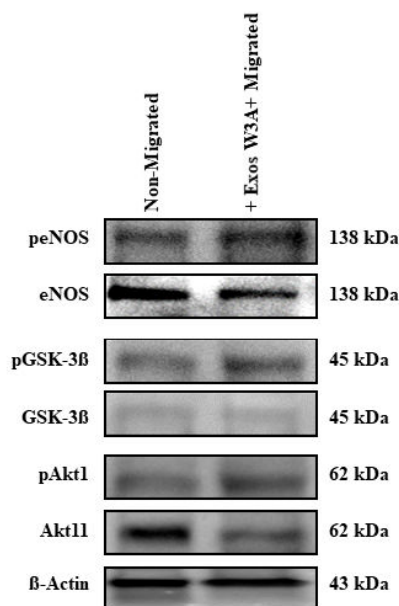
A



B



C



D

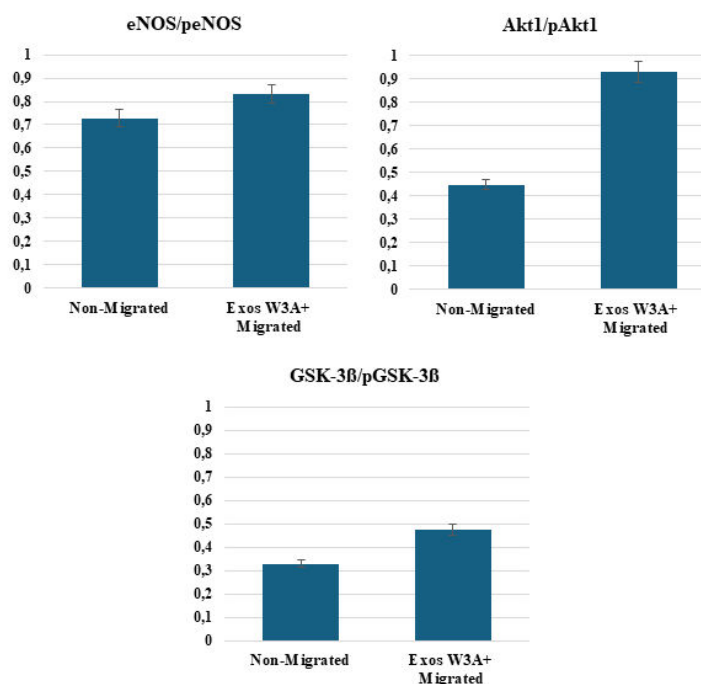


Fig. 35: Phosphokinase Arrays of Scratch Machine Assays. (A) Non-migrated and migrated PAECs were collected. Isolated protein solution was used for phosphokinase arrays at equal concentrations. 3 conditions were compared: Control (EV reduced FBS medium), W3A+ Ultra-centrifuged CM and addition of 1 $\mu\text{g/mL}$ EVPs from adipogenic-induced MSCs. (B) Significantly increased phosphorylated proteins were quantified. Dashed lines indicate base level of non-migrated PAECs. (C) Western blot analysis of phosphorylated signaling molecules. (D) Quantification of phospho-Western blots (ratio given on y-axis). NM: non-migrated; Mig: migrated; Significance: *** < 0,005.

The arrays (Fig. 37A) showed that 9 proteins were significantly increased in phosphorylation: endothelial nitric oxide synthase 3 (eNOS), the serine-threonine kinase glycogen synthase kinase-3 beta (GSK-3 β), protein kinase B isoforms 1-3 (Akt1/2/3), signal transducer and activator of transcription 3 and 5a/b (STAT3 & STAT5a/b), the serine-threonine kinase checkpoint kinase 2 (Chk-2), the transcription factor Jun (c-Jun), the protein kinase ribosomal s6 kinase (RSK p70 S6).

The eNOS signal more than doubled for migrated PAECs in the control and after the addition of EVPs whereas the signal in ultra-centrifuged medium without EVs did not exceed the base level threshold although it increased compared to the non-

migrated cells. GSK-3 β showed a significant increased signal in all migrated PAEC for all 3 conditions. The phosphorylation status of the migrated cells after adipogenic EVP addition was more than 3x higher than base level. For STAT5a/b signals under control conditions and after addition of EVPs were also very high and low in ultra-centrifuged medium. There was no difference between non-migrated and migrated PAEC after EVP addition. The signals for Akt1/2/3 increased in migrated PAECs under control conditions and after EVP addition, but not in ultra-centrifuged medium. The same results were observed for c-Jun and RSK1/2/3. For Chk-2, RSK1/2 and p70 S6 kinase an increased phosphorylation was detected in migrated cells after EVP addition, but not under control conditions. The signals in ultra-centrifuged conditions were lower than the base levels. Although the STAT3 signal increased in migrated PAECs after EVPs addition, non-migrated PAECs in ultra-centrifuged adipogenic-induced medium was already very high and decreased in migrated cells. The results of the phosphokinase array showed that phosphorylation of eNOS, Akt and GSK-3 β were highly increased in migrated PAECs after adipogenic EVP addition. To confirm these results phospho-Western blotting was conducted (Figures 37 C & D) showed that p-eNOS, pAkt and pGSK-3 β are upregulated after EVP addition.

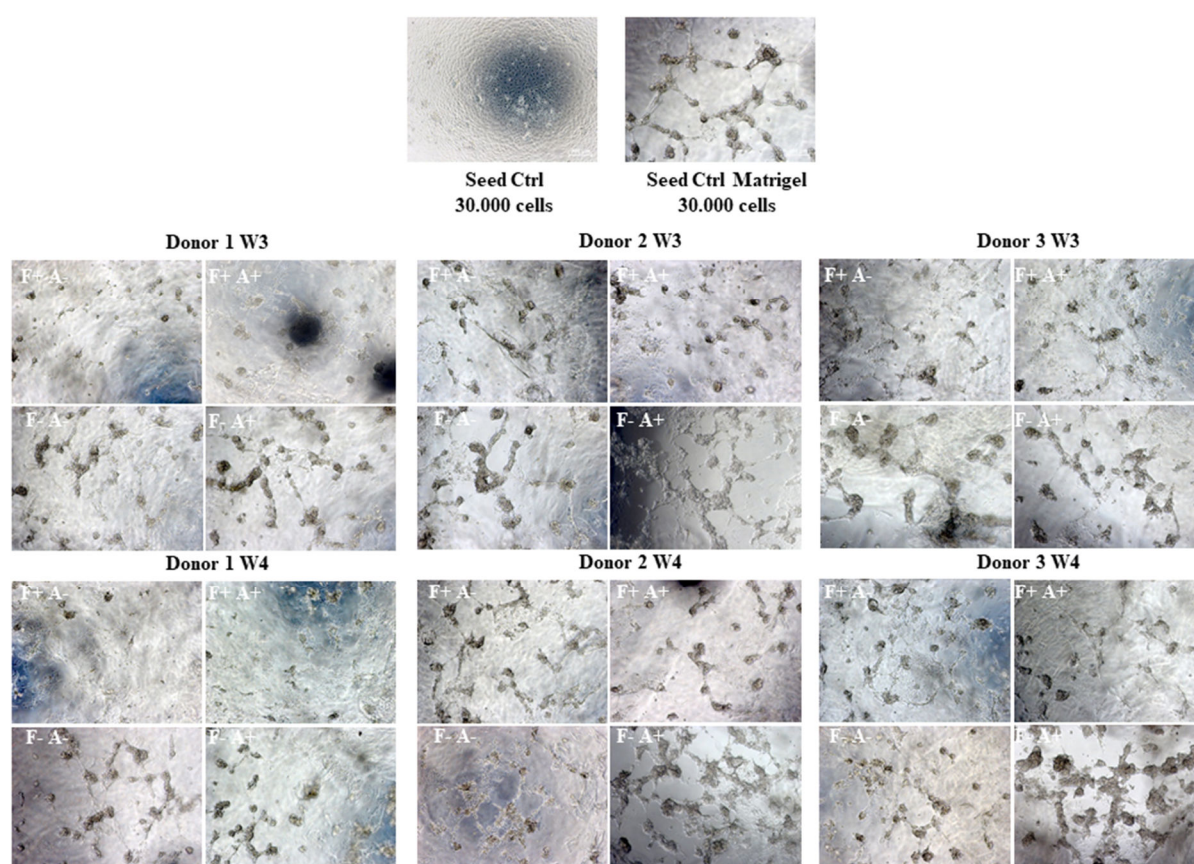
3.7 Angiogenesis Assays

Angiogenesis was investigated in Matrigel coated well plates in different formats. After the adjustment of the suitable seeding number of PAECs for 24-well and 12-well plates, angiogenesis with the addition of EVPs was studied. The quantification of network formation ability was done by using AngioQuant software to determine angiogenesis specific parameters: average lengths of structures, number of junctions and average size of structures.

3.7.1 Angiogenesis Assay with EVPs from Adipogenic Differentiations

The impact of all EVPs from the week 3 and week 4 differentiations of the 3 MSC donors was investigated by using Matrigel. EGM-2 medium served as control because DMEM lead to no network formation. EVPs at a final concentration of 1 $\mu\text{g/mL}$ were added to EGM-2 medium to investigate the impact of latter ones.

A



B

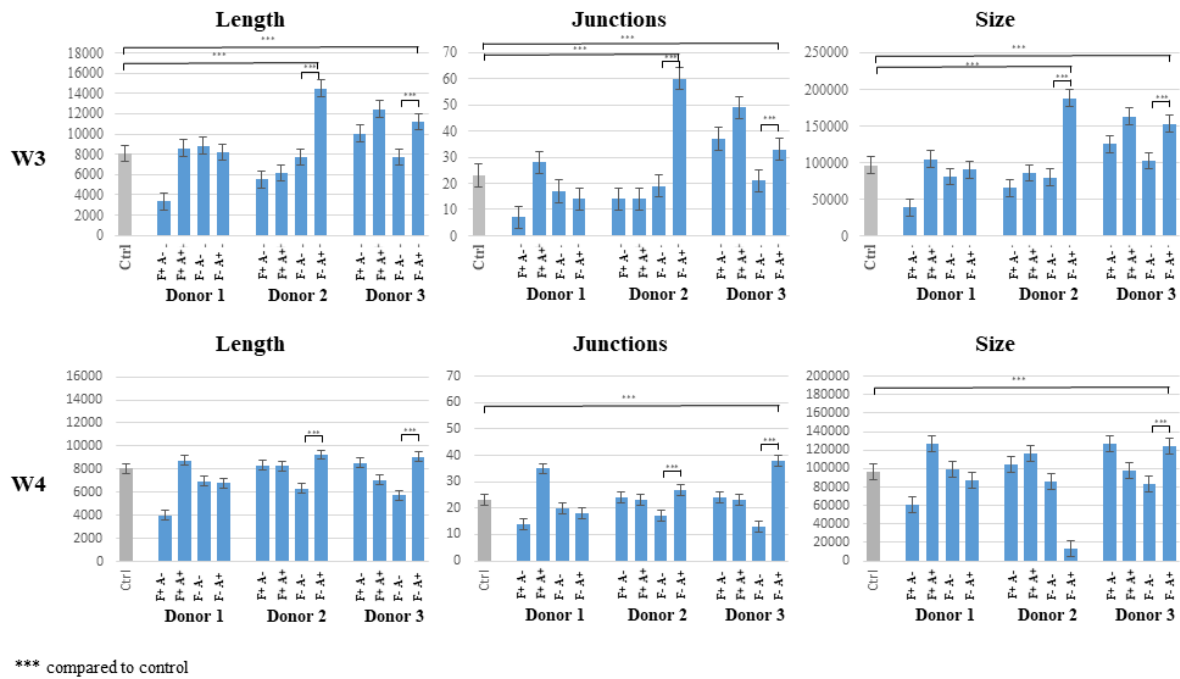


Fig. 36: Matrigel Assay with EVPs from Adipogenic Differentiations. (A) PAECs were seeded at a concentration of 30.000 cells per Matrigel coated 24-well. EGM-2 medium and EVPs from week 3 and week 4 of non-induced and adipogenic-induced MSCs were added at concentration of 1 μ g/mL. Pure EGM-2 medium served as control. After overnight incubation 3 random spots of the developed networks were imaged and (B) quantified with AngioQuant software to determine number of formed complexes, length, junctions and size of structures (y-axis: pixel values). Significance: *** < 0,005.

The Matrigel assay showed that addition of adipogenic-induced EVPs from week 3 had an impact on network formation. After addition of adipogenic EVPs from EV reduced FBS medium (F-A+) from donors 2 and 3 the lengths, number of junctions, and size of the network significantly increased. Compared to the addition of non-induced EVPs an increase in all 3 parameters was observed. For the addition of exosomes of week 4 the results were different. Here only adipogenic-induced exosomes from FBS-depleted conditions of donor 3 resulted in an increased size and number of junctions compared to the control. Due to these results, the following Matrigel assays were performed with exosomes from donor 3.

3.7.2 Angiogenesis Assay with EVPs from Week 3 of Adipogenic Differentiations

Stitched images of the control well (EGM-2 medium only) showed that the network was not completely developed after overnight incubation (whereas seeding of 250.000 cells resulted in a good developed network). The addition of 2 µg/mL non-induced EVPs from standard conditions resulted in an increase of length, size, and number of junctions. The values for the 3 angiogenic parameters could only be slightly elevated by 2 µg/mL adipogenic-induced EVPs from standard conditions. The addition of adipogenic EVPs from FBS-depleted conditions in concentrations of 1 and 2 µg/mL resulted in significant increased lengths, size, and number of junctions. By increasing the concentration to 5 µg/mL values dropped almost onto the level of control.

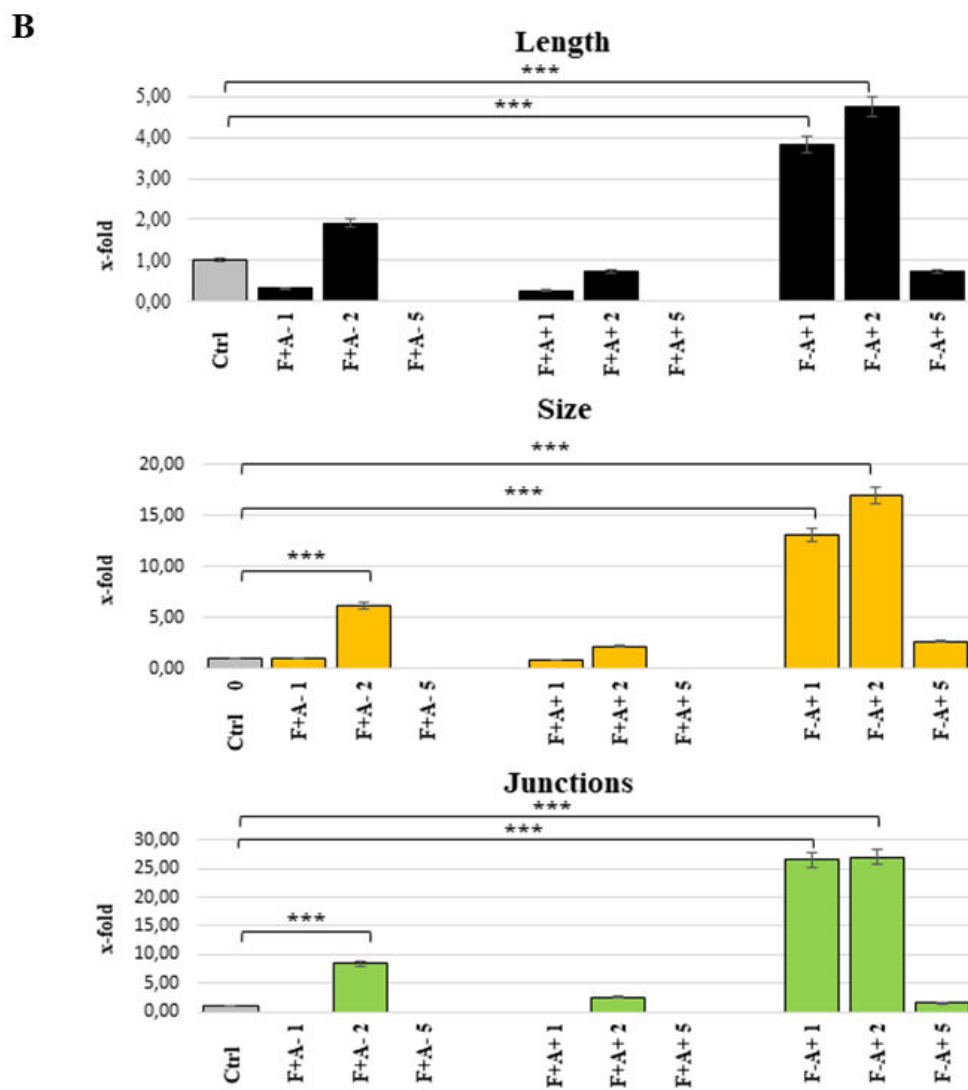
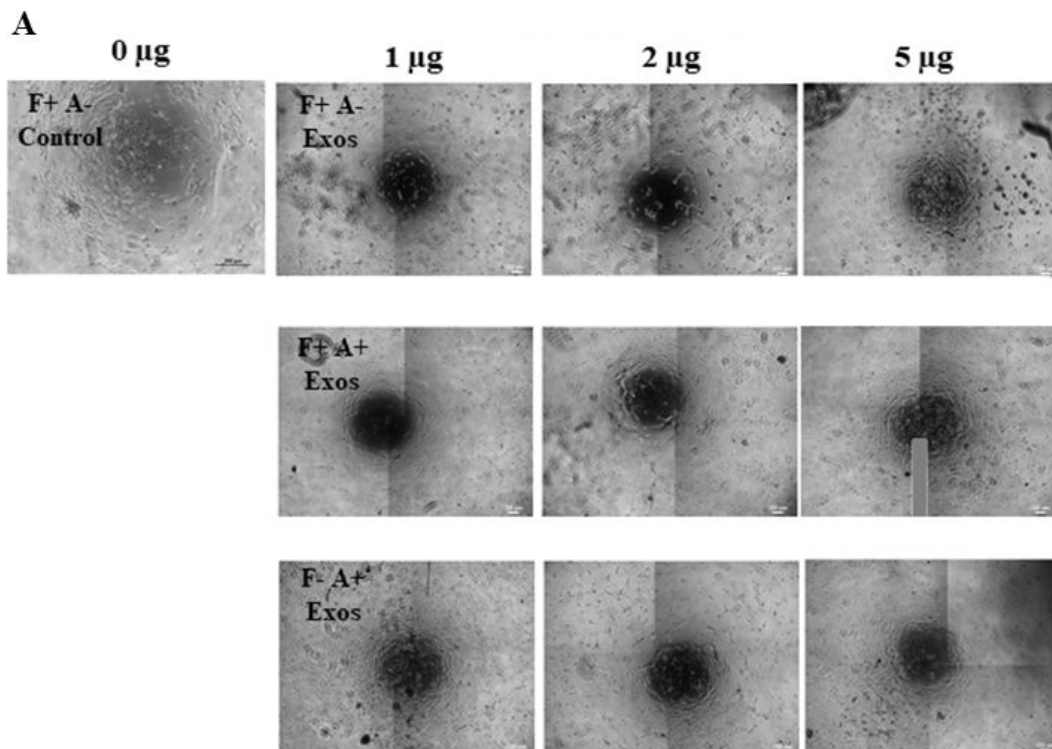
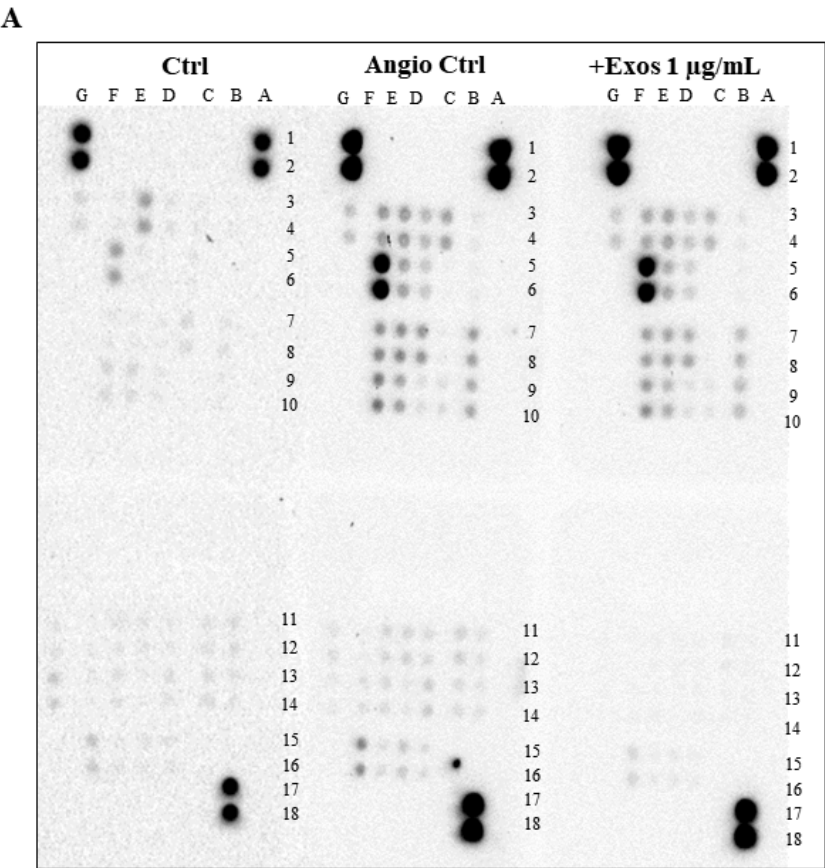


Fig. 37: Matrigel Assay with EVPs from Week 3 of Adipogenic Differentiations. (A) PAECs were seeded at a concentration of 100.000 cells per Matrigel coated 12-well. EGM-2 medium and EVPs from week 3 of donor 3 and of non-induced and adipogenic-induced MSCs were added at concentration of 1, 2 and 5 $\mu\text{g/mL}$ pure EGM-2 medium served as control. After overnight incubation, 4 images of 1 well were stitched to show network formation. (B) Quantification with AngioQuant software to determine number of formed complexes, length, junctions and size of structures. Significance: *** < 0,005.

In summary, the Matrigel assays showed that adipogenic EVPs increased network formation. The lengths of the structures increased by factor 5, while size increased up to 16x and the number of junctions increased up to 26x.

3.7.3 Signal Transduction in Angiogenesis

To investigate the signaling pathways of PAEC triggered by exosomes during angiogenesis, Matrigel assays in were conducted. The cells were collected and protein was isolated for further investigations.



B

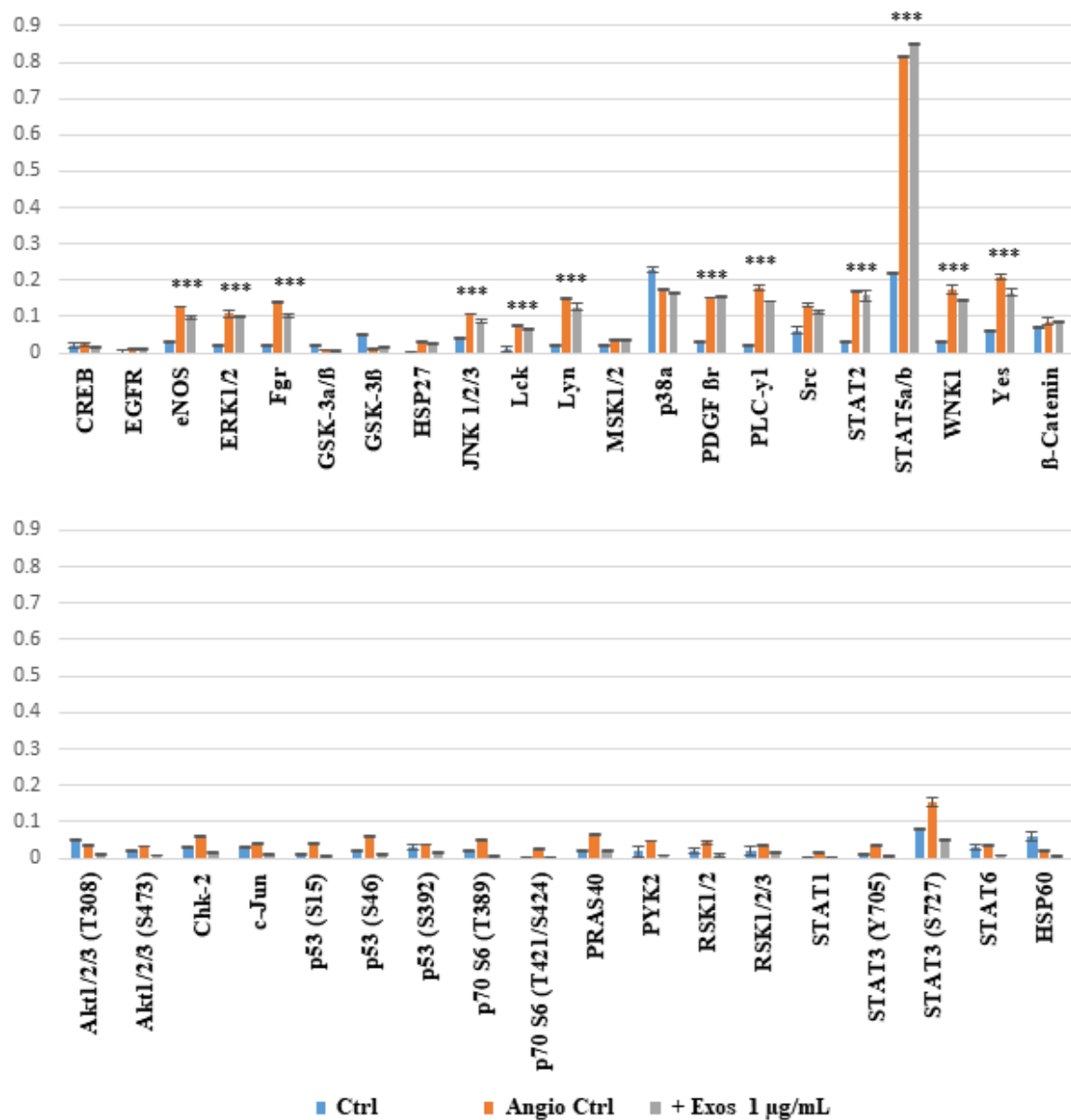


Fig. 38: Phosphokinase Array for Matrigel Assays of PAECs. (A) The network forming PAECs of Matrigel assays in EGM-2 (Angio Ctrl) and the addition of 1 µg/mL adipogenic-induced EVPs from FBS-depleted conditions of week 3 from donor 3 (+ Exos 1 µg/mL) were compared to control PAECs grown in F- medium (Ctrl). Protein concentration was equalized for all 3 membranes. (B) Quantification of the phosphokinase array. Significance: *** < 0,005.

The phosphokinase array showed a high number of phosphorylated proteins in networking-forming PAECs compared to control cells grown in EV reduced FBS medium (Ctrl). Significant increased signals were detected for: eNOS,

extracellular signal-regulated kinases 1/2 (ERK1/2), Src family tyrosine kinase Fgr, c-Jun n-terminal kinases 1-3 (JNK1/2/3), lymphocyte-specific protein tyrosine kinase (Lck), tyrosine kinase Lyn, platelet-derived growth factor receptor beta (PDGF- β R), phospholipase gamma 1 (PLC γ 1), signal transducer and activator of transcription 2 and 5a/b (STAT2, STAT5a/b), serine-threonine protein kinase lysine deficient protein kinase 1 (WNK1), and tyrosine-protein kinase Yes.

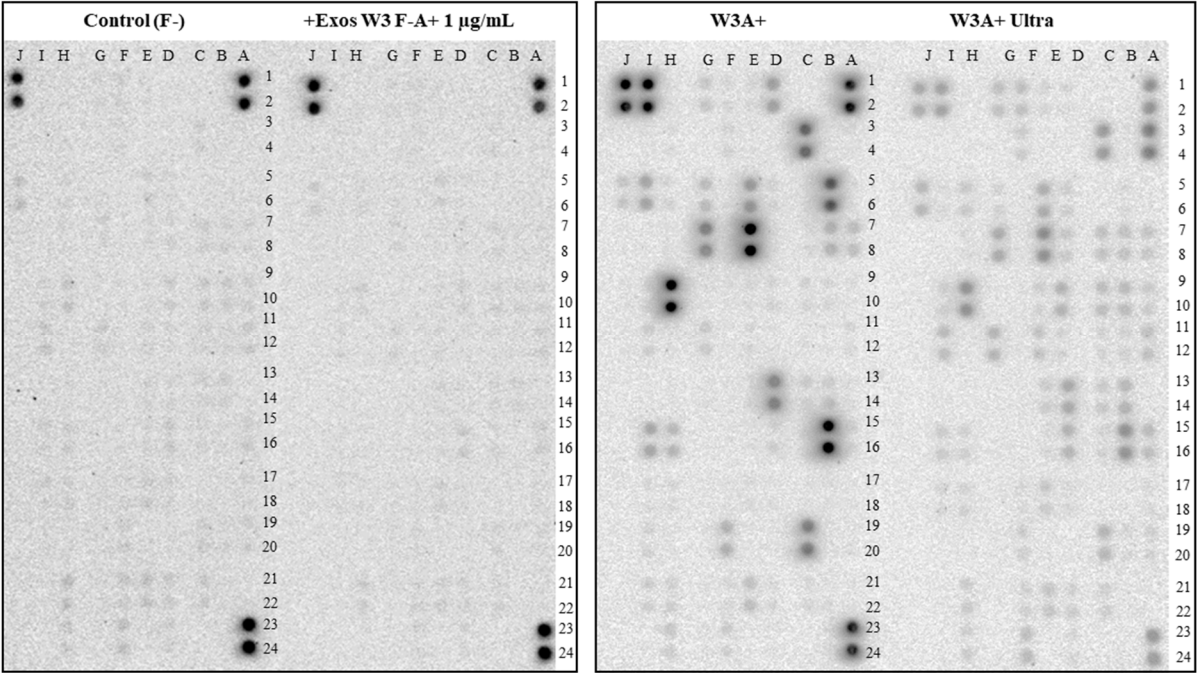
Comparing angiogenic PAECs (networking-forming) from EGM-2 medium and the angiogenic PAEC from EGM-2 medium supplemented with adipogenic EVPs, it can be seen that the signals for eNOS, Fgr, PLC γ 1, WNK1, and Yes decreased when EVPs were added. Only STAT 5a/b showed an increase after addition of EVPs.

3.8 Soluble Factors in Conditioned Medium

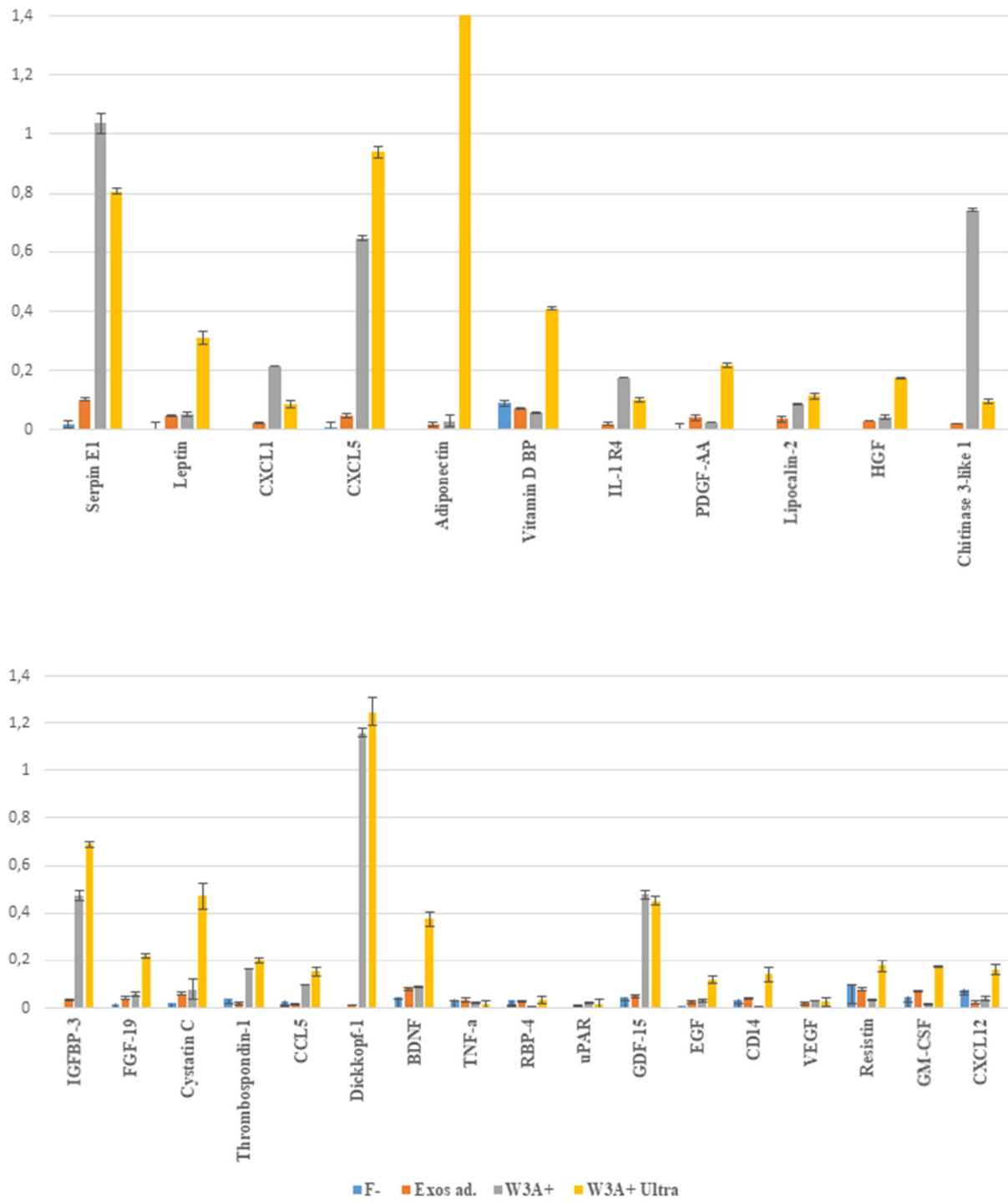
Next to EVs, soluble secreted factors during adipogenic differentiation were analyzed. Therefore, commercial cytokine and adipokine arrays were used. For both arrays, conditioned medium of week 3 from adipogenic differentiation under FBS-depleted conditions was compared to control medium (F-), adipogenic ultra-centrifuged conditioned medium of week 3 and control medium supplemented with 1 μ g/mL adipogenic-induced EVPs. The cytokine array showed that a high number of factors was upregulated in conditioned medium and ultra-centrifuged conditioned medium. The following cytokines showed strong increased signals in both compared to control and EVPs supplemented medium: Serpin E1, CXCL5, CCL2, Angiogenin, TSG14, IGFBP3, Dickkopf1, GDF-15, IL-6 and IL-8. Some cytokines were exclusively upregulated in ultra-centrifuged medium: Adiponectin, Vitamin D BP, PDGF-AA, HGF, ICAM-1, Fas-Ligand, Complement Factor D, Angiogenin, IFN- γ , FGF-2, C Reactive Protein,

Angiopoietin-1, CD71, MCS-F, Angiopoietin-1, FGF-19, Cystatin C, BDNF and interleukins (IL1a, 11, 15, 17a, 19, 22). Only Chitianse 3-like 1 showed an upregulation in conditioned medium of week 3 (Fig. 39).

A



B



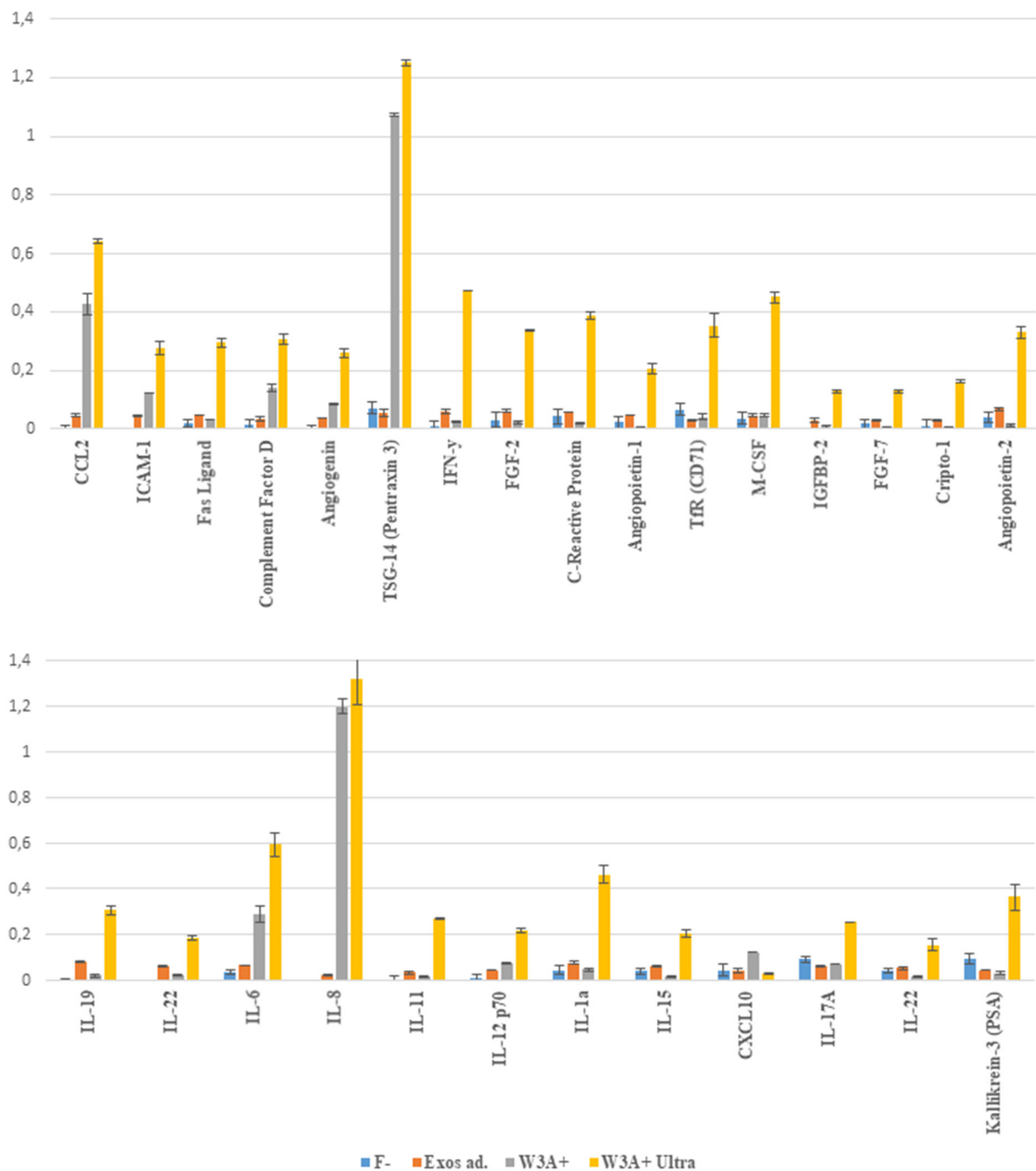
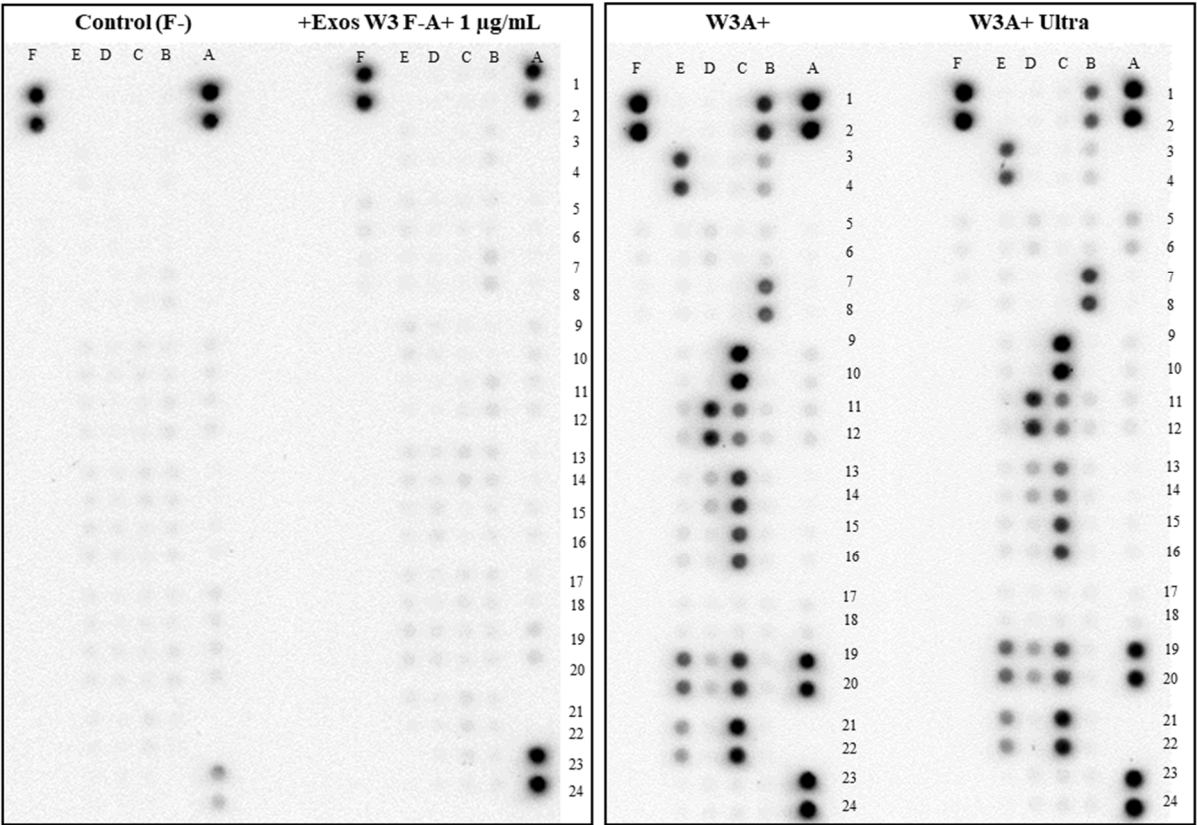
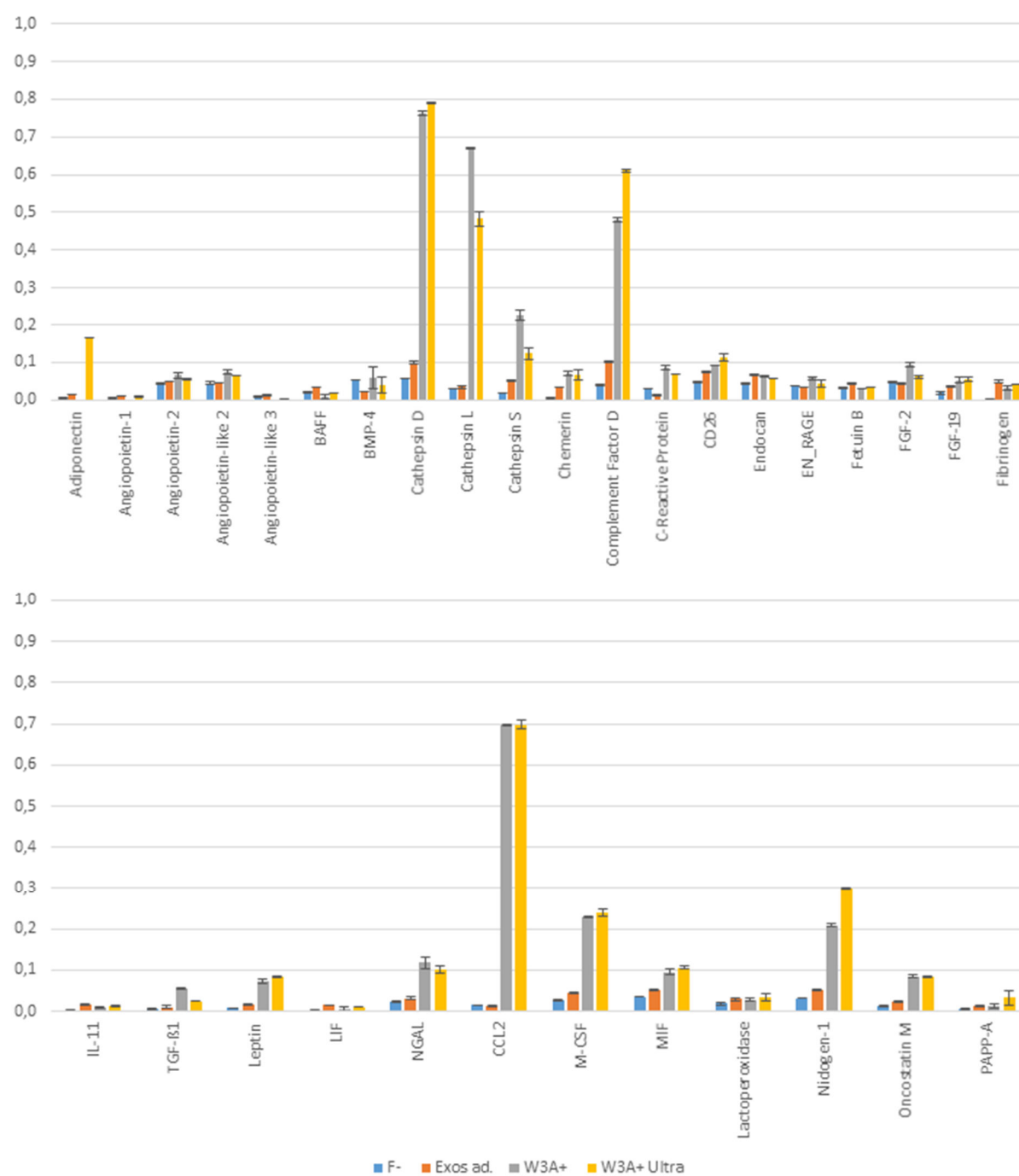


Fig. 39: Cytokine Array for Soluble Factors. (A) Membranes were incubated with conditioned medium from week 3 of adipogenic differentiation (F-), ultra-centrifuged conditioned medium from week 3 of adipogenic differentiation (F-), control medium (F-) and control medium supplemented with adipogenic-induced EVPs. (B) Quantification of cytokine arrays.

A



B



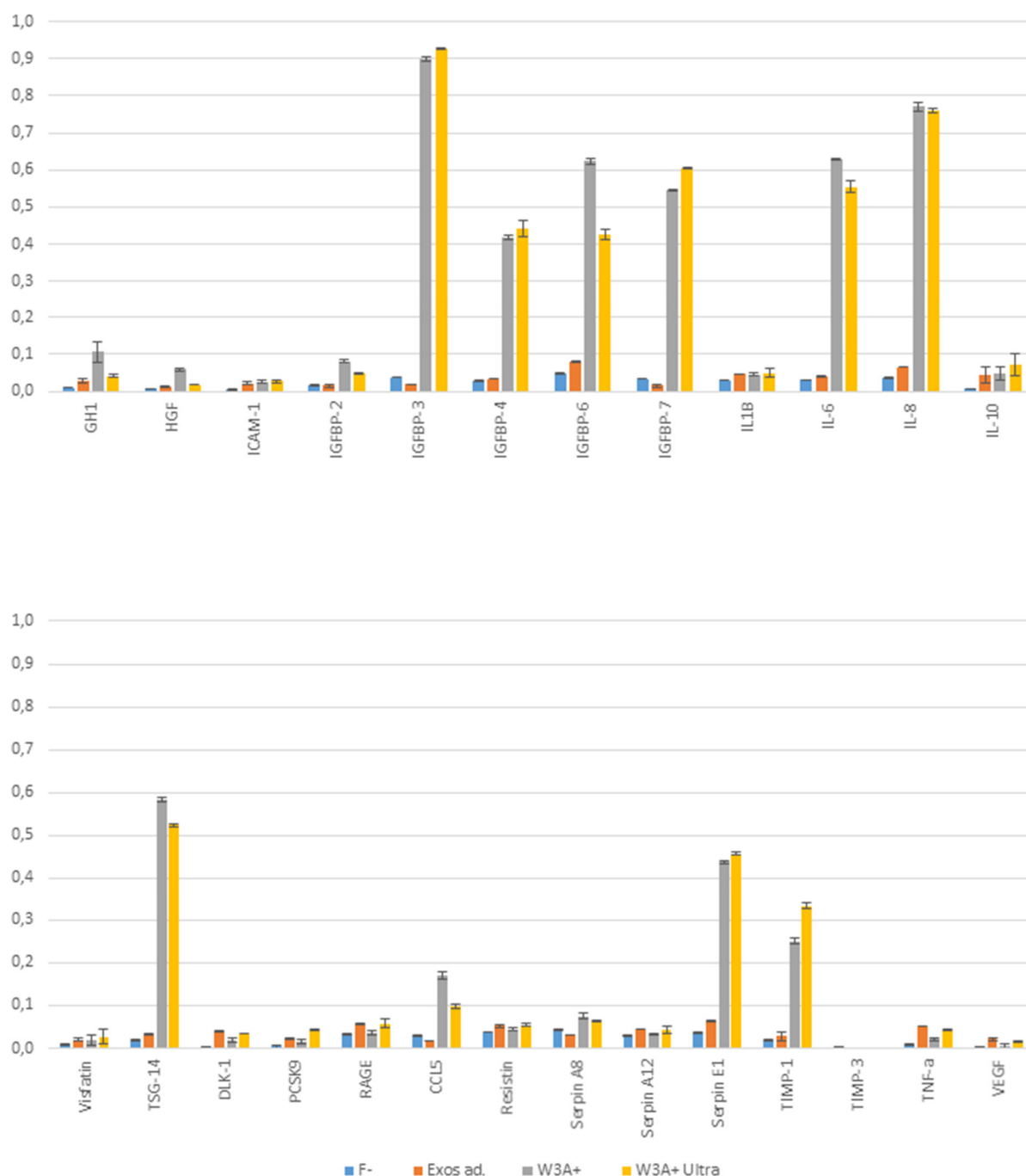


Fig. 40: Adipokine Array for Soluble Factors. (A) Membranes were incubated with conditioned medium from week 3 of adipogenic differentiation (F-), ultra-centrifuged conditioned medium from week 3 of adipogenic differentiation (F-), control medium (F-) and control medium supplemented with adipogenic-induced EVPs. (B) Quantification of adipokine arrays.

The adipokine arrays showed a similar result as the cytokine array. A high number of adipokines was upregulated in conditioned medium and the ultra-centrifuged conditioned medium. Following adipokines showed increased signals in both,

conditioned medium samples compared to control and EVPs supplemented medium: Cathepsin D & L, Complement Factor D, IGFBP3, 4, 6, and 7, NGAL, M-CSF, Nidogen -1, TSG-14, Serpin E1, TIMP1, and interleukins (IL4, 6, 7, 8). Only Adiponectin was exclusively upregulated in ultra-centrifuged medium (Fig. 40B).

4. DISCUSSION

4.1 Isolation and Characterization of Primary Cells

Due to the lack of human material, aortic endothelial cells were isolated from porcine aortas. The isolated PAECs showed as expected specific endothelial cell properties such as cobblestone patterned growth in cell culture (Fig. 9A) and the expression of the endothelial cell markers PECAM-1, KDR, and eNOS on gene level (Fig. 9B). On protein level the expression of eNOS, KDR, and PECAM-1 was confirmed by flow cytometry (Fig. 9C) and immunofluorescence staining (9D) (Zhang *et al.*, 2020). MSCs were obtained from human liposuction material. The isolated cells showed the ISCT-suggested features like capability of differentiation towards adipocytes, osteoblasts and chondrocytes *in vitro* confirmed by a specific staining (Fig. 10D; Dominici *et al.*, 2006). Flow cytometry analysis showed expression of the MSC markers CD44, CD73, CD90, and CD105, and the lacking expression of CD14, CD34, CD45, Vimentin as fibroblast marker was tested to exclude a contamination with fibroblasts, which have a similar morphology (Fig. 10C).

In addition, adipose tissue-derived MSCs (AT-MSCs) are a better cell source compared to MSCs derived from other body regions, because they are easily accessible in abundant numbers from liposuction material from healthy donors compared isolation from bone marrow (Aust *et al.*, 2004; Lin *et al.*, 2010). Secretome analysis even showed that adipose tissue plays a key role as a fat-storage depot and as an endocrine organ (Zhong *et al.*, 2010). Thus, as can be expected a comparative analysis of MSCs from bone marrow, adipose, and dermal tissues has shown that the highest amount of secreted cytokines such as VEGF-D can be detected in AT-MSCs (Hsiao *et al.*, 2012). The paracrine effect of MSCs lead to an increased interest in their secreted soluble factors and later, secreted

EVs like exosomes. Thus, the role of adipose tissue or adipogenic differentiating cells as potential source for these exosomes arose. In line with this, the work in hand focused on the impact of EVs derived from towards adipocytes differentiated MSCs on endothelial cells.

4.2 Adipogenic Differentiation of MSCs and EV Preparations

After the successful isolation and characterization of primary MSCs the cells were differentiated towards adipocytes under standard conditions and under FBS-depleted conditions. Specific staining for the adipogenic markers adiponectin, PPAR γ , and Leptin, as well as Oli Red O Stain for lipid droplets showed that all 3 donors differentiated towards adipocytes after 4 weeks. Also, there was no considerable difference in differentiation efficiency between standard conditions and FBS-depleted conditions (Fig. 11A). PCR analysis showed that expression of the adipogenic genes PPAR γ and LPL were upregulated and the expression of the mesenchymal stem cell markers CD73, CD90, and CD105 were down-regulated (Fig. 11B). Western blot analysis and flow cytometric analysis confirmed these results on protein level (Fig. 11C, D) (Dominici *et al.*, 2006). Donor 3 showed an earlier and stronger formation of lipid droplets after 3 weeks than the other younger donors. Our previous data showed AT-MSCs derived from older donors appeared to differentiate faster towards adipocytes while there was no decrease for the osteogenic differentiation potential compared to the cells from younger donors (Zippel *et al.*, 2012).

To investigate if the adipogenic differentiation and if the secreted adipogenic EVPs show similar results to adipocyte-derived exosomes, an additional differentiation was conducted. MSCs were differentiated towards adipocytes in adipogenic differentiation medium supplemented with 3% human platelet lysate instead of FBS to exclude effects of bovine exosomes. The efficiency was

comparable to the results of the 3 MSC donors before (Fig. 12). Since these results showed, that the adipogenic differentiations for all 3 donors and the controls were similar, the next step was to prepare EVs from collected supernatants of adipogenic-differentiated and non-induced MSCs supplemented with FBS and without FBS (see section 2.3.4).

Although the strategy of eliminating exosomes from FBS by ultra-centrifugation can be expected to be successful, it remains unclear if all EVs fractioned in the pellet. The possibility of a gradient consisting of remaining EVs has to be discussed. However, the use of supernatant without EVs is not free from handling errors, as it can result in a disruption of the EV pellet at the bottom of the centrifugation tubes. Thus, it cannot be ruled out that the lack of EVs has that been achieved and might have an impact. Therefore, the isolations were defined as extracellular vesicle preparations (EVPs).

The EVPs were obtained from supernatant of conditioned medium after each medium change from week 1 to week 4. The analysis showed the isolated EVs from MSCs (from week 4 of adipogenic differentiation) had a size range of 50 – 150 nm (Fig. 13A) which can be classified as endosomal exosomes (van der Pol *et al.*, 2012). The cells and the EVPs expressed the exosomal specific markers CD9, CD63, and CD81 (Fig. 13 B-D) and the DiL-pre-stained EVs were taken up by PAECs (Fig. 13E). The exosomal marker CD9, CD63, and CD81 are standard marker for exosomes. Some working groups have shown that MSC-EVs either express CD9 or CD81 but never simultaneously (Madel *et al.*, 2020). Our flow cytometric data showed that MSCs expressed all 3 standard exosomal markers. When capturing EVs with CD81-beads we could also show that the EVs expressed all 3 markers. Interestingly, on EVPs gained from MSCs under FBS-depleted conditions CD81 was expressed to a higher extend compared to MSCs grown under standard conditions (Fig. 13D). The data analysis of the flow cytometry experiments were performed at a time when histogram graphs where

commonly used in research. Nowadays, one would display the data in dot-blots to show the real partition and distribution of positive expressing surface markers.

The EVPs were also investigated with commercial available exosomes kit. The kit uses columns and capture beads to purify exosomes. EVPs from undifferentiated MSCs and from adipogenic-differentiated MSCs from week 3 were applied on the membranes in equal concentrations. Both EVPs were positive for exosomal standard markers like CD63, CD81, TSG101, FLOT1, and ALIX (Fig.16). The signal for CD81 was slightly stronger for adipogenic EVPs confirming our initial flow cytometry data (Fig. 13D). An explanation for this result might be the use of EV reduced FBS.

The concentration measurement of EVPs with BCA kits showed that until week 3 the concentration was between 100 and 200 ng/ μ L from 50 mL supernatants. There was no difference between adipogenic-induced EVPs and MSC-derived EVPs from standard conditions and from FBS-depleted conditions. The measured concentrations were similar to other studies. Gong and colleagues isolated 100 ng/ μ L from MSCs and the working group of Dougherty yielded the same amount from iPSCs (Gong *et al.*, 2017; Dougherty *et al.*, 2017). Interestingly, the concentration increased after 4 weeks of cultivation and differentiation up to 600 ng/ μ L for all conditions. MSCs from both, both adipogenic induced and non-induced cells, seem to be secretory very active between week 3 and week 4. This is in line with data from the working group of Zhong in 2010 who investigated the secretome of MSCs and identified 420 proteins (Zhong *et al.*, 2010). Another explanation for the high concentration of EVPs might be apoptosis. Due to lack of nutrition or rough handling before cell collection, e.g. for RNA or protein isolations, cells might go into apoptosis resulting in a higher concentration of protein during measurement after preparations of EVs.

To investigate the activity of the differentiating MSCs is also resulting in higher amounts of secreted adipokines, conditioned medium from week 3 and week 4

was investigated with cytokine arrays. The only significant increased cytokine was cystatin C, which is a constantly produced cysteine-protease inhibitor and is used as a marker in kidney malfunctions (Mussap *et al.*, 2004). However, no apoptotic cells were observed during and at the end of the adipogenic differentiation. Some cytokines were slightly elevated, e.g. FGF-19, GDF-15, CXCL1, IL-6 and IL-R4. FGF-19 is a high affinity, heparin dependent ligand for FGFR4. It was found to be reduced in in patients with metabolic syndrome (Stejskal *et al.*, 2008, Schreuder *et al.*, 2010). GDF-15 is an abbreviation for growth differentiation marker 15 and is proposed as a biomarker for mortality and disease progression in patients with atherosclerosis and/or cardiovascular disease (Schwarz *et al.* 2023). CXCL1 and IL-6 are well-known marker for inflammation, which emphasizes the pro-inflammatory state of adipogenic-differentiating MSCs.

4.3 Extracellular Vesicle Uptake

Most studies investigating the uptake of exosomes or extracellular vesicles in different cell types observed an uptake of pre-stained exosomes within a few hours. In this study MSC-derived EVPs were initially incubated with the membrane staining dye DiI and applied to PAECs for overnight incubation to check the, if the EVPs could be observed in or on the cells after washing. Immunofluorescence staining showed that after 1 hour EVs were visible in or on ECs (Fig.17). In order to quantify the potential uptake of adipogenic-induced and non-induced EVs, the EVPs from week 3 of adipogenic-differentiated MSCs under standard conditions and FBS-depleted conditions were pre-stained with the three exosomal marker antibodies against CD9, CD63 and CD81. After washing the EVPs were applied to PAECs for 1 – 4 hours. The pre-stained EVPs were then stained with a fluorochrome-labeled secondary antibody while the PAEC

exosomes were stained with the exosomal marker antibodies against CD9, CD63 and CD81 and another fluorochrome-labeled secondary antibody (Fig. 18). The images and quantification of the fluorescence signals showed that after 1 hour of incubation pre-stained EVPs were either taken up by PAECs or attached to the cells itself. All 3 markers were detectable, and the signal strength increased after 2 hours and 4 hours incubation. The signal of CD81 was elevated in F-adipogenic-induced EVPs after 2 and 4 hours compared to all other stainings, indicating a higher expression on F- adipogenic EVs, which was already observed in the characterization of EVPs (see section 3.4.1 Fig. 13C/D, section 3.4.2 Fig. 16B). The uptake experiments were repeated without counter stain of PAEC exosomes to exclude antibody cross-talk. The results were similar to the data mentioned above. The first fluorescence signals for the exosomal markers were detected after 1h of EVPs addition for both EVP types obtained from standard conditions (Fig. 19) and from FBS-depleted conditions with EV reduced FBS (Fig. 20). Additionally, the uptake of EVs was shown in HUVEC. EVPs from non-induced and adipogenic-induced MSCs were also detectable after 1 h by an increased fluorescence signal for the 3 exosomal markers (Fig. 21).

The investigation of uptake or internalization of exosomes or EVs into endothelial cells was part of many studies. Many working groups used PKH67 or DiI-labeled exosomes and incubated the recipient cells up to 24 hours. Exosomes could be detected *via* immunofluorescence microscopy near the perinuclear region of HUVECs after 2 hours by Liu and colleagues (Liu *et al.*, 2017). The working group of Hu also showed internalization of pre-stained exosomes in HUVECs *via* immunofluorescence microscopy (Hu *et al.*, 2019). A detection of MSC-derived exosomes after 3 hours was overserved by Vrijssen (Vrijssen *et al.*, 2016). Other groups incubated the endothelial cells with pre-stained exosomes for 24 hours and imaged the cells afterwards *via* confocal or immunofluorescence microscopy. The groups of Ren (confocal microscopy) and Zhao (immunofluorescence

microscopy) used AT-MSCs, whereas Yu (confocal microscopy) incubated HUVEC with pre-treated MSC-derived exosomes (Ren *et al.*, 2019; Zhao *et al.*, 2019; Yu *et al.*, 2020). The findings of early uptake of exosomes into ECs led to the question of the exact starting time-point. Some working groups conducted uptake studies in time-lapse experiments. Dougherty's group incubated bovine aortic endothelial cells (BAECs) with iPSC-derived exosomes for 6 hours and could show an increased uptake starting after 2 hours (Dougherty *et al.*, 2018). Gong and colleagues used MSC-derived exosomes in a 12 hour running network formation assays and saw the first exosomes after 4 hours (Gong *et al.*, 2017). The group of Gao imaged HUVEC exosomes uptake after 0,5, 1, 3, 6 and 24 hours and could already observe exosomes after 30 min. (Gao *et al.*, 2016). In this study, the uptake of EVs was investigated on PAECs and on porcine smooth muscle cells (PSMCs) in steps of 15 min for the first 90 min and in steps of 1 h steps from 2 hours to 6 hours. It could be shown that the uptake started around 45 min after EVPs addition and reached a plateau after 75 min (Fig. 22). For PSMCs the uptake started earlier, around 30 min after EVPs addition (Fig. 23). The question arose, if the EVs were internalized by the cells, or if they were just adherent on top of the cell membranes. However, some studies already showed that the added exosomes or EVs could be found inside the recipient cells. Dougherty's confocal images clearly showed the presence of exosomes in the perinuclear region of BAECs (Dougherty *et al.*, 2018). Zhang showed the uptake of osteoblast-derived exosomes in porcine osteoblasts (Zhang *et al.*, 2023). With respect to this, it can be assumed that the EVs in this study were internalized by PAECs and PSMCs as well.

The data revealed that the uptake of EVs happened fast and that the internalized EVs might have an impact on endothelial cell migration, which was later investigated in migration assays. Nevertheless, the effect on ECs either by

paracrine signals or uptake was reproducibly shown. As the main aim was to develop a potency assay to investigate if the addition of EVPs from adipogenic differentiated MSCs to ECs lead to activation, the focus was on migration assays and angiogenesis assays.

4.4 Migration Assays

Migration assays were conducted to investigate the activation of endothelial cells by conditioned medium and EVPs from adipogenic differentiation. The first experiments were performed with conditioned medium from all four weeks of the adipogenic differentiation for all donors. Previous experiments from our group showed that conditioned medium of week 3 from adipogenic differentiated MSCs had the highest impact on bovine endothelial cell (BAEC) migration (Lange, 2013). The investigated PAECs in this study showed similar results. Conditioned medium of week 3 and week 4 lead to the highest migration of PAECs (Fig. 24 & Fig. 25). To eliminate proliferation effects an inhibitor (hydroxyurea) was added to the migrating cells. The results were similar but the total number of migrated cells was lower, as expected. An enhanced migration rate of PAECs using conditioned medium from week 3 was demonstrated in section 3.6.1, prompting its selection for comparison between scratch assays and Boyden chamber migration assays.

Conditioned medium from week 3 of adipogenic differentiation under standard and FBS-depleted conditions was compared with week 3 medium from non-induced MSCs (W3C) and culture medium as control in these migration assays. The results consistently indicated that conditioned medium from week 3 of adipogenic-induced MSCs exerted the highest influence on PAEC migration. Additionally, migration was reduced when FBS-depleted medium was used for adipogenic-induced medium in both assay types. Notably, relative PAEC

migration was higher in scratch assays compared to Boyden chambers (Fig. 26). The analysis of conditioned medium (section 3.4.2) already showed that several cytokines were elevated in CM of adipogenic-induced MSCs (Fig. 15). These cytokines might be responsible for the increased EC migration. To investigate, if soluble factors or secreted EVs of the MSCs had the bigger impact on EC migration, further migration studies had to be conducted.

The EVPs impact on endothelial cell migration was initially explored with the use of Boyden chambers. MSC-derived EVPs were added either to the upper PAEC containing chamber or to the lower chamber with FBS-depleted DMEM in both. Adding EVPs to the top chamber resulted in higher migration rates compared to the bottom chamber. Increased EVPs concentrations led to PAEC clumping, most prominent at 2.5 $\mu\text{g/mL}$ and 5 $\mu\text{g/mL}$. Clumping was absent at 1 $\mu\text{g/mL}$ EVPs addition. The highest PAEC migration rate occurred when FBS was used as chemoattractant in the bottom chamber (Fig. 27). To mitigate clumping, subsequent scratch assays were conducted.

Migration assays were conducted to assess the impact of different conditions. For each donor, both standard and FBS-depleted medium and corresponding EVPs were applied to PAEC scratch assays in triplicates for 3 MSC donors (Fig. 28 - 30). The quantification of the migration assays showed that within ultra-centrifuged medium PAEC migration significantly decreased compared to CM, which was not centrifuged (Fig. 31). These findings could be observed for CM for the 3 donors in both conditions (F+ & F-), non-induced and induced supernatants, and for every week differentiation. Another result of the screening scratch experiments was the confirmation of the pro-migratory effect of adipogenic-induced CM from week 3 and week 4. Migration rates for standard conditions CM of week 3 raised up to 63% and up to 54% for week 4. Migration rates for EV-reduced FBS medium conditions raised up to 89% for week 3 CM and up to 73% for week 4 CM. The addition of non-induced EVPs as well as

adipogenic-induced EVPs increased PAEC migration for the tested 3 MSC donors significantly. These findings hint to pro-migratory features of EVPs from MSCs. It might be noted that non-centrifuged CM and the addition of EVPs always increased migration significantly compared to ultra-centrifuged CM. This lead to the conclusion that secreted soluble factors were not the main responsible factor for increased EC migration. To confirm the impact of MSC EVPs, adipogenic differentiations were conducted without FBS, using human platelet lysate instead. To do so week 3 and 4 supernatants were tested in scratch assays. Ultra-centrifuged medium reduced PAEC migration for both weeks. Adipogenic-induced medium from week 3 significantly increased migration. Additionally, the addition of adipogenic EVPs at 1 $\mu\text{g/mL}$ and 2 $\mu\text{g/mL}$ further enhanced migration. No significant difference in EC migration between non-induced and adipogenic-induced EVPs from both, standard conditions and FBS-depleted conditions was observed. Since the focus was to identify the MSC EVPs impact on EC activation, scratch assays with an own developed scratch machine were conducted with CM and EVPs obtained under FBS-depleted conditions. Week 3 conditioned medium from 3 donors was used in these scratch assays to collect migrated cells.

In summary, adipogenic EVPs increased PAEC migration significantly, compared to control, non-induced EVPs and ultra-centrifuged CM (Fig. 33 – 35). These findings were in line with the screening experiments before. The non-induced EVPs significantly led to a lower EC migration rate of approximately 50 %. Another interesting finding was made, when data was translated into traveled cells per hour (Fig. 36). Here, adipogenic-induced EVPs lead to the highest travelling rates after 48 h for the 3 MSC donors. This hints to a delayed PAEC migration. Nevertheless, EC migration was significantly enhanced after 24 h and the migrated cells were collected for molecular biological investigations. Numerous studies investigated the impact of exosomes or EVs on endothelial cell migration. The donor cells for exosomes varied from endothelial cells and endothelial

progenitor cells (EPCs) to MSCs from adipose tissue, bone marrow and placenta of different species to exosomes isolated from blood plasma. Ren was able to enhance HUVEC migration by applying AT-MSC-derived exosomes in a concentration of 5 and 10 $\mu\text{g/mL}$ (Ren *et al.*, 2019). While the group investigated migration in trans-well chambers, the presented study could clearly show an increased migration in scratch assays using lower concentrations of exosomes (1 – 2 $\mu\text{g/mL}$). Takeuchi and Salomon also used 5 – 10 $\mu\text{g/mL}$, but the donor cells were BM-MSCs and placental MSCs (Takeuchi *et al.*, 2019; Salomon *et al.*, 2013). Placental MSC-derived exosomes were also shown to enhance EC migration in lower concentrations of 1 $\mu\text{g/mL}$ by Komaki and colleagues (Komaki *et al.*, 2017). Han showed an increased migration after applying hypoxia-induced AT-MSC-derived exosomes onto microvascular brain endothelial cells (MBVEC) (Han *et al.*, 2018). Another strategy to influence the impact of exosomes on EC migration is pre-conditioning of donor cells. The group of Zhao used magniferin-stimulated rat AT-MSC to enhance rat aortic EC migration and Yu treated BM-MSCs with atorvastatin to enhance HUVEC migration (Zhao *et al.*, 2019; Yu *et al.*, 2020). Next to MSCs other cells served as source for exosomes and enhanced EC migration. Hu used HUVEC and EPC-derived exosomes and the impact of iPS-iMSC exosomes could be shown by Lin (Hu *et al.*, 2019; Liu *et al.*, 2016). Other exosomes used to trigger EC migration were derived from cardiomyocytes or osteoblasts (Vrijssen *et al.*, 2016; Tang *et al.*, 2019).

A quantitative comparison of the above listed studies and a comparison of the results from this study is very difficult. The assays used differed, and the type of migration measurement varied. Even comparing studies using scratch assays is not possible because there is no standard for the migration measurement. Counting migrated cells is depending on investigated scratch area and width and even magnification used for imaging. In this study, the migration effect was translated into percentage values compared to control conditions. With the use of

the selfmade scratch machine as a potency assay producing reproducible results, this study for the first time demonstrated the impact of EVPs derived from adipogenic-differentiated MSCs on EC migration in migrated and then re-collected cells. Thus, to uncover possible signal transduction pathways, protein isolated from the collected cells could be used for further investigations.

Phosphokinase arrays were employed on lysates from non-migrated and migrated cells with under three conditions: 1) addition of 1 $\mu\text{g/mL}$ adipogenic-induced EVPs in FBS-depleted medium, 2) ultra-centrifuged conditioned from adipogenic-induced MSCs, and 3) control in FBS-depleted medium. The arrays revealed that 9 proteins displayed a significantly increased phosphorylation: eNOS, GSK-3 β , Akt, STAT5a/b, Chk-2, c-Jun, RSK, p70 S6 kinase, and STAT3. The eNOS signal doubled in migrated cells with EVPs addition. Akt, Chk-2, c-Jun, RSK, and p70 S6 kinase signals increased significantly in migrated cells with addition of EVPs, though non-migrated and migrated cells in other conditions also exhibited high signals. RSK1/2/3 showed higher phosphorylation in migrated cells with EVPs addition. The STAT3 signal increased in migrated PAEC after EVPs addition, while STAT5a/b signals were high in all conditions, showing no difference between non-migrated and migrated PAEC. The GSK-3 β signal significantly increased in all migrated PAECs for all conditions. Western blot analysis confirmed these results for eNOS, GSK-3 β , and Akt1, whereas the other showed no statistically significant increase. The ability of the collection of migrated cells due to the own developed scratch machine enabled the investigation of activated signaling pathways including eNOS, Akt and GSK-3 β . The activation of Akt1/PI3K pathway was also shown by Liu (Liu *et al.*, 2016). Other groups also identified Akt signaling in migration. Vrijssen and colleagues showed Akt/Erk signaling in HMECs and the group of Yu identified the Akt/eNOS pathway after the addition of pre-conditioned BM-MSCs (Vrijssen *et al.*, 2016; Yu *et al.*, 2020). VEGF signaling is another key player, which was

shown by Wang and Tang (Wang *et al.*, 2021; Tang *et al.*, 2019). They detected increased VEGF levels resulting in activation of VEGF/Erk1/2 pathway of ECs after addition of EVs. In summary, the current study suggests a similar activation of pathways through adipogenic-derived EVPs. The activation and thus the migration of ECs is the first step to initiate the next step, which is the formation of tubular structures, and thus angiogenesis.

4.5 Angiogenesis Assays

Angiogenesis was assessed using Matrigel-coated plates with varying PAEC seeding numbers. AngioQuant software quantified network formation, evaluating average lengths, junction number, and average size. Since EVPs from week 3 and week 4 increased EC migration, these EVPs' effects on EC angiogenesis were first tested. Adipogenic-induced EVPs from FBS-depleted conditions of donors 2 and 3 notably increased lengths, size, and junctions, surpassing non-induced EVPs. These results were similar to the results of the scratch assays with the scratch machine. Week 4 EVPs had different outcomes; only donor 3's FBS-depleted adipogenic-induced EVPs increased size and junctions. These results are like the migration results and the reason might be a secretion of high amounts of pro-migratory cytokines and EVPs containing these.

Subsequently assays with further collected cells were done with donor 3's EVPs. EVPs from donor 3's week 3 were studied at various concentrations. Non-induced EVPs slightly raised angiogenic parameters such as length, size, and junctions, while adipogenic-induced EVPs from FBS-depleted conditions enhanced angiogenic parameters at 1 and 2 $\mu\text{g/mL}$.

Several studies investigated the role of exosomes and EVs in EC angiogenesis for different purposes, *e.g.* tumor development or wound healing. An enhanced angiogenesis through transferred miRNA after addition of MSC-derived

exosomes was observed by the working groups of Gong and by Ma (Gong *et al.*, 2017; Ma *et al.*, 2018). Shabbir showed a pro-angiogenic effect of MSC-derived exosomes in wound healing and Xue could show an enhanced angiogenesis of human brain microvascular ECs for future treatment of Parkinson's disease (Shabbir *et al.*, 2015; Xue *et al.*, 2021). Other working groups pre-treated MSCs, using hypoxia induction or treatment with drugs and overserved pro-angiogenic effects in ECs (Ding *et al.*, 2019; Liu *et al.*, 2019; Ge *et al.*, 2021). By applying MSC-derived exosomes several signaling pathways were identified, including VEGF, eNOS and Akt/Erk signaling. Elevated VEGF levels after addition of EVs from pretreated MSC were detected by different working groups. Han used hypoxia-induced AT-MSCs EVs whereas the group of Ren directly used EVs from adipogenic-induced MSCs Han *et al.*, 2019; Ren *et al.*, 2019). Chen and colleagues showed that MSC-derived miRNA-150-5p decreased migration and invasion in and downregulated tube formation in HUVECs by targeting MMP14 and VEGF (Chen *et al.*, 2018). These results are in line with the elevated VEGF levels after addition of EVPs from adipogenic-induced MSCs in this study. The addition of EVs from other MSCs also increased VEGF levels. Tang and colleagues saw increased VEGF levels after addition on EVs from mature osteoblasts in HUVECs and the group of Yu observed increased VEGF and eNOS levels in HUVECs after the addition of atorvastatin pre-treated MSCs (Tang *et al.*, 2019; Yu *et al.* 2020). This study identified activated eNOS, GSK-3 β , and Akt1 in migrated cells after exosomes addition and several phosphorylated proteins like eNOS, ERK1/2, PDGF- β R, STA2, and STAT5a/b.

In 2022, Tian and colleagues showed an improved migration and angiogenesis through miR-185-5p via Akt1/GSK-3 β /VEGF signaling, which correlates with our data (Tian *et al.*; 2022). Yu and colleagues identified the activation of eNOS/AKT pathway by using atorvastatin, as mentioned above (Yu *et al.* 2020). The working group of Ren also identified the activation of the AKT/ERK1/2

pathway after addition of AT-MMSC derived EVs. These results are in line with the phosphorylated proteins in the angiogenesis investigations of this study. Liang showed the activation of AKT/mTOR pathway by using dimethyloxaloylglycine (DMOG) pre-treated AT-MSCs (Ren *et al.*, 2019; Liang *et al.*, 2019). Other working groups showed the activation of HMGB1/AKT pathway by using BM-MSC-derived EVs or activation of PTEN/AKT pathway after addition of EVs from melatonin-stimulated MSCs (Gao *et al.*, 2020; Liu *et al.*, 2020). Ning showed that MSCs-derived exosomes with low-expressed miR-153-3p notably promotes the activation of ANGPT1 and the VEGF/VEGFR2/PI3K/Akt/eNOS pathways, thereby preventing the damages endothelial cells (Ning *et al.*, 2021). Summarizing the literature, AKT and ERK seem to be crucial molecules for angiogenesis after addition of exosomes or EVs. This study additionally identified STAT5a/b as a major player in angiogenesis after addition of adipogenic MSC-derived EVs. This molecule is known as a molecular regulator of proliferation, differentiation and apoptosis (Nosaka *et al.*, 1999). Additionally, it plays a role in cancer development (Tan *et al.*, 2008).

The major difference between this study and published data is the investigation of activated cells. Here, for the first time, migrated cells and tube forming cells after EV addition were investigated on the molecular level. All studies so far focused on migration and angiogenic properties of ECs after exosome addition. Signaling pathways and contributing proteins were investigated from static ECs after exosomes or EV addition. This might be an explanation for the identified pathways (AKT, eNOS, GSK-3 β) in migration, but not in angiogenesis. Next to AT-MSC-derived exosomes, other MSCs showed an impact on EC angiogenesis, like BM-MSCs, Human umbilical cord MSCs, human-placenta derived MSCs and iPSCs (Takeuchi *et al.*, 2019; Qiu *et al.*, 2020; Xiong *et al.*, 2018; Zhang *et al.*, 2021; Komaki *et al.*, 2017; Liu *et al.*, 2017). Recently, Huynh detected and upregulation of VWF and VEGFR-2 (Flk1) genes after addition of umbilical cord

MSC-derived exosomes (Huynh *et al.*, 2023). Not only MSCs enhance angiogenesis, the same effect was also shown for exosomes from cardiomyocytes, cardiomyocyte progenitor cells, ocells, and even osteoblasts (Vrijssen *et al.*, 2016; Dougherty *et al.*, 2018; Wang *et al.*; 2021; Takeuchi *et al.*, 2019). The use of a Matrigel assay as potency assays is controversial. The coating of the wells is not always identical and the formed networks have to be handled very carefully because of their instable adherence. Additionally, a huge number of cells and Matrigel is needed to perform molecular biological experiments in networking forming ECs. During the implementation of experiments for the study, Matrigel was not available in sufficient amounts on the world market. The data of the phosphokinase assay give hints that in angiogenesis different pathways than in EC migration were activated after EVPs addition. To confirm these preliminary results further investigations, have to be done.

4.6 Soluble Factors in Conditioned Medium

Soluble secreted factors alongside EVPs during adipogenic differentiation were investigated by using commercial cytokine and adipokine arrays. Conditioned medium from week 3 of adipogenic differentiation under FBS-depleted conditions was compared to control medium, adipogenic ultra-centrifuged conditioned medium of week 3, and control medium supplemented with 1 µg/mL adipogenic-induced EVPs. Both arrays revealed numerous upregulated cytokines in conditioned and ultra-centrifuged medium. Not surprisingly, adipogenic marker proteins like Leptin and Adiponectin were upregulated in adipogenic CM and ultra-centrifuged adipogenic CM. The cytokine array also identified strong signals for molecules involved in immune response and inflammatory signaling. Among them were IL-6, IL-8, CXCL5 (C-X-C motif chemokine 5) a chemokine for immune response, CCL2 (monocyte chemoattractant protein 1) a cytokine in

inflammation, and GDF-15, regulating inflammatory pathways (Carr *et al.*, 1994; Zimmer *et al.*, 2005; Luttmann, 2014). Others are known to contribute to growth of cells, like IGFBP3 (insulin-like growth factor-binding protein-3) known for promoting growth by transactivating the EGF receptor or participating in angiogenesis, like Angiogenin (ribonuclease 5) (Granta *et al.*, 2004; Gao *et al.*, 2008). Additionally, Serpin E1 (plasminogen activator inhibitor-1) responsible for coagulation of blood and TSG14 (pentraxin 3) a tumor necrosis factor were detected (Binder *et al.*, 2002; Lee *et al.*, 1993).

Exclusive upregulations in ultra-centrifuged medium included Vitamin D-binding protein and the growth factors PDGF-AA, Ang-1, Ang-2 and HGF (Gallagher and Lyon, 2000; Thurston *et al.*, 2003; Fredriksson *et al.*, 2004; Norman *et al.*, 2008). Molecules participating in immune response were interleukins (1a, 11, 17, 19, 22), ICAM-1, a regulator in immune response and IFN γ , a key player in inflammation (Schroder *et al.*, 2004; Bui *et al.*, 2020). Additionally, Complement Factor D, which has a high level of expression in fat, suggesting a role for adipose tissue in immune system biology was increased (Ronti *et al.*, 2006).

The detected increased are not known to play crucial roles in EC migration or EC activation. In conclusion, the enhanced migration of ECs after addition of EVPs can be explained by secreted EVs and not by secreted factors. Especially ultra-centrifuged CM was shown to decrease EC migration significantly.

The adipokine array mirrored the cytokine array findings. Numerous adipokines were upregulated in adipogenic CM and ultra-centrifuged adipogenic CM including Cathepsins (D, L, S), Complement Factor D, IGFBP (3, 4, 6, 7), interleukins (6 & 8), CCL2, M-CSF, Nidogen-1, TSG-14, Serpin-E1 and TIMP-1. As already discussed, most of the upregulated secreted adipokines are related to immune response and inflammation but are not known to activate migration signal transduction.

In summary, the secreted soluble factors of adipogenic-differentiated MSCs were presumably not the major players in EC migration. While the addition of EVPs lead to higher migration rates, leading to the hypothesis of EVs carrying bioactive cargo for this. The cargo of these EVs had an impact on signaling cascades in EC migration, either by their internalization into the cells, or the release of their cargo into cells by surface binding or just by surface binding. Several pathways are known to activate EC migration. Many of them are activated by cytokines such as VEGF, PDGF, hFGF or HGF. In this work 3 downstream phosphorylated proteins during EC migration were identified after EVPs addition (eNOS, Akt and GSK-3 β). eNOS and Akt have been shown to be activated by VEGF and PDGF supporting these findings. Additionally, eNOS and Akt pathway were shown to be activated by EVs in previous studies indicating to carry VEGF and PDGF as EV cargo.

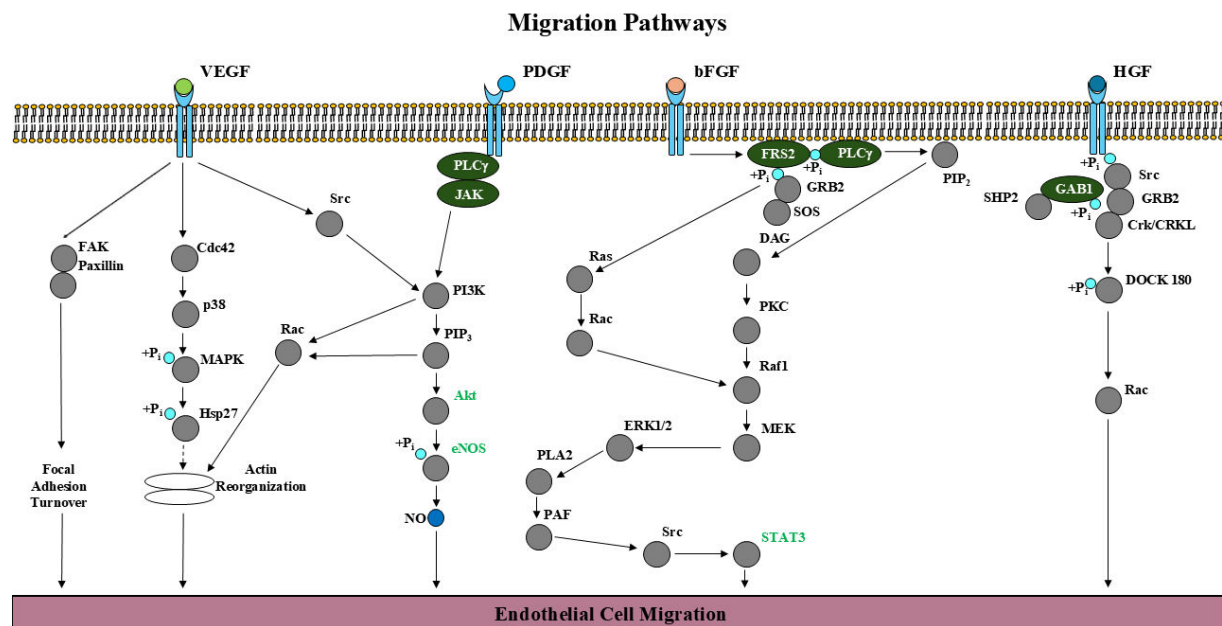


Fig. 41: Signaling Pathways in EC Migration. The cytokines VEGF, PDGF, bFGF, and HGF activated EC migration via different signaling pathways. Akt and eNOS pathways can be activated by VEGF and PDGF.

Here for the first time migrated ECs were investigated by re-collecting the cells after activation via added EVPs. The results showed that EVs were taken up or bound by the recipient cells within 15 minutes and reaching a maximum plateau after 75 minutes. Since the EVPs were labeled with antibodies against the exosomal markers CD9, CD63 and CD81 it cannot be ruled out if these binding blocked the uptake. Nevertheless, the addition of EVPs clearly showed an increased EC migration in the developed potency assays. The investigation of the activated and migrated ECs revealed the activation of eNOS and Akt signaling. Also, the addition of adipogenic EVPs lead to an increased network formation in angiogenesis assays, indicating the bioactive potency of these EVPs.

4.7 Future Work and Perspectives

The results of this work show that EVPs from adipogenic-differentiated MSCs significantly enhance endothelial cell migration and angiogenesis. These two parameters are signs for endothelial cell activation, which is an important step in early atherosclerosis. Since obesity and the connected metabolic syndrome are risk factors for the development of atherosclerosis, the findings of this study might shed a better light on the mechanisms and lead therefore to new approaches for drug development in the future. The new findings that not only secreted factors from developing adipocytes during the process of gaining weight might activate endothelial cells, but also secreted exosomes contribute to the activation is of interest for new approaches in healing this disease.

Next to this more molecules have been found which might be included these processes.

In detail in this study, it was shown that adipogenic EVPs of the late differentiation state of MSCs towards adipocytes significantly triggered endothelial cell activation via pathways well known for migration and angiogenesis such as Akt, eNOS and GSK-3 β -pathways highlighting the importance of these signaling pathways in the early development of EC activation which might lead to atherosclerosis.

Future works should focus on the identification of the concrete EVs cargo to reveal the specific molecules which might be growth factors, mi-RNA or other proteins of these adipogenic EVs which are triggering these devastating long-term effects such as heart attacks or strokes.

References

- Ahrens, M., Ankenbauer, T., Schroder, D., Hollnagel, A., Mayer, H., Gross, G. (1993) Expression of human bone morphogenetic proteins-2 or -4 in murine mesenchymal progenitor C3H10T1/2 cells induces differentiation into distinct mesenchymal cell lineages. *DNA Cell. Biol.*, 12, 871-880.
- Andreu, Z., and Yáñez-Mó, M. (2014). Tetraspanins in extracellular vesicle formation and function. *Front. Immunol.*, 5, 442.
- Autieri, M.V. (2012) Pro- and Anti-Inflammatory Cytokine Networks in Atherosclerosis. *ISRN Vas. Med.*, 17, 987629.
- Aust, L., Devlin, B., Foster, S.J., Halvorsen, Y.D., Hicok, K., du Laney, T., Sen, A., Willingmyre, G.D., Gimble, J.M. (2004) Yield of human adipose-derived adult stem cells from liposuction aspirates. *Cytotherapy*, 6, 7-14.
- Banas, A., Teratani, T., Yamamoto, Y., Tokuhara, M., Takeshita, F., Quinn, G., Okochi, H., Ochiya, T. (2007) Adipose tissue-derived mesenchymal stem cells as a source of human hepatocytes. *Hepatology*, 46, 219-228.
- Bhatta, M. P., Won, G. W., Lee, S. H., Choi, S. H., Oh, C. H., Moon, J. H., Hoang, H. H., Lee, J., Lee, S. D., & Park, J. I. (2024). Determination of adipogenesis stages of human umbilical cord-derived mesenchymal stem cells using three-dimensional label-free holotomography. *Methods*, 231, 204 - 214.
- Binder, B., Christ, G., Gruber, F., Grubic, N., Hufnagl, P., Krebs, M., Mihaly, J., Prager, G. (2002) Plasminogen activator inhibitor 1: physiological and pathophysiological roles. *News Physiol. Sci.*, 17, 56-61.
- Bruno, S., Grange, C., Deregibus, M.C., Calogero, R.A., Saviozzi, S., Collino, F. (2009) Mesenchymal stem cell-derived microvesicles protect against acute tubular injury. *J. Am. Soc. Nephrol.*, 20, 1053-1067.
- Bui, T., Wiesolek, H.L., Sumagin, R. (2020) ICAM-1: A master regulator of cellular responses in inflammation, injury resolution, and tumorigenesis. *J. Leukoc. Biol.*, 108, 787-799.
- Butterwith, S.C., Wilkie, R.S., Clinton, M. (1996) Treatment of pluripotential C3H 10T1/2 fibroblasts with bone morphogenetic protein-4 induces adipocyte commitment. *Biochem. Soc. Trans.*, 24, 163-164.
- Carmeliet P. (2003) Angiogenesis in health and disease. *Nat. Med.*, 9, 653 – 660.

Carmeliet, P, De, S.F., Loges, S., Mazzone, M. (2009) Branching morphogenesis and antiangiogenesis candidates: tip cells lead the way. *Nat. Rev. Clin. Oncol.*, 6, 315 – 326.

Carr, M.W., Roth, S.J., Luther, E., Rose, S.S., Springer, T.A. (1994) Monocyte chemoattractant protein 1 acts as a T-lymphocyte chemoattractant. *Proc. Natl. Acad. Sci. USA*. 91, 3652-3656.

Cerutti, C., Ridley, A.J. (2017) Endothelial cell-cell adhesion and signaling. *Exp. Cell. Res.*, 358, 31-38.

Chahine, M.N., Blackwood, D.P., Dibrov, E., Richard, M.N., Pierce, G.N. (2008) Oxidized LDL affects smooth muscle cell growth through MAPK-mediated actions on nuclear protein import. *J. Mol. Cell. Cardiol.*, 46, 431-441.

Chargaff, E., and West, R. (1946) The biological significance of the thromboplastic protein of blood. *J. Biol. Chem.* 166, 189 – 197.

Chen, Z., Wang, H., Xia, Y., Yan, F., Lu, Y. (2018) Therapeutic Potential of Mesenchymal Cell-Derived miRNA-150-5p-Expressing Exosomes in Rheumatoid Arthritis Mediated by the Modulation of MMP14 and VEGF. *J. Immunol.*, 201, 2472 – 2482.

Choi, D. S., Kim, D. K., Kim, Y. K., and Gho, Y. S. (2013). Proteomics, transcriptomics and lipidomics of exosomes and ectosomes. *Proteomics*, 13, 1554 – 1571.

Cines, D.B., Pollak, E.S., Buck, C.A., Loscalzo, J., Zimmerman, G.A., McEver, R.P., Pober, J.S., Wick, T.M., Konkle, B.A., Schwartz, B.S., Barnathan, E.S., McCrae, K.R., Hug, B.A., Schmidt, A.M., Stern, D.M. (1998) Endothelial cells in physiology and in the pathophysiology of vascular disorders. *Blood*, 91, 3527-2561.

Colombo, M., Raposo, G., and Théry, C. (2014) Biogenesis, secretion, and intercellular Interactions of exosomes and other extracellular vesicles. *Annu. Rev. Cell Dev. Biol.*, 30, 255 – 289.

Console, L., Scalise, M., Indiveri, C. (2019) Exosomes in inflammation and role as biomarkers. *Clin. Chim. Acta.*, 488, 165 – 171.

De Caterina, R., Libby, P., Peng, H.B., Thannickal, V.J., Rajavashisth, T.B., Gimbrone, M.A. Jr, Shin, W.S., Liao, J.K. (1995) Nitric oxide decreases cytokine-induced endothelial activation. Nitric oxide selectively reduces endothelial expression of adhesion molecules and proinflammatory cytokines. *J. Clin. Invest.* 96, 60 – 68.

de Gassart, A., Geminard, C., Fevrier, B., Raposo, G., Vidal, M. (2003) Lipid raft-associated protein sorting in exosomes. *Blood*, 102, 4336 – 4344.

Dejana, E., Orsenigo, F., Molendini, C., Baluk, P., McDonald, D.M. (2009) Organization and signaling of endothelial cell-to-cell junctions in various regions of the blood and lymphatic vascular trees. *Cell Tissue Res.*, 335, 17 – 25.

De Ugarte, D.A., Alfonso, Z., Zuk, P.A., Elbarbary, A., Zhu, M., Ashjian, P., Benhaim, P., Hedrick, M.H., Fraser, J.K. (2003a) Differential expression of stem cell mobilization-associated molecules on multi-lineage cells from adipose tissue and bone marrow. *Immunol. Lett.*, 89, 267 – 270.

Ding, D.C., Shyu, W.C., Lin, S.Z. (2011) Mesenchymal stem cells. *Cell Transplant.*, 20, 5 – 14.

Ding, J., Wang, X., Chen, B., Zhang, J., and Xu, J. (2019) Exosomes derived from human bone marrow mesenchymal stem cells stimulated by deferoxamine accelerate cutaneous wound healing by promoting angiogenesis. *Biomed. Res. Int.*, 5, 9742765.

Dominici, M., Le Blanc, K., Mueller, I., Slaper-Cortenbach, I., Marin, F., Krause, D., Deans, R., Keating, A., Prockop, D.J., Horwitz, E. (2006) Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. *Cytotherapy*, 8, 315 – 317.

Dougherty, J.A., Kumar, N., Noor, M., Angelos, M.G., Khan, M., Chen, C.A., Khan, M. (2018) Extracellular Vesicles Released by Human Induced-Pluripotent Stem Cell-Derived Cardiomyocytes Promote Angiogenesis. *Front Physiol.*, 9, 1794.

Duijvesz, D., Luiders, T., Bangma, C.H., Jenster, G. (2011) Exosomes as biomarker treasure chests for prostate cancer. *Eur Urol.*, 59, 823 – 831.

Eckel, R.H., Grundy, S.M., Zimmet, P.Z. (2005) The metabolic syndrome. *Lancet*, 365, 1415 – 1428.

- Fadini, G.P., Pauletto, P., Avogaro, A., Rattazzi, M. (2007) The good and the bad in the link between insulin resistance and vascular calcification. *Atherosclerosis*, 193(2), 241-244
- Fang, Y., Zhang, Y., Zhou, J., Cao, K. (2019) Adipose-derived mesenchymal stem cell exosomes: a novel pathway for tissues repair. *Cell Tissue Bank*, 20, 153 – 161.
- Fernández-Veledo, S., Hernandez, R., Teruel, T., Mas, J., Ros, M., Lorenzo, M. (2006) Ceramide mediates TNF-alpha-induced insulin resistance on GLUT4 gene expression in brown adipocytes. *Arch. Physiol. Biochem.*, 112, 13 – 22.
- Ferrari, G., Cusella-De Angelis, G., Coletta, M., Paolucci, E., Stornaiuolo, A., Cossu, G., Mavilio, F. (1998) Muscle regeneration by bone marrow-derived myogenic progenitors. *Science*, 279, 1528 – 1530.
- Ferguson, J.E. III, Kelley, R.W., Patterson, C. (2005) Mechanisms of endothelial differentiation in embryonic vasculogenesis. *Arterioscler. Thromb. Vasc. Biol.*, 25, 2246 – 2254.
- Finney, A.C., Stokes, K.Y., Pattillo, C.B., Orr, A.W. (2017) Integrin signaling in atherosclerosis. *Cell. Mol. Life. Sci.*, 74, 2263 – 2282.
- Fredriksson L, Li H, Eriksson U. (2004) The PDGF family: four gene products form five dimeric isoforms. *Cytokine Growth Factor Rev.*, 15, 197 – 204.
- Galic, S., Oakhill, J.S., Steinberg, G.R. (2009) Adipose tissue as an endocrine organ. *Mol. Cell. Endocrinol.*, 25, 316, 129 – 139.
- Gallagher, J.T, Lyon, M. (2000). “Molecular structure of Heparan Sulfate and interactions with growth factors and morphogens”. In Iozzo MV (ed.). *Proteoglycans: structure, biology and molecular interactions*. Marcel Dekker Inc. New York, New York. pp. 27 – 59.
- Gao, W., Liang, T., He, R., Ren, J., Yao, H., Wang, K., Zhu, L., Xu Y. (2021) Exosomes from 3D culture of marrow stem cells enhances endothelial cell proliferation, migration, and angiogenesis via activation of the HMGB1/AKT pathway. *Stem Cell Res.*, 50, 102122.
- Gao X, Xu Z. (2008) Mechanisms of action of angiogenin. *Acta. Biochim. Biophys. Sin.*, 40, 619 – 624.

Ge, L., Xun, C., Li, W., Jin, S., Liu, Z., Zhuo, Y., Duan, D., Hu, Z., Chen, P., Lu, M. (2021) Extracellular vesicles derived from hypoxia-preconditioned olfactory mucosa mesenchymal stem cells enhance angiogenesis via miR-612. *J. Nanobiotechnology*, 19, 380.

Germain, S., Monnot, C., Muller, L., Eichmann, A. (2010) Hypoxiadriven angiogenesis: role of tip cells and extracellular matrix scaffolding. *Curr. Opin. Hematol.* 17, 245 – 251.

Gimbrone, M.A. Jr, Nagel, T., Topper, J.N. (1997) Biomechanical activation: An emerging paradigm in endothelial adhesion biology. *J. Clin. Invest.*, 99, 1809 – 1813.

Gong, M., Yu, B., Wang, J., Wang, Y., Liu, M., Paul, C., Millard, R.W., Xiao, D.S., Ashraf, M., Xu, M. (2017) Mesenchymal stem cells release exosomes that transfer miRNAs to endothelial cells and promote angiogenesis. *Oncotarget*, 11, 8, 45200 – 45212.

Granata R, Trovato L, Garbarino G, Taliano M, Ponti R, Sala G, Ghidoni R, Ghigo E. (2004) Dual effects of IGFBP-3 on endothelial cell apoptosis and survival: involvement of the sphingolipid signaling pathways. *FASEB J.*, 18, 1456 – 1458.

Grimmig, R., Babczyk, P., Gillemot, P., Schmitz, K.P., Schulze, M., Tobiasch, E. (2019) Development and Evaluation of a Prototype Scratch Apparatus for Wound Assays Adjustable to Different Forces and Substrates. *Appl. Sci.*, 9, 4414.

Gronthos, S., Franklin, D.M., Leddy, H.A., Robey, P.G., Storms, R.W., Gimble, J.M. (2001) Surface protein characterization of human adipose tissue-derived stromal cells. *J. Cell Physiol.*, 189, 54 – 63.

Gupta, M. P., Tandalam, S., Ostrager, S., Lever, A. S., Fung, A. R., Hurley, D. D., *et al.* (2019) Non-reversible tissue fixation retains extracellular vesicles for in situ imaging. *Nat. Methods*, 16, 1269 – 1273.

Gurunathan, S., Kang, M., Jeyaraj, M., Qasim, M., and Kim, J. (2019) Review of the isolation, characterization, biological function, and multifarious therapeutic approaches of exosomes. *Cells*, 8, 307.

Han, Y., Ren, J., Bai, Y., Pei, X., and Han, Y. (2019) Exosomes from hypoxia-treated human adipose-derived mesenchymal stem cells enhance angiogenesis through VEGF/VEGF-R. *Int. J. Biochem. Cell Biol.*, 109, 59 – 68.

Harding, C., Heuser, J., and Stahl, P. (1984) Endocytosis and intracellular processing of transferrin and colloidal gold-transferrin in rat reticulocytes: demonstration of a pathway for receptor shedding. *Eur. J. Cell Biol.*, 35, 256 – 263.

Hass, R., Kasper, C., Böhm, S., Jacobs, R. (2011) Different populations and sources of human mesenchymal stem cells (MSC): a comparison of adult and neonatal tissue derived MSC. *Cell Commun. Signal.*, 9, 12.

Hauner, H. (2005) Secretory factors from human adipose tissue and their functional role. *Proc. Nutr. Soc.*, 64, 163 – 169.

Haynesworth, S.E., Baber, M.A., Caplan, A.I. (1996) Cytokine expression by human marrow-derived mesenchymal progenitor cells in vitro: effects of dexamethasone and IL-1 alpha. *J. Cell Physiol.*, 166, 5, 85 – 92.

Hsiao, S.T., Asgari, A., Lokmic, Z., Sinclair, R., Disting, G.J., Lim, S.Y., Dilley R.J. (2012) Comparative analysis of paracrine factor expression in human adult mesenchymal stem cells derived from bone marrow, adipose, and dermal tissue. *Stem Cells Dev.*, 21, 2189 – 2203.

Hu, H., Jiang, C, Li, R., Zhao, J. (2019) Comparison of endothelial cell- and endothelial progenitor cell-derived exosomes in promoting vascular endothelial cell repair. *Int. J. Clin. Exp. Pathol.*, 1, 12, 2793 – 2800.

Hu, Y., Zhang, R., Chen, G.. (2020) Exosome and secretion: action on? *Adv. Exp. Med. Biol.*, 1248, 455 – 483.

Hu, X., Ning, X., Zhao, Q., Zhang, Z., Zhang, C., Xie, M., Huang, W., Cai ,Y., Xiang, Q., Ou, C. (2022) Islet-1 Mesenchymal Stem Cells-Derived Exosome-Incorporated Angiogenin-1 Hydrogel for Enhanced Acute Myocardial Infarction Therapy. *ACS Appl. Mater. Interfaces*, 17, 14, 36289 – 36303.

Huang, P.L. (2009) A comprehensive definition for metabolic syndrome. *Dis. Model. Mech.*, 2, 231 – 237.

Huynh, P.D., Van Pham, P., Vu N.B. (2023) Exosomes Derived from Human Umbilical Cord Mesenchymal Stem Cells Enhance Angiogenesis Through Upregulation of the VWF and Flk1 Genes in Endothelial Cells. *Adv. Exp. Med. Biol.*, Online ahead of print

Hynes, R.O., Bader, B.L, Hodivala-Dilke, K. (1999) Integrins in vascular development. *Braz. J. Med. Biol. Res.*, 32, 501 – 510.

Iraci, N., Leonardi, T., Gessler, F., Vega, B., Pluchino, S. (2016) Focus on Extracellular Vesicles: Physiological Role and Signalling Properties of Extracellular Membrane Vesicles. *Int. J. Mol. Sci.*, 17, 171.

Jeppesen, D.K., Fenix, A.M., Franklin, J.L., Higginbotham, J.N., Zhang, Q., Zimmerman, L.J., Liebler, D.C., Ping, J., Liu, Q., Evans, R., Fissell, W.H., Patton, J.G., Rome, L.H., Burnette, D.T., Coffey, R.J. (2019) Reassessment of Exosome Composition. *Cell*, 4, 177, 428 – 445.

Johnstone, R.M., Adam, M., Hammond, J.R., Orr, L., Turbide, C. (1987) Vesicle formation during reticulocyte maturation. Association of plasma membrane activities with released vesicles (exosomes). *J. Biol. Chem.*, 262, 9412 – 9420.

Joo, H.S., Suh, J.H., Lee, H.J., Bang, E.S., Lee, J.M. (2020) Current Knowledge and Future Perspectives on Mesenchymal Stem Cell-Derived Exosomes as a New Therapeutic Agent. *Int. J. Mol. Sci.*, 21, 727.

Juan, T., and Fürthauer, M. (2018) Biogenesis and function of ESCRT-dependent extracellular vesicles. *Semin. Cell Dev. Biol.*, 74, 66 – 77.

Kern, S., Eichler, H., Stoeve, J., Kluter, H., Bieback, K. (2006) Comparative analysis of mesenchymal stem cells from bone marrow, umbilical cord blood, or adipose tissue. *Stem Cells*, 24, 1294 – 1301.

Khan, D., Kleinfeld, C., Winter, M., Tobiasch, E. (2012) Oral tissues as source for bone regeneration in dental implantology. In: Davies J., editor. Tissue regeneration – From basic biology to clinical application. *InTech: Rijeka*, 325 – 335.

Kingsley, K., Huff, J.L., Rust, W.L., Carroll, K., Martinez, A.M., Fitchmun, M., Plopper, G.E. (2002) ERK1/2 mediates PDGF-BB stimulated vascular smooth muscle cell proliferation and migration on laminin-5. *Biochem. Biophys. Res. Commun.*, 10, 293, 1000 – 1006.

Kiosses, W.B., Shattil, S.J., Pampori, N., Schwartz, M.A. (2001) Rac recruits high-affinity integrin α v β 3 to lamellipodia in endothelial cell migration. *Nat. Cell. Biol.*, 3, 316 – 320.

Kitagawa, Y., Kobori, M., Toriyama, K., Kamei, Y., Torii, S. (2006) History of discovery of human adipose-derived stem cells and their clinical application. *Japan. J. Plastic Reconstruct. Sur.*, 49, 1097 – 1104.

Klagsbrun, M., and D'amore, P. J. (1991) Regulators of angiogenesis. *Annu. Rev. Physiol.*, 53, 217 – 239.

Komaki, M., Numata, Y., Morioka, C., Honda, I., Tooi, M., Yokoyama, N., Ayame, H., Iwasaki, K., Taki, A., Oshima, N., Morita, I. (2017) Exosomes of human placenta-derived mesenchymal stem cells stimulate angiogenesis. *Stem Cell Res. Ther.*, 3, 8, 219.

Kopen, G.C., Prockop, D.J., Phinney, D.G. (1999) Marrow stromal cells migrate throughout forebrain and cerebellum, and they differentiate into astrocytes after injection into neonatal mouse brains. *Proc. Natl. Acad. Sci. USA*, 96, 10711 - 10716.

Kreimer, S., Belov, A. M., Ghiran, I., Murthy, S. K., Frank, D. A., and Ivanov, A. R. (2015) Mass-spectrometry-based molecular characterization of extracellular vesicles: lipidomics and proteomics. *J. Proteome Res.*, 14, 2367 – 2384.

Kubes, P., Suzuki, M., Granger, D.N. (1991) Nitric oxide: An endogenous modulator of leukocyte adhesion. *Proc. Natl. Acad. Sci.*, 88, 4651 – 4655.

Kuhlencordt, P.J., Gyurko, R., Han, F., Scherrer-Crosbie, M., Aretz, T.H., Hajjar, R., Picard, M.H., Huang P.L. (2001) Accelerated atherosclerosis, aortic aneurysm formation, and ischemic heart disease in apolipoprotein E/endothelial nitric oxide synthase double-knockout mice. *Circulation*, 24, 104, 448 – 454.

Lange, M. (2013) Artery Specific Differences in Cell Cycle Regulation are Associated with Serum Induced Proliferation of Vascular Smooth Muscle Cells. *B.Sc. Thesis*, Hochschule Bonn-Rhein-Sieg.

Lee, G.W., Lee, T.H., Vilcek, J. (1993) TSG-14, a tumor necrosis factor- and IL-1-inducible protein, is a novel member of the pentaxin family of acute phase proteins. *J. Immunol.*, 150, 1804 – 1812.

Lee, R.H., Kim, B., Choi, I., Kim, H., Choi, H.S., Suh, K., Bae, Y.C., Jung, J.S. (2004) Characterization and expression analysis of mesenchymal stem cells from human bone marrow and adipose tissue. *Cell. Physiol. Biochem.*, 14, 311 – 324.

Lertkiatmongkol, P., Liao, D., Mei, H., Hu, Y., Newman, P.J. (2016) Endothelial functions of platelet/endothelial cell adhesion molecule-1 (CD31). *Curr Opin Hematol.*, 23, 253 – 259.

Li, H., Cybulsky, M.I., Gimbrone, M.A. Jr, Libby, P. (1993) An atherogenic diet rapidly induces vcam-1, a cytokine- regulatable mononuclear leukocyte adhesion molecule, in rabbit aortic endothelium. *Arterioscler. Thromb.*, 13, 197 – 204.

- Liang, B., Liang, J.M., Ding, J.N., Xu, J., Xu, J.G., Chai, Y.M. (2019) Dimethylloxaloylglycine-stimulated human bone marrow mesenchymal stem cell-derived exosomes enhance bone regeneration through angiogenesis by targeting the AKT/mTOR pathway. *Stem. Cell. Res. Ther.*, 10, 335.
- Liao, J.K., Shin, W.S., Lee, W.Y., Clark, S.L. (1995) Oxidized low-density lipoprotein decreases the expression of endothelial nitric oxide synthase. *J. Biol. Chem.*, 270, 319 – 324.
- Liao, J.K. (2013) Linking endothelial dysfunction with endothelial cell activation. *J Clin Invest.*, 123(2), 540-541
- Libby, P., Ridker, P.M., Maseri, A. (2002) Inflammation and atherosclerosis. *Circulation*, 105(9), 1135–1143
- Libby, P. (2005) The forgotten majority: unfinished business in cardiovascular risk reduction. *J. Am. Coll Cardiol.*, 46, 1225 – 1228.
- Lin, C.S., Xin, Z.C., Deng, C.H., Ning, H., Lin, G., Lue, T.F. (2010) Defining adipose tissue-derived stem cells in tissue and in culture. *Histol. Histopathol.*, 25, 807 – 815.
- Liu, M., Han, Z. (2008) Mesenchymal stem cells: biology and clinical potential in type 1 diabetes therapy. *J. Cell. Mol. Med.*, 12, 1155 – 1168.
- Liu, S. S., Fang, X., Wen, X., Liu, J. S., Alip, M., Sun, T., Wang, Y. Y., & Chen, H. W. (2024). How mesenchymal stem cells transform into adipocytes: Overview of the current understanding of adipogenic differentiation. *World journal of stem cells*, 16, 245 - 256.
- Liu, X., Li, Q., Niu, X., Hu, B., Chen, S., Song, W., Ding, J., Zhang, C., Wang, Y. (2017) Exosomes Secreted from Human-Induced Pluripotent Stem Cell-Derived Mesenchymal Stem Cells Prevent Osteonecrosis of the Femoral Head by Promoting Angiogenesis. *Int. J. Biol. Sci.*, 13, 232 – 244.
- Liu, W., Li, L., Rong, Y., Qian, D., Chen, J., Zhou, Z., Luo, Y., Jiang, D., Cheng, L., Zhao, S., Kong, F., Wang, J., Zhou, Z., Xu, T., Gong, F., Huang, Y., Gu, C., Zhao, X., Bai, J., Wang, F., Zhao, W., Zhang, L., Li, X., Yin, G., Fan, J., Cai, W. (2020) Hypoxic mesenchymal stem cell-derived exosomes promote bone fracture healing by the transfer of miR-126. *Acta. Biomater.*, 103, 196 – 212.
- Liu, W., Yu, M., Xie, D., Wang, L., Ye, C., Zhu, Q., Liu, F., Yang, L. (2020) Melatonin-stimulated MSC-derived exosomes improve diabetic wound healing through regulating macrophage M1 and M2 polarization by targeting the PTEN/AKT pathway. *Stem Cell Res Ther.*, 11, 259.

Lugano, R., Ramachandran, M., Dimberg, A. J. C., and Sciences, M. L. (2020) Tumor angiogenesis: Causes, consequences, challenges and opportunities. *Cell. Mol. Life Sci.*, 77, 1745 – 1770.

Lund, A.W., Yener, B., Stegemann, J.P., Plopper, G.E. (2009) The natural and engineered 3D microenvironment as a regulatory cue during stem cell fate determination. *Tissue Eng. Part B Rev.*, 15, 371 – 380.

Luttmann, W. Der Experimentator: Immunologie. Springer-Verlag, 2014, ISBN 978-3-642-41899-0, S. 278 – 280.

Ma, T., Chen, Y., Chen, Y., Meng, Q., Sun, J., Shao, L., Yu, Y., Huang, H., Hu, Y., Yang, Z., Yang, J., Shen, Z. (2018) MicroRNA-132, Delivered by Mesenchymal Stem Cell-Derived Exosomes, Promote Angiogenesis in Myocardial Infarction. *Stem Cells Int.*, 9, 3290372.

Madel, R. J., Börger, V., Dittrich, R., Bremer, M., Tertel, T., Phuong, N. N. T., Baba, H. A., Kordelas, L., Staubach, S., Stein, F., Haberkant, P., Hackl, M., Grillari, R., Grillari, J., Buer, J., Horn, P. A., Westendorf, A. M., Brandau, S., Kirschning, C. J., & Giebel, B. (2023) Independent human mesenchymal stromal cell-derived extracellular vesicle preparations differentially attenuate symptoms in an advanced murine graft-versus-host disease model. *Cytotherapy*, 25, 821 – 836.

Mayo Clinic: <https://www.mayoclinic.org/diseases-conditions/metabolic-syndrome/symptoms-causes/syc-20351916> (accessed: July, 29th, 2023)

Merino-González, C., Zuñiga, F.A., Escudero, C., Ormazabal, V., Reyes, C., Nova-Lamperti, E., Salomón, C., Aguayo, C. (2016) Mesenchymal Stem Cell-Derived Extracellular Vesicles Promote Angiogenesis: Potencial Clinical Application. *Front Physiol.*, 9, 24.

Michaelis, U.R. (2014) Mechanisms of endothelial cell migration. *Cell. Mol. Life Sci.*, 71, 4131 – 4148.

Mingrone, G., Rosa, G., Di Rocco, P., Manco, M., Capristo, E., Castagneto, M., Vettor, R., Gasbarrini, G., Greco, A. (2002) Skeletal muscle triglycerides lowering is associated with net improvement of insulin sensitivity, TNF-alpha reduction and GLUT4 expression enhancement. *Int. J. Obes. Relat. Metab. Disord.*, 26, 1165 – 1172.

- Mueller, S.M., Glowacki, J. (2001) Age-related decline in the osteogenic potential of human bone marrow cells cultured in three-dimensional collagen sponges. *J. Cell Biochem.*, 82, 583 – 590.
- Mussap, M., Plebani, M. (2004) Biochemistry and clinical role of human cystatin C. *Crit. Rev. Clin. Lab. Sci.*, 41, 467 – 550.
- Narayanan, K., Kumar, S., Padmanabhan, P., Gulyas, B., Wan, A.C.A., Rajendran, V.M. (2018) Lineage-specific exosomes could override extracellular matrix mediated human mesenchymal stem cell differentiation. *Biomaterials*, 182, 312 – 322.
- Ning, W., Li, S., Yang, W., Yang, B., Xin, C., Ping, X., Huang, C., Gu, Y., Guo, L. (2021) Blocking exosomal miRNA-153-3p derived from bone marrow mesenchymal stem cells ameliorates hypoxia-induced myocardial and microvascular damage by targeting the ANGPT1-mediated VEGF/PI3k/Akt/eNOS pathway. *Cell Signal.*, 77, 109812.
- Nosaka T, Kawashima T, Misawa K, Ikuta K, Mui AL, Kitamura T. (1999) STAT5 as a molecular regulator of proliferation, differentiation and apoptosis in hematopoietic cells. *EMBO J.*, 18, 4754 – 4765.
- Norman AW. (2008) From vitamin D to hormone D: fundamentals of the vitamin D endocrine system essential for good health. *Am. J. Clin. Nutr.*, 88, 491 – 499.
- Ohara, Y., Peterson, T.E., Harrison, D.G. (1993) Hypercholesterolemia increases endothelial superoxide anion production. *J Clin. Invest.*, 91, 2546 – 2551.
- Orr, A.W., Lee, M.Y., Lemmon, J.A., Yurdagul, A. Jr., Gomez, M.F., Bortz, P.D., Wamhoff, B.R. (2009) Molecular mechanisms of collagen isotype-specific modulation of smooth muscle cell phenotype. *Arterioscler. Thromb. Vasc. Biol.*, 29, 225 – 231.
- Otto, T.C., Lane, M.D. (2005) Adipose Development: From Stem Cell to Adipocyte. *Crit. Rev. Biochem. Mol. Biol.*, 40, 229 – 242.
- Owens, G.K., Kumar, M.S., Wamhoff, B.R. (2004) Molecular regulation of vascular smooth muscle cell differentiation in development and disease. *Physiol. Rev.*, 84, 767 – 801.
- Pan, B. T., Johnstone, R. M. (1983) Fate of the transferrin receptor during maturation of sheep reticulocytes in vitro: selective externalization of the receptor. *Cell*, 33, 967 – 978.

Pan, Q., Wang, Y., Lan, Q., Wu, W., Li, Z., Ma, X., Yu, L. (2019) Exosomes Derived from Mesenchymal Stem Cells Ameliorate Hypoxia/Reoxygenation-Injured ECs via Transferring MicroRNA-126. *Stem Cells Int.*, 2, 2831756.

Pansky, A., Roitzheim, B., Tobiasch, E. (2007) Differentiation potential of adult human mesenchymal stem cells. *Clin. Lab.*, 53, 81 – 84.

Parithimarkalaignan, S., and Padmanabhan, T. (2013) Osseointegration: An update. *J. Indian Prosthodont. Soc.*, 13, 2 – 6.

Planat-Bénard, V., Menard, C., André, M., Puceat, M., Perez, A., Garcia-Verdugo, J.M., Pénicaud, L., Casteilla, L. (2004) Spontaneous cardiomyocyte differentiation from adipose tissue stroma cells. *Circ. Res.*, 94, 223 – 229.

Potente, M., Gerhardt, H., Carmeliet, P. (2011) Basic and therapeutic aspects of angiogenesis. *Cell*, 146, 873 – 887.

Pournasr, B., Mohamadnejad, M., Bagheri, M., Aghdami, N., Shahsavani, M., Malekzadeh, R., Baharvand, H. (2011) *In vitro* differentiation of human bone marrow mesenchymal stem cells into hepatocyte-like cells. *Arch. Iran Med.*, 14, 244 – 249.

Qiu, X., Liu, J., Zheng, C., Su, Y., Bao, L., Zhu, B., Liu, S., Wang, L., Wang, X., Wang, Y., Zhao, W., Zhou, J., Deng, Z., Liu, S., Jin, Y. (2020) Exosomes released from educated mesenchymal stem cells accelerate cutaneous wound healing via promoting angiogenesis. *Cell Prolif.*, 53, e12830.

Rajala, M.W., Scherer, P.E. (2003) Minireview: The adipocyte--at the crossroads of energy homeostasis, inflammation, and atherosclerosis. *Endocrinology*, 144, 3765 – 3773.

Rani, S., Ryan, A.E., Griffin, M.D., Ritter, T. (2015) Mesenchymal Stem Cell-derived Extracellular Vesicles: Toward Cell-free Therapeutic Applications. *Mol Ther.*, 23, 812 – 823.

Raposo, G., Nijman, H. W., Stoorvogel, W., Leijendekker, R., Harding, C. V., Melief, C. J. M. (1996) B lymphocytes secrete antigen-presenting vesicles. *J. Exp. Med.*, 183, 1161–1172.

Ratajczak, J., Miekus, K., Kucia, M., Zhang, J., Reca, R., Dvorak, P., Ratajczak, M.Z. (2006) Embryonic stem cell-derived microvesicles reprogram hematopoietic progenitors: evidence for horizontal transfer of mRNA and protein delivery. *Leukemia*, 20, 847 – 856.

Ren, S., Chen, J., Duscher, D., Liu, Y., Guo, G., Kang, Y., Xiong, H., Zhan, P., Wang, Y., Wang, C., Machens, H.G., Chen, Z. (2019) Microvesicles from human adipose stem cells promote wound healing by optimizing cellular functions via AKT and ERK signaling pathways. *Stem Cell Res. Ther.*, 31, 10, 47.

Robertson, S.A., Rae, C.J., Graham, A. (2009) Induction of Angiogenesis by Murine Resistin: Putative Role of PI3-kinase and NO-dependent Pathways. *Regul. Pept.*, 152, 41 – 47.

Rutkowski, J.M., Davis, K.E., Scherer, P.E. (2009) Mechanisms of obesity and related pathologies: the macro- and microcirculation of adipose tissue. *FEBS J.*, 276, 5738 – 5746.

Ronti, T., Lupattelli, G., Mannarino, E. (2006) The endocrine function of adipose tissue: an update. *Clin. Endocrinol (Oxf)*, 64, 355 – 365.

Salomon, C., Ryan, J., Sobrevia, L., Kobayashi, M., Ashman, K., Mitchell, M., Rice, G.E. (2013) Exosomal signaling during hypoxia mediates microvascular endothelial cell migration and vasculogenesis. *PLoS One*, 8, e68451.

Schreuder, T.C., Marsman, H.A., Lenicek, M., van Werven, J.R., Nederveen, A.J., Jansen, P.L., Schaap, F.G. (2010) The hepatic response to FGF19 is impaired in patients with nonalcoholic fatty liver disease and insulin resistance. *Am. J. Physiol. Gastrointest. Liver. Physiol*, 298, 440 – 445.

Schroder, K., Hertzog, P.J., Ravasi, T., Hume, D.A. (2004) Interferon-gamma: an overview of signals, mechanisms and functions. *J. Leukoc Biol.*, 75, 163 – 189.

Schwarz, A., Kinscherf, R., Bonaterra, G.A. (2023) Role of the Stress- and Inflammation-Induced Cytokine GDF-15 in Cardiovascular Diseases: From Basic Research to Clinical Relevance. *Rev Cardiovasc. Med.*, 6, 24, 81.

Schwartz, S.M. (1978) Selection and characterization of bovine aortic endothelial cells. *In Vitro.*, 14, 966 – 980.

Shabbir, A., Cox, A., Rodriguez-Menocal, L., Salgado, M., and Van Badiavas, E. (2015) Mesenchymal stem cell exosomes induce proliferation and migration of normal and chronic wound fibroblasts, and enhance angiogenesis In Vitro. *Stem Cells Dev.*, 24, 1635 – 1647.

Seo, M.J., Suh, S.Y., Bae, Y.C., Jung, J.S. (2005) Differentiation of human adipose stromal cells into hepatic lineage *in vitro* and *in vivo*. *Biochem. Biophys. Res. Commun.*, 328, 258 – 264.

Serini, G., Valdembrì, D., Bussolino, F. (2006) Integrins and angiogenesis: a sticky business. *Exp. Cell. Res.*, 312, 651- 658.

Sethi, J.K., Vidal-Puig, A.J. (2007) Thematic review series: adipocyte biology. Adipose tissue function and plasticity orchestrate nutritional adaptation. *J. Lipid. Res.*, 48, 1253 – 1262.

Simons, M., Raposo, G. (2009) Exosomes – vesicular carriers for intercellular communication. *Curr. Opin. Cell. Biol.*, 21, 575 – 581.

Sommer, G., Kralisch, S., Stangl, V., Vietzke, A., Köhler, U., Stephan, H., Faber, R., Schubert, A., Lössner, U., Bluher, M., Stumvoll, M., Fasshauer, M. (2009) Secretory products from human adipocytes stimulates proinflammatory cytokine secretion from human endothelial cells. *J. Cell. Biochem.*, 106, 729 – 737.

Stary, H.C., Chandler, A.B., Dinsmore, R.E., Fuster, V., Glagov, S., Insull, W. Jr., Rosenfeld, M.E., Schwartz, C.J., Wagner, W.D., Wissler, R.W. (1995) A definition of advanced types of atherosclerotic lesions and a histological classification of atherosclerosis. A report from the Committee on Vascular Lesions of the Council on Arteriosclerosis, American Heart Association. *Circulation*, 92, 1355 – 1374.

Stejskal, D., Karpísek, M., Hanulová, Z., Stejskal, P. (2008) Fibroblast growth factor-19: development, analytical characterization and clinical evaluation of a new ELISA test. *Scand. J. Clin. Lab. Invest.*, 68, 501 – 507.

Stenderup, K., Justesen, J., Clausen, C., Kassem, M. (2003) Aging is associated with decreased maximal life span and accelerated senescence of bone marrow stromal cells. *Bone*, 33, 919 -926.

Suzuki E, Fujita D, Takahashi M, Oba S, Nishimatsu H. Therapeutic Effects of Mesenchymal Stem Cell-Derived Exosomes in Cardiovascular Disease. *Adv Exp Med. Biol.*, 998. 179 – 185.

Swift, M.R., Weinstein, B.M. (2009) Arterial-venous specification during development. *Circ. Res.*, 104, 576 – 588.

Takeuchi, R., Katagiri, W., Endo, S., Kobayashi, T. (2019) Exosomes from conditioned media of bone marrow-derived mesenchymal stem cells promote bone regeneration by enhancing angiogenesis. *PLoS One.*, 21, 14, e0225472.

Tan, S.H., Nevalainen, M.T. (2008) Signal transducer and activator of transcription 5A/B in prostate and breast cancers. *Endocr. Relat. Cancer.*, 15, 367 – 390.

Tang, H., He, Y., Li, L., Mao, W., Chen, X., Ni, H., Dong, Y., Lyu, F. (2019) Exosomal MMP2 derived from mature osteoblasts promotes angiogenesis of endothelial cells via VEGF/Erk1/2 signaling pathway. *Exp. Cell. Res.*, 15, 383.

Templeton, D. M., and Liu, Y. (2003) Genetic regulation of cell function in response to iron overload or chelation. *Biochim. Biophys. Acta. Gen. Subj.*, 1619, 113 – 124.

Thery, C., Ostrowski, M., Segura, E. (2009) Membrane vesicles as conveyors of immuneresponses. *Nat. Rev. Immunol.*, 9, 581 – 593.

Théry, C., Witwer, K. W., Aikawa, E., Alcaraz, M. J., Anderson, J. D., and Andriantsitohaina, R. (2018) Minimal information for studies of extracellular vesicles 2018 (MISEV2018): a position statement of the international society for extracellular vesicles and update of the MISEV2014 guidelines. *J. Extracell. Vesicles* 7, 1535750.

Thurston, G. (2003) Role of Angiopoietins and Tie receptor tyrosine kinases in angiogenesis and lymphangiogenesis. *Cell Tissue Res.*, 314, 61 – 68.

Tian, J., Cheng, L., Kong, E., Gu, W., Jiang, Y., Hao, Q., Kong, B., Sun, L. (2022) linc00958/miR-185-5p/RSF-1 modulates cisplatin resistance and angiogenesis through AKT1/GSK3 β /VEGFA pathway in cervical cancer. *Reprod. Biol. Endocrinol.*, 2, 20, 132.

Tobiasch, E. (2010) Differentiation potential of adult human mesenchymal stem cells. In: Artmann, G.M., Hescheler, J., Minger, S., editors. Stem cell engineering. Berlin: Springer-Verlag, 61 – 77.

Tsao, C.R., Liao, M.F., Wang, M.H., Cheng, C.M., Chen, C.H. (2014) Mesenchymal Stem Cell Derived Exosomes: A New Hope for the Treatment of Cardiovascular Disease? *Acta. Cardiol. Sin.*, 30, 395 – 400.

Valadi, H., Ekström, K., Bossios, A., Sjöstrand, M., Lee, J. J., and Lötvall, J. O. (2007) Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells. *Nat. Cell Biol.*, 9, 654 – 659.

van der Pol, E., Böing, A. N., Harrison, P., Sturk, A., and Nieuwland, R. (2012) Classification, functions, and clinical relevance of extracellular vesicles. *Pharmacol. Rev.*, 64, 676 – 705.

Virmani, R., Burke, A.P., Farb, A., Kolodgie, F.D. (2006) Pathology of the vulnerable plaque. *J. Am. Coll. Cardiol.*, 18, 47, 13 – 18.

Vitorino, P., Hammer, M., Kim, J., Meyer, T. (2011) A steering model of endothelial sheet migration recapitulates monolayer integrity and directed collective migration. *Mol. Cell. Biol*, 31, 342 – 350.

Vrijzen, K.R., Maring, J.A., Chamuleau, S.A., Verhage, V., Mol, E.A., Deddens, J.C., Metz, C.H., Lodder, K., van Eeuwijk, E.C., van Dommelen, S.M., Doevendans, P.A., Smits, A.M., Goumans, M.J., Sluijter, J.P. (2016) Exosomes from Cardiomyocyte Progenitor Cells and Mesenchymal Stem Cells Stimulate Angiogenesis Via EMMPRIN. *Adv. Healthc. Mater.*, 5, 2555 – 2565.

Wakitani, S., Saito, T., Caplan, A.I. (1995) Myogenic cells derived from rat bone marrow mesenchymal stem cells exposed to 5-azacytidine. *Muscle Nerve*, 18, 1417 – 1426.

Wang, Z., Yuan, Y., Ji, X., Xiao, X., Li, Z., Yi, X., et al. (2021) The Hippo-TAZ axis mediates vascular endothelial growth factor C in glioblastoma-derived exosomes to promote angiogenesis. *Cancer Lett.*, 513, 1 – 13.

Wolf, P. (1967) The nature and significance of platelet products in human plasma. *Br. J. Haematol.*, 13, 269 – 288.

Wolk, R., Deb, A., Caplice, N.M., V.K., S. (2005) Leptin receptor and functional effects of leptin in human endothelial progenitor cells. *Atherosclerosis*, 183, 131 – 139.

Wosnitza, M., Hemmrich, K., Groger, A., Gräber, S., Pallua, N. (2007) Plasticity of human adipose stem cells to perform adipogenic and endothelial differentiation. *Differentiation*, 75, 12 – 23.

Wubbolts, R., Leckie, R.S., Veenhuizen, P.T., Schwarzmann, G., Möbius, W., Hoernschemeyer, J., Slot, J.W., Geuze, H.J., Stoorvogel, W. (2003) Proteomic and biochemical analyses of human B cell-derived exosomes. Potential implications for their function and multivesicular body formation. *J. Biol. Chem.*, 278, 10963 – 10972.

Xiong, Z.H., Wei, J., Lu, M.Q., Jin, M.Y., Geng, H.L. (2018) Protective effect of human umbilical cord mesenchymal stem cell exosomes on preserving the morphology and angiogenesis of placenta in rats with preeclampsia. *Biomed. Pharmacother.*, 105, 1240 – 1247.

Xue, C., Shen, Y., Li, X., Li, B., Zhao, S., Gu, J., Chen, Y., Ma, B., Wie, J., Han, Q., Zhao R.C. (2018) Exosomes Derived from Hypoxia-Treated Human Adipose Mesenchymal Stem Cells Enhance Angiogenesis Through the PKA Signaling Pathway. *Stem Cells Dev.*, 27, 456 – 465.

Yáñez-Mó, M., Siljander, P. R., Andreu, Z., Zavec, A. B., Borràs, F. E., Buzas, E. I., Buzas, K., Casal, E., Cappello, F., Carvalho, J., Colás, E., Cordeiro-da Silva, A., Fais, S., Falcon-Perez, J. M., Ghobrial, I. M., Giebel, B., Gimona, M., Graner, M., Gursel, I., Gursel, M., De Wever, O. (2015) Biological properties of extracellular vesicles and their physiological functions. *J. of extr. vesicles*, 4, 27066.

Yu, M., Liu, W., Li, J., Lu, J., Lu, H., Jia, W., Liu, F. (2020) Exosomes derived from atorvastatin-pretreated MSC accelerate diabetic wound repair by enhancing angiogenesis via AKT/eNOS pathway. *Stem Cell Res. Ther.*, 12, 350.

Zhang, Y., Babczyk, P., Pansky, A., Kassack, M.U., Tobiasch, E. (2020) P2 Receptors Influence hMSCs Differentiation towards Endothelial Cell and Smooth Muscle Cell Lineages. *Int. J. Mol. Sci.* 27, 21, 6210.

Zhang, Y., Xie, Y., Hao, Z., Zhou, P., Wang, P., Fang, S., Li, L., Xu, S., Xia, Y. (2021) Umbilical Mesenchymal Stem Cell-Derived Exosome-Encapsulated Hydrogels Accelerate Bone Repair by Enhancing Angiogenesis. *ACS Appl. Mater. Interfaces*. 28, 13, 18472 – 18487.

Zhang, X., Zhao, Q., Zhou, N., Liu, Y., Qin, K., Buhl, E.M., Wang, X., Hildebrand, F., Balmayor, E., Greven, J. (2023) Osteoblast derived extracellular vesicles induced by dexamethasone: A novel biomimetic tool for enhancing osteogenesis in vitro. *Front in Bioengineering and Biotechnology*, 11, 1160703.

Zhao, Q., Yang, J., Liu, B., Huang, F., Li, Y. (2019) Exosomes derived from mangiferin-stimulated perivascular adipose tissue ameliorate endothelial dysfunction. *Mol. Med. Rep.*, 19(6), 4797 – 4805.

Zhong, J., Krawczyk, S.A., Chaerkady, R., Huang, H., Goel, R., Bader, J.S., Wong, G.W., Corkey, B.E., Pandey, A. (2010) Temporal profiling of the secretome during adipogenesis in humans. *J. Proteome. Res.* 1, 9, 5228 – 5238.

Ziemke, F., Mantzoros, C.S. (2010) Adiponectin in insulin resistance: lessons from translational research. *Am. J. Clin. Nutr.*, 91, 258 – 261.

Zimmers TA, Jin X, Hsiao EC, McGrath SA, Esquela AF, Koniaris LG. (2005) Growth differentiation factor-15/macrophage inhibitory cytokine-1 induction after kidney and lung injury. *Shock.*, 23, :543 – 548.

Zippel, N., Scholze, N.J., Limbach, C.A., Urban, C., Luparello, C., Pansky, A., Kassack, M.U., Tobiasch, E. (2012) Purinergic receptors influence the differentiation of human mesenchymal stem cells. *Stem Cells Dev.*, 21, 884 – 900.

Zuk, P., Zhu, M., Mizuno, H., Huang, J., Futrell, J., Katz, A., Benhaim, P., Lorenz, H., Hedrick, M. (2001) Multilineage cells from human adipose tissue: implications for cell-based therapies. *Tissue Eng.*, 7, 211 – 228.

Zuk, P., Zhu, M., Ashjian, P., De Ugarte, D., Huang, J., Mizuno, H., Alfonso, Z., Fraser, J., Benhaim, P., Hedrick, M. (2002) Human adipose tissue is a source of multipotent stem cells. *Mol. Biol. Cell.*, 13, 4279 – 4295.

Zuyong, W., Wanqi, Z., Chao, X., Feng, W., Chao, M., Nan, L., Eng, S.T., Xianwei, W. (2019) Geometric anisotropy on biomaterials surface for vascular scaffold design: engineering and biological advances. *J. Phys. Mater.*, 2, 3.

Curriculum Vitae

Personal data

Date and place of birth	25. August 1982, Bad Arolsen, Deutschland
Nationality	German
Family state	Single

Education and Academic Career

04/2023 – 07/2023	Scholarship student at HBRS, Rheinbach
09/2022 – 05/2023	Scientific Associate at HBRS, Rheinbach Project: Hybrid-KEM
03/2021 – 09/2022	Scientific Associate at HBRS, Rheinbach Lecturer and IT support Online Exams
04/2019 – 09/2022	Scientific Associate at HBRS, Rheinbach IT support & Technical Service Lab Devices
10/2017 – 03/2023	Scientific Associate at HBRS, Rheinbach Lecturer Molecular Genetics, Cell Culture
04/2016 – 09/2017	Scholarship student at HBRS, Rheinbach
04/2013 – 03/2016	Scientific Associate at HBRS, Rheinbach Project: MeMoAthero
10/2005 – 03/2012	Diploma Degree in Biology; RWTH Aachen, Germany
09/2003 – 06/2005	Biological Assistant Technician, Olsberg, Germany

Tutoring/Mentoring

6x UniStemDay

6x Girls & Boys Day

3x Studieninforamtionstag

4x Mentoring DAAD RISE students

3x Mentoring NRW Scholarship Holders for Middle East

2x Mentoring Bachelor Thesis students

Publication List

Original Publications

1. Weinandy S*, **Babczyk P***, Dreier A, Unger RE, Flanagan TC, Kirkpatrick CJ, Zenke M, Klee D, Jockenhoevel S. Ovine carotid artery-derived cells as an optimized supportive cell layer in 2-D capillary network assays. *PLoS One*. 2014 Mar 12;9(3). IF₂₀₁₄: 3,234
2. Grimmig R#, **Babczyk P#**, Gillemot P, Schmitz KP, Schulze M*, Tobiasch E*. Development and Evaluation of a Prototype Scratch Apparatus for Wound Assays Adjustable to Different Forces and Substrates. *Appl. Sci*. 2019, 9(20), 4414; doi.org/10.3390/app9204414. IF₂₀₁₉: 2,474
3. Zhang Y, **Babczyk P**, Pansky A, Kassack MU, Tobiasch E. P2 Receptors Influence hMSCs Differentiation towards Endothelial Cell and Smooth Muscle Cell Lineages. *Int J Mol Sci*. 2020, 21, 6210. IF₂₀₂₀: 4,556
4. **Babczyk, P.**, Winter, M., Kleinfeld, C., Pansky, A., Oligschleger, C., Tobiasch, E. Examination of the Quality of Particulate and Filtered Mandibular Bone Chips for Oral Implants: An In Vitro Study. *Appl. Sci*. 2022, 12, 2031. IF₂₀₂₂: 2,679

Reviews

1. **Babczyk P**, Conzendorf C, Klose J, Schulze M, Harre K, Tobiasch E. Stem Cells on Biomaterials for Synthetic Grafts to Promote Vascular Healing. *J Clin Med*. 2014 Jan 15;3(1):39-87. Review. IF₂₀₁₄: 5,583
2. Kaebisch C, Schipper D, **Babczyk P**, Tobiasch E. The Role of Purinergic Receptors in Stem Cell Differentiation Comput Struct Biotechnol J. 2014 Nov 7;13:75-84. IF₂₀₁₄: 4,148
3. Witzler M, Büchner D, Shoushrah SH, **Babczyk P**, Baranova J, Witzleben S, Tobiasch E, Schulze M. Polysaccharide-Based Systems for Targeted Stem Cell Differentiation and Bone Regeneration. *Biomolecules*. 2019 Dec 6;9(12):840. IF₂₀₁₉: 4,650

4. Tonk CH, Shoushrah SH, **Babczyk P**, El Khaldi-Hansen B, Schulze M, Herten M, Tobiasch E. Therapeutic Treatments for Osteoporosis-Which Combination of Pills Is the Best among the Bad? *Int J Mol Sci.* 2022 Jan 26;23(3):1393. IF₂₀₂₂: 5,924
5. Lange M, **Babczyk P**, Tobiasch E. Exosomes: A New Hope for Angiogenesis-Mediated Bone Regeneration. *Int. J. Mol. Sci.* 2024, 25, 5204. IF₂₀₂₄: not announced yet

Bookchapter

1. Schipper D, **Babczyk P**, El-Sayed F, Klein SE, Schulze M, Tobiasch E. The Effect of Nanostructured Surfaces on Stem Cell Fate. In “Set Nanostructures for Novel Therapy”. Editors: A. M. Grumezescu, D. Ficiu. Elsevier [erste Auflage: 2017]; ISBN: 978-0-32346-148-1.

Poster/Talks

1. Kombinatorische Biokatalyse zur In-vitro-Synthese von UDP-Glucuronsäure. Engels, L; **Babczyk, P**; Elling, L. Chemie Ingenieur Technik Vol. 82, 9 (2010)
2. Weinandy, S*.; **Babczyk, P***.; Schiklenk, C.; Unger, R.; Kirkpatrick, C.J.; Flanagan, T.; Sachweh, J.S.; Zenke, M.; Klee, D.; Jockenhoevel, S. Ovine Carotid Artery-Derived Cells as an Optimal Supportive Layer in 2-D Capillary Network Assays International Vascular Biology Meeting (IVBM), Wiesbaden, June 2012.
3. Conzendorf C, Klose J, **Babczyk P**, Harre K, Schulze M, Tobiasch E. Modified Cryoprocessed Collagen Scaffolds for Future Use of Mesenchymal Stem Cells Differentiated Towards Endothelial Cells. IIR-Workshop (International Institute of Refrigeration) on Cryoprocessing of Biopharmaceuticals and Biomaterials. Dresden, Germany, September 4th - 5th, 2013.
4. **Babczyk P** and Tobiasch E. Development of a Test Model System for Drug Development in Early Atherosclerosis. University of Palermo, Italy, November 4th, 2013. *Invited Talk*

5. Zhang Y, Kaebisch C, **Babczyk P**, Pansky A, Kassack M, Tobiasch E. Human Mesenchymal Stem Cell Differentiation Towards Endothelial and Smooth Muscle Cells are Regulated *via* Different P2 Receptors and Their Underlying Signalling Pathways. 1st Annual Conference of the German Stem Cell Network (GSCN) Berlin, Germany, November 11th - 13th 2013.
6. **Babczyk P**, Lange M, Schipper D, Tobiasch E. Development of a Test Model System for Drug Improvement to Prevent Stenosis in Early Atherosclerosis. Bund (BMBF)-Länder-Gespräch zu „Forschung an Fachhochschule“, St. Augustin, Germany, 22nd May 2014.
7. Schipper D, Zhang Y, **Babczyk P**, Seifert A, Tobiasch E. An *in vitro* System to Study the Molecular Mechanisms of Restenosis. Bund (BMBF)-Länder-Gespräch zu „Forschung an Fachhochschule“, St. Augustin, Germany, 22nd May 2014.
8. Zhang Y, Kaebisch C, Schipper D, **Babczyk P**, Gueneri D, Tobiasch E. Purinergic signalling in mesenchymal stem cell proliferation and differentiation. Purines 2014 – International Conference on Nucleotides, Nucleosides and Nucleobases. Bonn, Germany, July 23rd - 27th, 2014.
9. Zhang, Y Kaebisch C, Schipper C, **Babczyk P**, Gueneri D, Tobiasch E. Purinergic signalling in mesenchymal stem cell proliferation and differentiation. Purines 2014 – International Conference on Nucleotides, Nucleosides and Nucleobases. Bonn, Germany, July 23rd - 27th, 2014
10. **Babczyk P**, Schipper D, Zhang Y, Knapp S, Seifert A, Tobiasch E. Purinergic Receptor Regulation in Activated Endothelial Cells of Obesity Triggered Early Atherosclerosis. Purines 2014 – International Conference on Nucleotides, Nucleosides and Nucleobases. Bonn, Germany, July 23rd - 27th, 2014.
11. Kaebisch C, Zhang Y, Winter M, Schipper D, **Babczyk P**, Schulze M, Tobiasch E. Ectomesenchymal stem cells are comprised of a higher portion of osteo-committed cells than mesenchymal stem cells. Stem Cells and Cancer, 8th International Heinrich F. C. Behr-Symposium, Heidelberg, Germany, September 28th - 30th, 2014.
12. Werheid D, Knapp S, **Babczyk P**, Seifert A, Lange M, Khan D, Pansky A, Tobiasch E. Adipogenic-induced endothelial cell migration is linked to HOXC8 induction. Stem Cells and Cancer, 8th International Heinrich F.C. Behr Symposium, Heidelberg, Germany, September 28th - 30th, 2014.

13. Knapp S, Lange M, **Babczyk P**, Seifert A, Werheid D, Pansky A, Tobiasch E. Adipogenesis-mediated endothelial cell migration is not regulated *via* slit2. Stem Cells and Cancer, 8th International Heinrich F. C. Behr-Symposium, Heidelberg, Germany, September 28th - 30th, 2014.
14. Schipper D, Gueneri D, **Babczyk P**, Zhang Y, Elsayed F, Tobiasch E. The Purinergic Receptors P1A2A and P1A2B Play a Role in hMSC Differentiation towards Endothelial Cells. 8th Annual World Congress of Regenerative Medicine & Stem Cell. Theme: Light the Hope of Incurable Diseases. Busan, South Korea, March 23rd - 25th, 2015.
15. **Babczyk P**; Schipper, D. Investigation of the Molecular Mechanisms Underlying Atherosclerosis. 20th Anniversary HBRS, Sankt Augustin, June, 19th, 2015.
16. **Babczyk P**, Rösler K, Lange M, Garnett L, Harre K, Tobiasch E. Conditioned Medium of Developing Fat Cells Activates Endothelial Cells – A Prerequisite of Early Atherosclerosis. Recent Advances & Challenges in Molecular Biology, Biochemistry and Biotechnology. Abbottabad, Pakistan, August 25th - 27th, 2016.
17. Zhang Y, Schipper D, **Babczyk P**, Ottensmeyer PF, Elsayed F, Tobiasch E. P2 Receptor Signalling Controls Human Mesenchymal Stem Cell Differentiation Towards Endothelial and Smooth Muscle Cells. Jahrestagung der Deutschen Gesellschaft für Klinische Mikrozirkulation und Hämorheologie (DGKMH). Satellitensymposium der Deutschen Gesellschaft für Zelluläre Biotechnologie. Mainz, Germany, November 04th - 05th, 2016. Poster award
18. Tobiasch E, Schipper D, Ottensmeyer P, **Babczyk P**, Hescheler J. Purinergic receptor expression differs in iPS Cells and MSCs. Stem Cell Community Day. Hamburg, Germany April 4th, 2017.
19. Tobiasch E, Ottensmeyer P, Schipper D, **Babczyk P**, Elsayed F, Witzler M, Schulze, M. HOX A7 expression linked to osteogenesis differs in mesenchymal stem cells isolated from various body sites. Stem Cell Community Day. Hamburg, Germany April 4th, 2017.
20. Ottensmeyer P, Schipper D, **Babczyk P**, Witzler M, Elsayed F, Götz W, Schulze M, Tobiasch E. HOX A7 is Downregulated During *in vitro* Differentiation of Mesenchymal Stem Cells Towards Osteoblasts. 9th International Meeting of the Stem Cell of the Stem Cell Network North Rhine-Westphalia. Münster, Germany, May 16th - 17th, 2017.

21. **Babczyk P**, Schipper D, Ottensmeyer P, Götz W, Tobiasch E. Differentiation Potential of Dental Neural Crest-Derived Progenitor Cells. 9th International Meeting of the Stem Cell of the Stem Cell Network North Rhine-Westphalia. Münster, Germany, May 16th - 17th, 2017.
22. Schipper D, Ottensmeyer P, **Babczyk P**, Hescheler J, Tobiasch E. Direct Differentiation of Induced Pluripotent Stem Cells into Osteoblasts and Endothelial Cells. 9th International Meeting of the Stem Cell of the Stem Cell Network North Rhine-Westphalia. Münster, Germany, May 16th - 17th, 2017.
23. Hielscher D, Ottensmeyer P, **Babczyk P**, Kannan A, Hescheler J, Tobiasch E. Stronger Osteogenesis of Induced Pluripotent Stem Cell-derived MSCs as Compared to Dental Follicle Stem Cells. Euro Dentistry Congress, Dublin, Ireland, September 20th - 21st, 2017.
24. Ottensmeyer P, Shaaban S, Hielscher D, **Babczyk P**, Warmer J, Baranova J, Tobiasch E. HOX A11 Preselected Human Mesenchymal Stem Cells from Different Body Parts for Enhanced Osteoblastogenesis. Euro Dentistry Congress, Dublin, Ireland, September 20th - 21st, 2017.
25. **Babczyk P**, Hielscher D, Ottensmeyer P, Webster S, Aljabali S, Götz W, Tobiasch E. Dental Neural Crest-Derived Progenitor Cells as a Better Source for Jawbone Repair. Euro Dentistry Congress, Dublin, Ireland, September 20th - 21st, 2017.
26. Hielscher D, Ottensmeyer P, **Babczyk P**, Hescheler J, Tobiasch E. Purinergic Signaling in iPS Cells. Interne Klausurtagung des Kompetenznetzwerk Stammzellforschung NRW, Bonn, Germany, May 4th - 5th, 2018.
27. Tobiasch E, Hielscher D, Ottensmeyer P, **Babczyk P**, Zhang Y, Schulze, M Purinergic signaling in mesenchymal stem cell differentiation and iPS. Purines in Brazil 2018, Foz do Iguacu, Brazil, June 19th - 22nd, 2018.
28. **Babczyk, P**. Investigation on Atherosclerosis. Tag der Forschung; HBRS, Rheinbach December 10th, 2021
29. **Babczyk P**. Exosomes from Mesenchymal Stem Cell-Derived Pre-Adipocytes Enhance Endothelial Cell Migration and Angiogenesis. Tag der Forschung 2023, HBRS, Sankt Augustin, June 21st, 2023

30. **Babczyk, P**, Odefey U., Tobiasch E. Testing the Potency of Extracellular Vesicles derived from Differentiating Mesenchymal Stem Cells with an Adapted Wound Assay. The First Conference on Targeting Extracellular Vesicles, Mitochondria and Microbiota: Crucial Impact. Malta, October 17th – 18th, 2024. talk

University Thesis

1. Diplomarbeit: 2D-Vaskularisierung – Die Entwicklung tubulärer Strukturen *in vitro*. Helmholtzinstitut für Angewandte Medizintechnik. Tissue Engineering and Textile Implants (Prof. Dr. med. Stefan Jockenhövel); March 2012.