Establishment of a Blood Vessel Cell Culture Model, Testing its Applicability in Microgravity and Drug Research and Examination of the Underlying Signaling Pathways

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ABSTRACT

During space missions astronauts suffer from cardiovascular deconditioning, when they are exposed to microgravity conditions. Until now, no specific drugs are available for effective countermeasures, since the underlying mechanism is not completely understood. Endothelial cells (ECs) and smooth muscle cells (SMCs) play crucial roles in a variety of cardiovascular functions, many of which are regulated *via* P2 receptors. However, their function in ECs and SMCs under microgravity condition is still unknown. In this study, ECs and SMCs were isolated from bovine aorta and differentiated from human mesenchymal stem cells (hMSCs), respectively. Subsequently, the cells were verified based on specific markers. An altered P2 receptor expression pattern was detected during the commitment of hMSC towards ECs and SMCs. The administration of natural and artificial P2 receptor agonists and antagonists directly affected the differentiation process. By using EC growth medium as conditioned medium, a vessel cell model was created to culture SMCs and *vice versa*. Within this study, we were able to show for the first time that the expression of some P2 receptors were altered in ECs and SMCs grown for 24h under simulated microgravity conditions. On the other hand, in some P2 receptor expressions such as P2X7 conditioned medium compensated this change.

In conclusion, our data show that P2 receptors play an important functional role in hMSC differentiation towards ECs and SMCs. Since some P2 receptor artificial ligands are already used as drugs for patients with cardiovascular diseases, it is reasonable to assume that in the future they might be promising candidates for treating cardiovascular deconditioning.

ZUSAMMENFASSUNG

Während Weltraummissionen tritt bei Astronauten aufgrund der Schwerelosigkeit eine Dekonditionierung der Kreislauffunktion auf. Bisher gibt es keine spezifischen Medikamente die diesem Prozess effektiv entgegenwirken, da der zugrundeliegende Mechanismus noch nicht vollständig verstanden ist. Endothelzellen (ECs) sowie glatte Muskelzellen (SMCs) spielen eine wichtige Rolle bei einer Reihe von vaskulären Funktionen, die häufig durch P2 Rezeptoren reguliert werden. Ihre genauen Funktionen in diesen Zellen in der Schwerelosigkeit sind bislang jedoch noch nicht bekannt.

In dieser Arbeit wurden Endothel- sowie glatte Muskelzellen sowohl aus bovinen Aorten isoliert als auch aus humanen mesenchymalen Stammzellen (hMSC) differenziert. Die Verifizierung der Zellen erfolgte anschließend anhand von spezifischen Marker. Veränderungen im Muster der P2-Expression konnten während der Spezialisierung der hMSCs in Richtung ECs und SMCs gefunden werden. Natürliche und artifizielle Agonisten und Antagonisten von P2 Rezeptoren hatten dabei einen direkten Einfluss auf den Differenzierungsprozess. Unter Verwendung von endothelialem Wachstumsmedium, welches als konditioniertes Medium verwendet wurde, konnte ein Modell eines Blutgefäßes etabliert werden. Dies diente der Kultivierung von SMCs und umgekehrt. Zum ersten Mal konnte ein verändertes P2 Rezeptor Expressionsmuster in ECs und SMCs nach einem Wachstumszeitraum von 24 Stunden unter simulierten Schwerelosigkeitsbedingungen festgestellt werden. Konditioniertes Medium kompensierte diesen Effekt allerdings für einige P2 Rezeptoren, wie z.B. P2X7.

Zusammenfassend zeigen die erhaltenen Ergebnisse deutlich, dass P2 Rezeptoren eine wichtige funktionale Rolle bei der Differenzierung von hMSCs in Richtung ECs und SMCs spielen. Da eine Reihe von künstlichen P2 Rezeptorliganden bereits als Medikamente für Patienten mit kardiovaskulären Erkrankungen auf dem Markt verfügbar sind, ist es anzunehmen, dass P2 Rezeptorliganden in Zukunft vielversprechende Kandidaten für die Behandlung von kardiovaskulärer Dekonditionierung darstellen.

1. INTRODUCTION

1.1 Cardiovascular Deconditioning

Cardiovascular deconditioning is defined as a state in which the cardiovascular system does not work as efficiently as it could. It is usually caused by a change of environment, such as in patients with long periods of bed rest or astronauts during space travel. With prolonged cardiovascular deconditioning there is an excessive rise in low blood volume, cardiac atrophy, heart rate during orthostatic stress, reduced exercise capacity, muscle atrophy, and many other wide ranging structural and metabolic alterations (Joyner & Masuki, 2008). Next to this cardiovascular deconditioning was also found to be associated with diabetes mellitus, hyperinsulinemia and respiratory compromise in severe obesity patients (body mass index ≥ 40 kg/m^2), who often showed high prevalence of cardiovascular risk factors (Gidding *et al.*, 2004). Patients and astronauts who suffer from cardiovascular deconditioning are lacking effective treatments to improve cardiovascular functions and thus prevent subsequently risky diseases such as heart attack, heart failure, and stroke.

1.1.1 Cardiovascular Deconditioning in Microgravity

Cardiovascular deconditioning is often observed in the astronauts when they are exposed to microgravity conditions during space missions, besides other health issues caused by microgravity, such as bone loss, muscle atrophy, and decreased immune activity. (Coupé *et al.*, 2009; Hargens, Richardson 2009; Pietsch *et al.*, 2011). The response of cardiovascular system to microgravity is complicated and not completely understood. The main reason leading to cardiovascular deconditioning is redistribution of body fluids from legs toward the head and upper

body. However, other reasons such as the alteration of cardiac functions, peripheral vasculature, and nervous system cannot be ignored as well (Williams *et al.*, 2009). During space missions, the volume in the lower part of astronaut's body decreases by about an average of 10% compared to pre-flight (Charles & Lathers, 1991). This short-term effect is defined as the "puffy face–bird leg" syndrome (see Fig. 1).

In addition, there are also long-term effects of cardiovascular deconditioning when the body adapts to the microgravity environment after approximately 3 weeks (Williams *et al.*, 2009). A reduced stroke volume and cardiac output, decreased red blood cell mass, reduced plasma volume (17%) and a disturbed endocrine system were reported (Williams *et al.*, 2009). Astronauts also suffer from post-flight effects of cardiovascular deconditioning, which is called orthostatic intolerance. About 25% astronauts are unable to stand quietly for 10 min continuously within several hours after landing. This is because the body cannot adapt to the redistribution of body fluids so quickly and drive the blood back from lower body to the heart which is also associated with the reduced blood volume, and thus causes syncope, heart palpitations and light-headedness (Buckey, 2006).

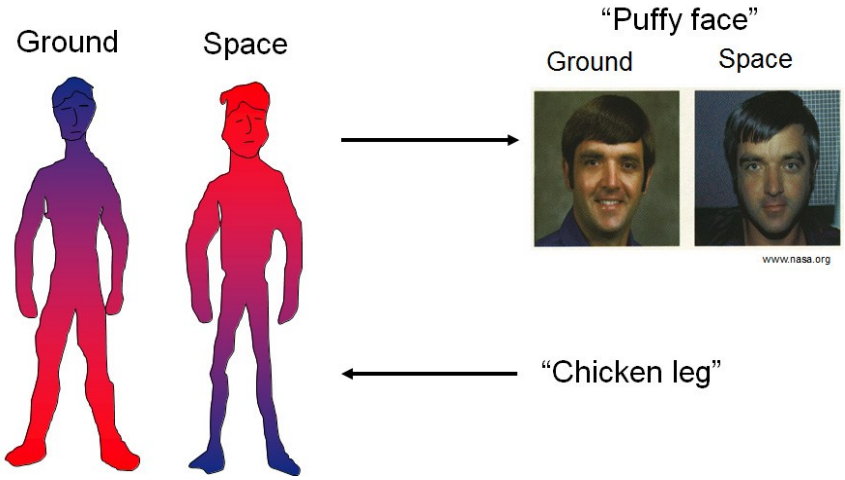


Figure 1. Short-Term Effect of Microgravity on the Cardiovascular System. Microgravity leads to a redistribution of blood in astronauts so that most of the blood shifts from the low part to the upper part of the body. The typical appearance can be observed as "puffy face" and "chicken leg" on the astronaut's body. The red color in the body refers a large portion of the total blood distribution whereas the blue color means a small percentage of blood distribution. The photo from astronaut was taken from www.nasa.org.

The countermeasures during space flight mainly focus on performing exercises with different techniques and using devices to improve the cardiovascular function to reduce or slow the effect of body fluid redistribution. However, the efficiency is very low and the outcome is poor (Schmidt & Goodwin, 2013). In addition, no effective drugs are available for the treatment of cardiovascular deconditioning in astronauts. The reason for that is that the underlying mechanism of vascular cells responding to microgravity is not clear. Therefore, more investigations on the cellular level are required in order to select effective exercises and develop drugs against the negative effect of vascular deconditioning.

1.1.2 Vascular Cells in the Blood Vessels

Blood vessels are mainly classified into arteries and veins. Both of them consist of three layers: *tunica intima*, *tunica media*, and *tunica adventitia* with the media layer being thicker in the arteries than in the veins. The intima is composed out of a monolayer of endothelial cells (ECs) kept together by a polysaccharide intercellular matrix. The media is rich in vascular smooth muscle cells (SMCs), which control the caliber of the vessel. The adventitia is mainly made of connective tissue composed out of fibroblasts. The media and adventitia layer are separated by a thick elastic band namely the external elastic lamina (see in Fig. 2) (Cines *et al.*, 1998). Among these cells, endothelial and smooth muscle cells are found to play the crucial role in maintaining vascular functions. ECs usually functions as a barrier between blood and tissue, playing a vital role in protecting against vascular injury and upholding blood fluidity (Woolf, 2000). Next to this, ECs secrete cytokines that inhibit SMC proliferation into the healthy vessels (Limbach *et al.*, 2010). SMCs are arranged between the fibers in the media layer supporting the EC monolayer and also providing contraction and relaxation of the vessels (Schiffrin, 1994). Cardiovascular diseases such as atherosclerosis, restenosis, hypertension, and stroke are usually triggered from the dysfunction or damage of endothelial and smooth muscle cells

(Rivard and Andrés, 2000; Deanfield et al., 2007).

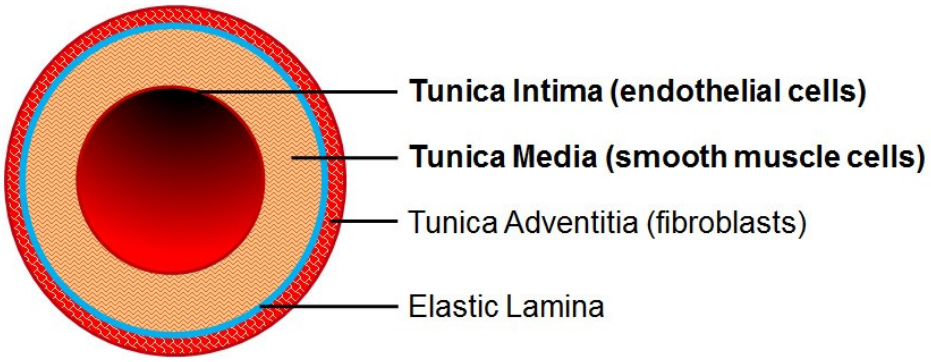


Figure 2. Vascular Cells in the Blood Vessels.
The healthy vessel consists of three layers: the *tunica intima*, a single layer of endothelial cells; the *tunica media* that is rich of smooth muscle cells; and the *tunica adventitia* that is mainly composed out of fibroblasts. The media and adventitia are separated by an enriched elastin layer called elastic lamina.

1.1.3 Endothelial and Smooth Muscle Cell Dysfunction in Microgravity

The cardiovascular deconditioning is likely to be caused by the dysfunction of ECs and/or SMCs. Recent publications have shown that ECs are sensitive to gravity changes. For example the cytoskeleton arrangement and gene expressions in ECs were altered after a parabolic flight and 22 seconds exposure to microgravity (Grosse *et al.,* 2012; Wehland *et al.,* 2013). Long-term exposure of 24h to microgravity revealed an altered expression of the extracellular matrix and cell surface adhesion molecules (Grimm *et al.,* 2010; Zhang *et al.,* 2010). Furthermore ECs formed tubes only delayed after 7 days of culture in a Random Positioning Machine (RPM), a ground-based facility which is used to simulate microgravity conditions on earth (Grimm *et al.,* 2010). Some cytokines secreted by primary human umbilical ECs such as basic fibroblast growth factor, interleukin-1 and interleukin-8 decreased, whereas others such as rantes and eotaxin increased after 96h of treatment within the RPM (Griffoni *et al.,* 2011). Compared to data on ECs,

there is only few data regarding SMC morphology and functional changes under microgravity conditions. SMCs showed suppressed proliferation and an enhanced rate of apoptosis in simulated microgravity, supporting the observation that their phenotype was transferred from a synthetic (proliferative) to a quiescence (contractive) state 72h after exposure to simulated microgravity by a rotating wall vessel (RWV) (Kang *et al.,* 2013).

The activation or inhibition of cell signaling transduction pathways can be considered as the underlying mechanisms of EC and SMC functional changes under microgravity. For example, some evidence showed that simulated microgravity promotes human umbilical endothelial cell angiogenesis *via* the PI3K-Akt-eNOS signaling pathway (Siamwala *et al.,* 2010). However, the numbers of these studies are very limited and the underlying mechanisms are still mainly unclear.

1.2 Purinergic Receptors

Purinergic receptors are one of the evolutionary oldest receptor families. They are activated by extracellular nucleosides and nucleotides (Burnstock, 1978) and crucial players in regulating a series of physiological and pathological cardiovascular processes such as atherosclerosis, hypertension and vascular pain (Burnstock, 2006a; Burnstock, 2010). In recent years some potent and selective artificial P2 receptor ligands have been synthesized as drugs to treat patients suffering from cardiovascular diseases (Ralevic & Burnstock, 2013). For example, clopidogrel (trade name Plavix), the highly potent and selective P2Y12 antagonists, is now the second-top selling drug in the world as antiplatelet drugs against heart attack. Therefore, it is reasonable to investigate the role of purinergic receptors and their underlying signaling pathways on endothelial and smooth muscle cell function under microgravity conditions as well.

Purinergic receptors are classified into two families: the P1 receptors (also named adenosine receptor) that response predominately to adenosine and the P2

receptors that are most responsive to purines and pyrimidines such as ATP (Burnstock, 1978). The P1 receptors belong to the G protein-coupled receptors and are subdivided into four subtypes A1, A2A, A2B and A3. The P2 receptors are subdivided into the ionotrophic P2X and the metabotrophic P2Y-receptors. P2X-receptors form ligand-gated ion channels that regulate influx and efflux of cations. P2Y receptors are G protein-coupled receptors that trigger a series of intracellular signaling cascades (Abbracchio *et al.*, 2006; Erb *et al.*, 2006). Until now, seven P2X (P2X1-7) and eight P2Y receptor subtypes (P2Y1, 2, 4, 6, 11, 12, 13 and 14) have been characterized (Jacobson *et al.*, 2002). (for an overview see Fig. 3).

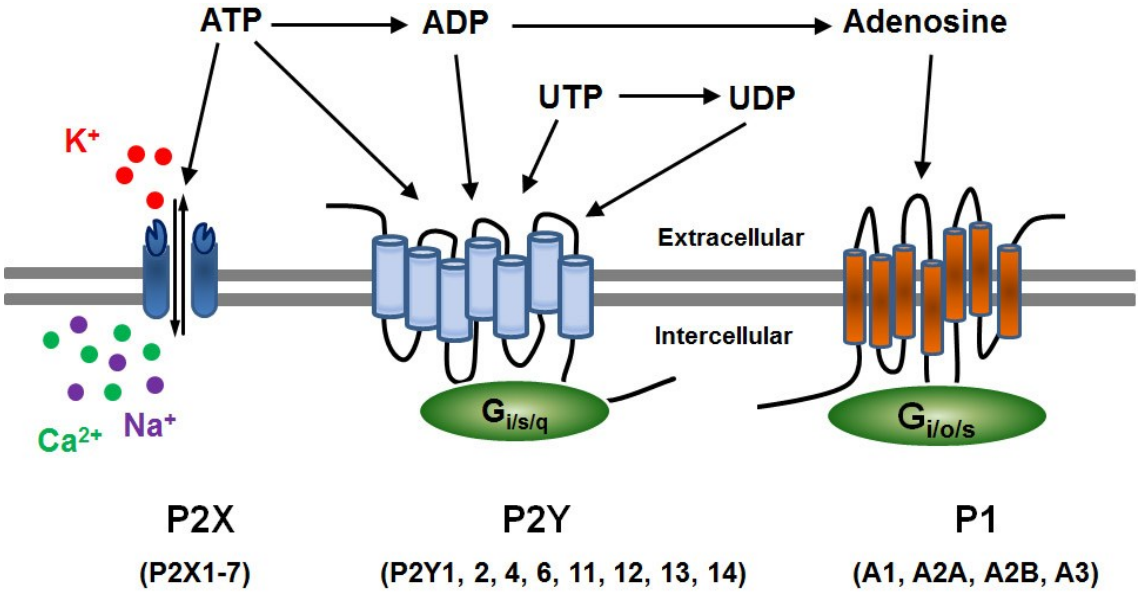


Figure 3. Purinergic Receptor Family and Its Subtypes.
The purinergic receptors can be classified into two subfamilies: the P1 receptors that response predominately to adenosine and the P2 receptors that are most responsive to ATP, UTP, ADP and UDP. The P2 receptors are subdivided into seven ion channel (P2X1-7) and eight G coupled protein receptors (P2Y1, 2, 4, 6, 11, 12, 13, 14).

P2 receptors have been shown to participate in different metabolic processes of various tissues and cell types (Burnstock & Knight, 2004; Burnstock, 2006a). The influence is usually regulated by a combination of different subtypes. For example in the short term, ATP released from sympathetic nerves in the adventitia acts as a

cotransmitter at smooth muscle P2X1, P2X2, P2X4 and P2Y2 and P2Y6 receptors leading to vasoconstriction. ATP released from sensory-motor nerves during axon reflex activity acts on P2Y receptors on smooth muscle cells, resulting in either vasoconstriction or vasodilatation. In addition ATP and UTP secreted by endothelial cells under shear stress and hypoxia condition act on P2Y1, P2Y2, and sometimes P2Y4, P2Y11, and P2X4 receptors causing the production of nitric oxide (NO) and subsequent vasodilatation. In the long term ATP released from both, sympathetic nerves and sensory-motor nerves activates smooth muscle cell proliferation *via* P2Y2 and P2Y4 receptors and their subsequent mitogen-activated protein kinase (MAPK) cascades. In detail ATP and UTP secreted from endothelial cells stimulate endothelial and smooth muscle cell proliferation *via* P2Y1, P2Y2 and P2Y4 receptors (Burnstock, 2010)

P2 receptors have been also shown to regulate important functions in other cells. For example, several publications showed the functional role of P2 receptor in stem cell function: extracellular ATP regulates embryonic stem (ES) cells (Heo & Han, 2006), induced pluripotent stem (iPS) cells (Mastrangelo *et al.*, 2012), hematopoietic stem cells (HSC) (Demoli *et al.*, 2004; Rossi *et al.*, 2007; Rossi *et al.*, 2012), and neural stem cells (Mishra *et al.*, 2006; Lin *et al.*, 2007) proliferation, migration and differentiation. Recently, data from our laboratory showed for the first time that P2 receptors are not only expressed in hMSCs but also directly influencing hMSC differentiation towards adipocytes and osteoblasts (Zippel *et al.*, 2012). Also the expression and activation of P1 receptors influences the osteogenic and adipogenic differentiation in rat bone marrow mesenchymal stem cells (BM-MSC) (Gharibi *et al.*, 2011). Pre-incubation with ATP promoted human BM-MSC osteogenic differentiation *via* its degradation product adenosine but enhanced adipogenic differentiation *via* its triphosphate form (Ciciarello *et al.*, 2013). The P2Y13 receptor plays an important role in the balance of osteoblast and adipocyte terminal differentiation of bone marrow progenitors in a P2Y13 deficient mouse model (Biver *et al.*, 2013).

1.2.1 P2X Receptors and their Downstream Signaling Pathways

P2X receptors are widely expressed in tissues and organs throughout the body, mainly in endothelium, muscle, nervous tissue, epithelium, hematopoietic system or bone tissue (Erb *et al.*, 2006). Functional P2X receptors assemble as homo- and heterotrimers to form pores (Burnstock, 2004). As ligand-gated ion channels, P2X receptors mediate sodium and calcium influx and potassium efflux followed by a depolarization of the cell membrane (Erb *et al.*, 2006). The membrane depolarization activates voltage-gated calcium channels, leading to an intracellular calcium accumulation (Fig. 4). A unique P2X receptor subtype is P2X7 that has a large and unselective pore which allows molecules up to 900 Da size to pass (Erb *et al.*, 2006). Each P2X receptor subunit consists of two transmembrane domains TM1 and TM2, a large extracellular ligand-binding loop and intracellular amino and carboxy termini (Roberts *et al.*, 2006).

The detailed mechanisms of downstream signaling pathways have not been demonstrated clearly for most P2X receptor subtypes, but it is well-established that cytoplasmic Ca^{2+} can trigger various intracellular events *via* activating MAPKs, PKC, and calmodulin (Burnstock, 2006b). For example the activation of P2X7 lead to the subsequent activation of ERK1/2 and p38 *via* Src and PI3K kinase activity, as well as cytoplasmic Ca^{2+} and PKC activity in astrocytes (Panenka *et al*., 2001). ERK1/2 are also found to be activated through a PKC- and Ca^{2+}-dependent pathway *via* P2X1 in platelets (Oury *et al*., 2002), P2X2 in PC12 cells (Swanson et al., 1998). Among the P2X receptors, P2X7 has a unique long intracellular tail that can regulate various cellular functions (Burnstock, 2006a). For example this tail contains a conserved death domain mediating apoptosis *via* controlling caspase activity and a Src homology binding domain regulating membrane blebbing *via* Rho or other small G proteins (Denlinger *et al*., 2001). It might also modulate an inflammatory response *via* activating JAK or JNK pathway (Denlinger *et al*., 2003).

1.2.2 P2Y Receptors and their Downstream Signaling Pathways

P2Y receptors belong to the G protein-coupled receptors that are composed out of seven transmembrane domains. They have an intracellular C terminus that can activate several intracellular signaling cascades (Erb *et al.*, 2006). According to the subunit formation (as shown in Fig. 4), P2Y receptor can be classified as:

i) G_q-protein coupled (P2Y1, P2Y2, P2Y4, P2Y6, P2Y11, P2Y14) that signals *via* phosphoinositide-dependent phospholipase C (PLC) pathway with subsequent formation of inositol 1,4,5-triphosphate (IP3) and Ca^{2+} mobilization from intracellular stores to activate ERK1/2 and subsequently c-jun and c-fos. They can also activate the PI3K/NF-κB pathway and c-fos, or the ASK1/p38 pathway and subsequently CREB;

ii) G_s-protein-coupled (P2Y11) that mediates the stimulation of adenylate cyclase with subsequent elevation of the cAMP level to activate ERK1/2 and subsequently c-myc and c-jun;

iii) G_i-protein-coupled (P2Y4, P2Y12, P2Y13) that activates the STAT3 pathway.

Some P2Y receptors have the unique ability to couple the two different G proteins, such as P2Y11 (G_q and G_s) and P2Y4 (G_q and G_i). P2Y receptors are widely expressed throughout the nervous, bone or muscle tissue, endothelial cells, and immune cells, where they play a functional role in cell growth, proliferation, apoptosis, migration and differentiation *via* activating the above mentioned signaling pathways (Abbracchio *et al.*, 2006).

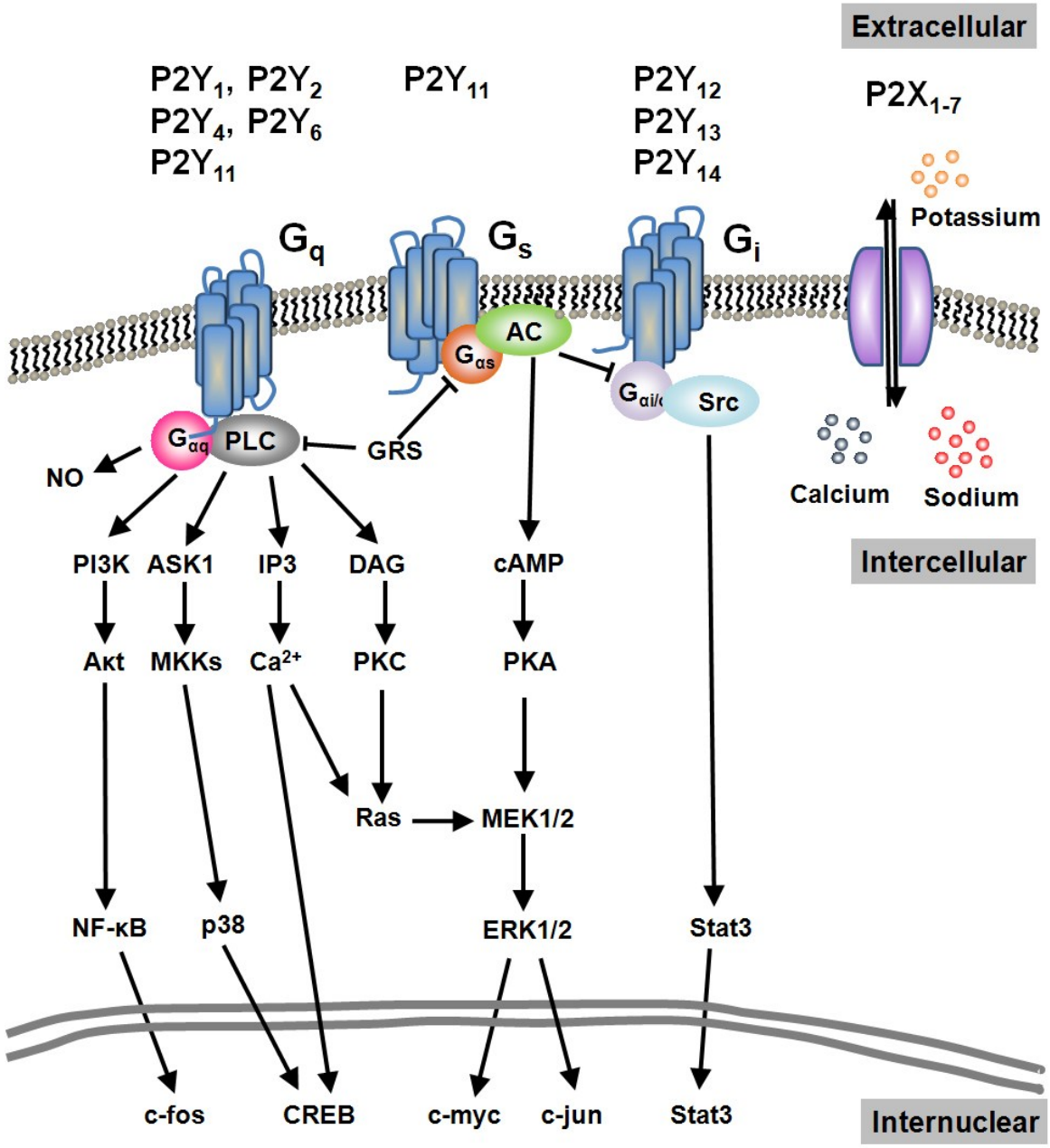


Figure 4. P2 Receptor Downstream Singaling Pathways.
The P2X receptors are ion channels that exchange intracellular calcium or sodium with extracellular potassium. The P2Y receptor subtypes P2Y1, P2Y2, P2Y4, P2Y6 and P2Y11 are coupled to Gq protein and activate the PLC and subsequent MKK/p38/CREB, Akt/NF-κB/c-fos, Ca^{2+}/CREB, and PKC/ERK/c-myc/c-jun pathways. In addition, the activation of Gq protein-coupled receptors could lead to a release of NO. P2Y11 can couple to Gs protein and stimulate the adenylate cyclase and its subsequent PKA/ERK/c-myc/c-jun pathway. The subtypes P2Y12, P2Y13 and P2Y14 coupled to Gi protein which causes an activation of the STAT3 pathway.

1.2.3 P2 Receptor Agonists and Antagonists

The purine nucleotide ATP is well-known as a key molecule in intracellular energy transmission (Burnstock, 1972). On the other hand it acts as a ligand for purinergic receptors. In addition, the breakdown products of ATP such as ADP, AMP, adenosine and the pyrimidines UTP, UDP also displayed their functions as ligands for some purinergic receptors (Burnstock, 2007).

P2X receptors are usually sensitive to ATP and with minor potency to ADP, UTP and UDP. On the other hand, specific P2Y receptor subtypes differ in their sensitivity for the different natural purinergic ligands (see Table 1). For example, P2Y2 and P2Y11 are predominately sensitive to ATP; whereas P2Y1, P2Y12 and P2Y13 response mainly to ADP. Both, P2Y2 and P2Y4 are activated by UTP and P2Y6 is activated by UDP. Particularly P2Y2 shows equal sensitivity to ATP and UTP. Whereas ATP acts as an agonist for P2Y4 in rat, but as an antagonist in human (Abbracchio *et al.*, 2006). P2Y14 on the other hand is activated by ribose-nucleotides, such as UDP-glucose and UDP-galactose (Abbracchio *et al.*, 2006). Most P2 receptor antagonists are more likely to be universally binding to several subtypes rather than to inhibit only one particular subtype. For example the symmetric polysulfonated trypanozid suramin, the pyridoxalphosphate derivate PPADS, and the histochemical dye reactive blue 2 inhibit most of the P2 receptors. Recently, more artificial P2 receptor ligands have been synthesized and are listed in table 1. The ligands with bold and italic characters especially are potent and selective.

In this study some of the above mentioned natural and artificial ligands were utilized. The artificial ligands were applied to analyze specific P2-receptor subtype functions. For example A-740003 is an artificial highly potent and selective P2X7 antagonists (IC_{50}= 40 nM) (TOCRIS). MRS2365 (EC_{50}= 0.4 nM) and MRS2500 tetraammonium salt (IC_{50}= 0.5 nM) are potent and selective agonist and antagonist of P2Y1 (TOCRIS). The structures of the natural and artificial ligands used in this study are given in figure 5.

Table 1. Potent P2 Receptor Agonists and Antagonists.
Source: Modified after tocris.com/ehandbook_2012 and (Burnstock *et al.*, 2006a; Jackobsen *et al.*, 2012; Burnstock & Ralevic, 2013)

Receptor Subtype	*Potent agonists*	*Potent antagonists*
P2X1	BzATP; ATP; 2-MeSATP; α, β-MeSATP; CTP	***NF449***; IP5I; TNP-ATP; ***Ro 0437626***; ***NF279***; ***NF023***; ***PPNDS***
P2X2	ATP; 2-MeSATP; α, β-MeSATP	RB2; isoPPADS; PPADS; suramin
P2X3	ATP; 2-MeSATP; Ap4A; α, β-MeATP	TNP-ATP; isoPPADS; A317491; ***NF110***; PPADS; ***Ro51***; ***RO-3***
P2X4	ATP; α, β-MeATP; CTP; ATPγS	TNP-ATP; PPADS; BBG; phenolphthalein
P2X5	ATPγS; ATP; α, β-MeATP; GTP; BzATP	BBG; PPADS; suramin
P2X6	(does not function as homomultimer)	-
P2X7	BzATP; ATP; 2-MeSATP; α, β-meATP	BBG; KN62; ***A804598***; ***AZ11645373***; ***A438079***; ***A-740003***
P2Y1	***MRS2365***; 2-MeSADP; ADPβS; ADP; ATP;	***MRS2500; MRS2279***; ***MRS2179;*** BzATP; PPADS, suramin
P2Y2	UTP; MRS2698; ATP; INS 365; UTPγS; ***MRS2768; PSB 1114; 2-ThioUTP tetrasodium salt***	ARC126313; suramin; RB2; MRS2576
P2Y4	UTPγS; UTP; Up4U; Ap4A; CTP; INS365; ***MRS4062***	ATP; RB2; MRS2577; PPADS; suramin
P2Y6	***MRS2957; MRS2693***; UDPβS; ***PBS0474***; INS48823; UDP; UTP	***MRS2578***; RB2; PPADS; MRS2575
P2Y11	ATPβS; BzATP; ATP; ***NF546***	***NF157***; suramin; RB2; ***NF340***
P2Y12	2-MeSADP; ADP; IDP	***Clopidogrel***; ***Ticlopidine***; ***AR-C66096***; AZD6140; ***PSB0739***; RB2; 2- MeSAMP
P2Y13	ADP; 2-MeSADP; 2-MeSATP; ADPβS; ATP	AR-C69931MX; AR-C67085; ***MRS2211***; 2-MeSAMP; PPADS
P2Y14	***MRS2690***; UDP; UDP-glucose; UDP-galactose	***PPTN***

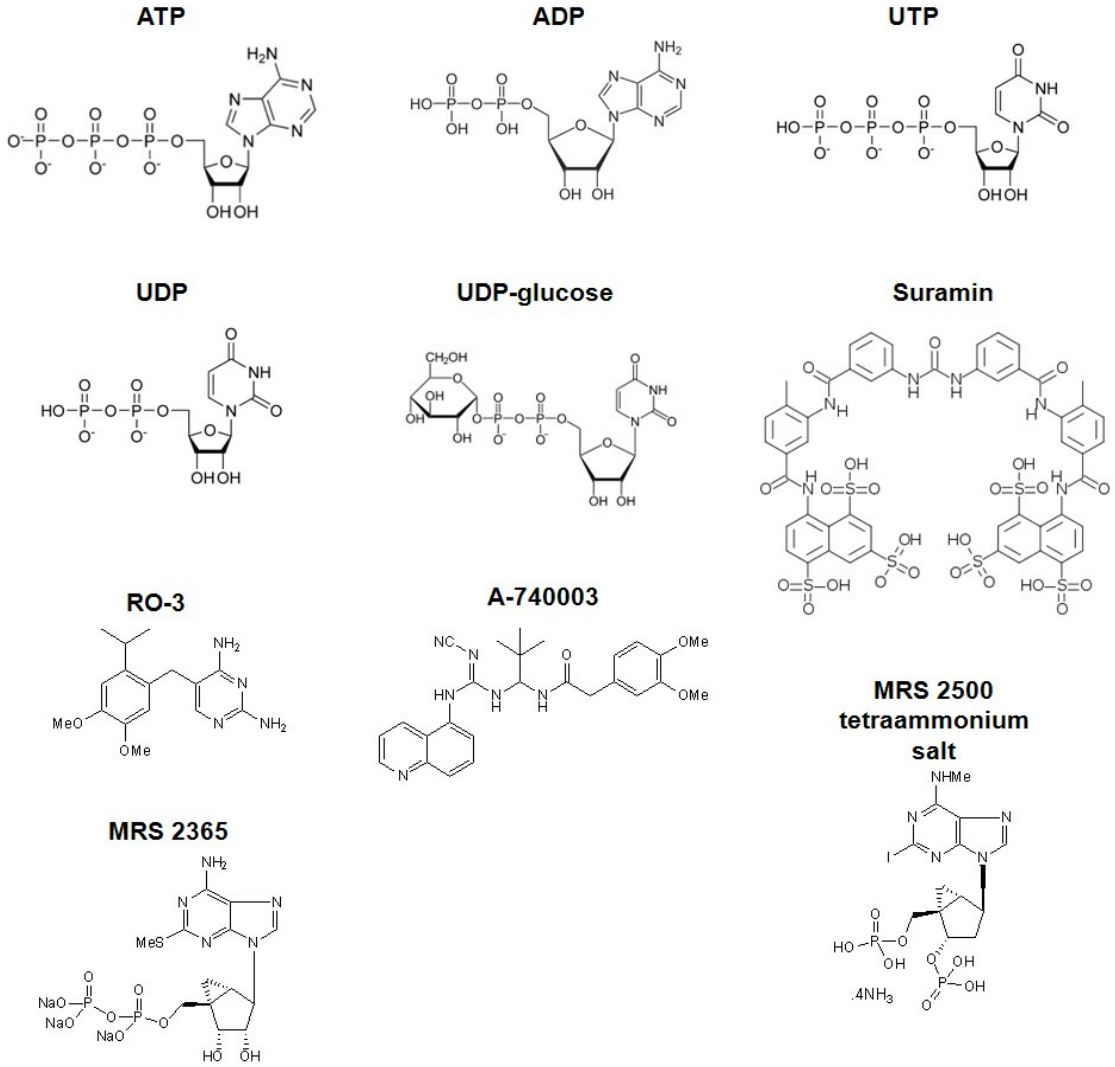


Figure 5. P2 Receptor Agonists and Antagonists.
P2Y2 and P2Y11 are most sensitive to ATP, whereas P2Y1, P2Y12 and P2Y13 response predominately to ADP, and P2Y6 and P2Y14 are mainly activated by UDP and UDP-glucose, respectively. Suramin is a universal antagonist. RO-3, A-740003, and MRS 2500 tetraammonium are artificial, potent and selective antagonist for P2X3, P2X7, P2Y1 respectively, and MRS2365 is the artificial, potent and selective agonist for P2Y1. The structure of the P2 receptor agonists and antagonists were taken from Tocris Bioscience (www.tocris.com).

1.3 Vascular Cell Co-Culture Models

Some morphological and functional alterations were already found in ECs and SMCs under microgravity conditions (Zhang *et al*., 2010; Kang *et al*., 2013).

Purinergic signaling is a reasonable candidate to investigate a possible underlying mechanism due to their functions. However, the mentioned findings were encountered when only single cell type such as endothelial or smooth muscle cells were cultured under real or simulated microgravity conditions. But vascular cells live and grow in a native three-dimensional (3D) environment *in vivo*. This environment is composed out of extracellular matrix, mechanical stimulation, cell-cell communication and positioning. This single cell type model is lacking most of the above mentioned important factors. Thus, its outcome might be very different from the one found *in vivo*. Different co-culture models have been developed to mimic *in vivo* environment in blood vessels (Kirkpatrick *et al.*, 2011). These models can be classified into two-dimensional (2D) models that are usually made by a co-culturing system using conditioned medium, or cell-cell contact, or a transwell system and 3D models that are achieved by culturing cells on and in scaffolds.

1.3.1 Two-Dimensional Models

Two-dimensional vascular cell co-culture models can be divided into paracrine model and cell-cell contact model. In a paracrine model, cells are reacted with cytokines and growth factors that are released from the same cell type or from another cell type. Several models have been developed to achieve this goal, e.g. conditioned medium could be collected from medium in which endothelial cell have grown and can be used to culture smooth muscle cells and *vice versa*. This provides the opportunity to investigate unknown molecules secreted by a specific cell type on the target cells. The simultaneous paracrine effect between ECs and SMCs can be studied *via* a transwell system by culturing SMCs on the bottom and ECs on the surface of a membrane. A cell-cell contact model is used to study both, paracrine signals and the cell-cell physical contact as impact on the cells. It can be simply set by directly co-culturing both cell types together. Later the different cell types can be separated by FACS sorting or magnetic beads with specific markers. Another

technique that allows the cell-cell contact is to culture one cell type on the upper surface and the other cell type on the lower surface of the membrane in the transwell system. Although this transwell system gives the possibility to investigate the cell-cell interaction simultaneously, it is difficult to separate the one cell type influence on the other and vice versa. Next to this, culturing cells in the clinostat requires airtight condition which is difficult for the transwell system, thus the method of using conditioned medium is a simple and fast way to investigate paracrine effect under simulated microgravity.

1.3.2 Three-Dimensional Models with Scaffolds

A large amount of useful data has been generated from the above mentioned 2D models, however, it has limitations at some points. It lacks the complex spatial and temporal patterning that is found *in vivo*. In addition, recent evidence hints that the 3D environment is essential to maintain cellular function and develop physiologically relevant structures (Schulze & Tobiasch, 2012). For example, mature cells grown in 3D matrices exhibited altered phenotypes that inhibited their proliferative nature and enhanced their capacity to form higher ordered structures (Meng *et al.*, 2014). For example vascular endothelial cells can form capillary-like structures with a lumen when they were cultured on basement membrane gels in an angiogenesis study (Benton *et al*., 2009). This is because various factors within 3D matrices are different from those in 2D such as the structural, mechanical and biochemical make-up of the cellular microenvironment and the bidirectional interplay between the cell and its surroundings during development *in vitro* and *in vivo* (Lund *et al.*, 2009). For example EC migration distance is much smaller in an *ex vivo* artery injury model than in an *in vitro* cell culture model and SMC proliferation was significantly inhibited in the EC reendothelialization or intact areas than in the EC denuded areas (Lee *et al*., 2010)

The interaction between cells and their microenvironment (see Fig. 6 for an overview)

can be classified as:

i) Soluble signals: paracrine and endocrine molecules *via* growth factors, cytokines. For example, purine derivates are very broadly affecting molecular group. ATP itself is not only an energy molecule, but also can activate subsequent signaling transduction pathways to control cell morphology and function. In the vascular system, purines and pyrimidenes act as the important paracrine soluble factors in controlling the vascular tone and remolding (Burnstock & Ralevic, 2013).

ii) Solid-state signals: physical contact *via* cell-extracellular matrix interactions, cell-cell interactions, cell-scaffold interactions. A typical example for interactions between cells and extracellular matrix is the action *via* cell adhesion molecules and integrins binding to insoluble ECM proteins (Discher *et al.*, 2009). The binding of the ligand causes a rearrangement of the actin fibers and subsequent change of the cell shape (Tanentzapf *et al.* 2007). Other factors such as transforming growth factor-β (TGF-β), tension induced proteins (TIPs) and non-muscle myosin, ultimately influence cell shape and function *via* activating signaling pathways (Dennis *et al.* 2009; Vicente-Manzanares *et al.* 2009). Interestingly, the elasticity of scaffolds has been found to influence cell behavior as well. For example, mesenchymal stem cells cultured on a stiff surface are more easily differentiated into hard tissue lineages such as osteoblasts, and with decrease of elasticity, MSCs are prone to differentiate towards myogenic, adipogenic and neurogenic lineages, respectively (McBeath *et al.*, 2004; Engler *et al.*, 2006; Guilak *et al.*, 2009).

iii) Chemical and physical signals: pH, oxygen, NO. For example in blood vessel, the endothelium uses NO as a signal molecule to relax the surrounding smooth muscle cells resulting in vasodilation and increasing blood flow (Knowles & Moncada, 1992).

iv) Mechanical signals: shear stress, pressure. For example, endothelial cells are very sensitive to blood shear stress which can stimulate cellular responses for

maintaining endothelial cell function and are atheroprotective (Traub & Berk, 1998).

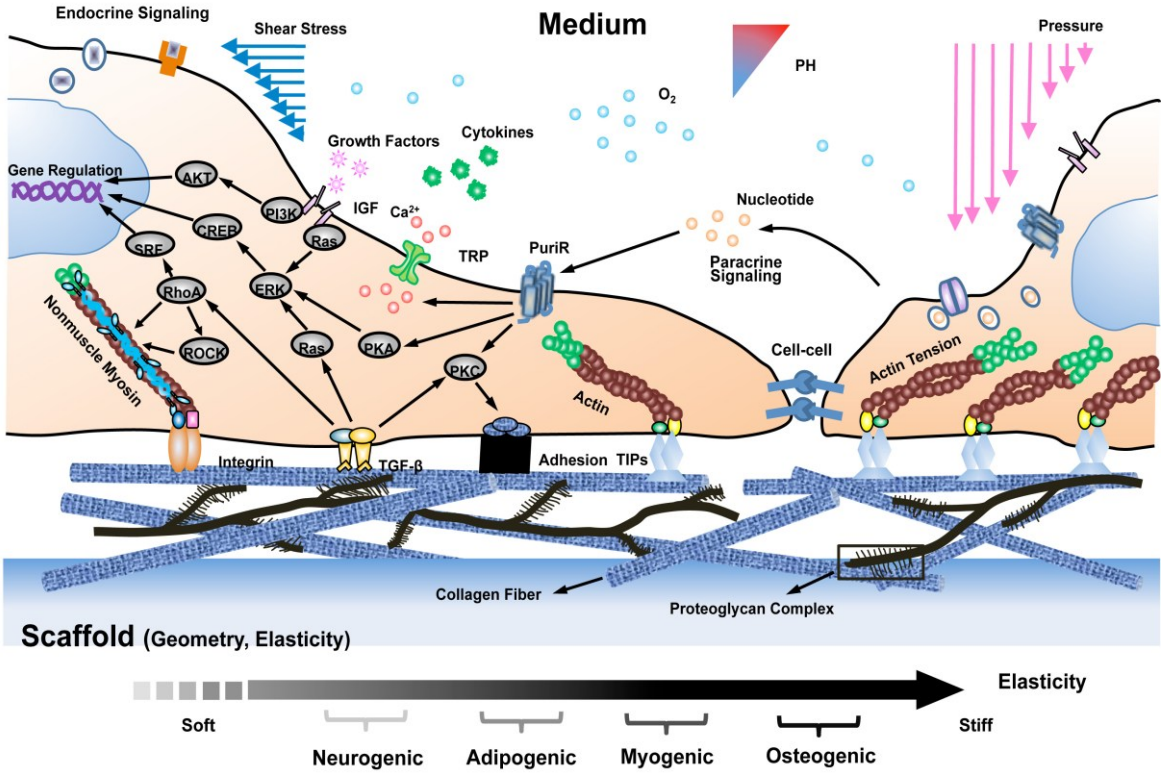


Figure 6. Various Factors Define the 3D Cell Microenvironment.
Cells are living and growing in a 3D environment that is defined by extracellular matrix properties such as elasticity and geometry, molecules with connection to the extracellular matrix such as intergrins, transforming growth factor-β (TGF-β), tension induced proteins (TIPs), and transient receptor potential (TRP) that regulate gene expression and focal adhesion *via* cytoskeleton tension and the activation of a series of mechanical transduction events. Simultaneously, a large number of soluble factors such as growth factors, cytokines and extracellular nucleotides influence cell behavior. In addition, mechanical forces such as shear stress, blood pressure as well as chemical and physical factors like pH or oxygen can also regulate cell morphology and function (Taken from Schulze & Tobiasch, 2012).

A variety of different scaffolds has been created to mimic a 3D environment *in vitro*. Generally, scaffolds can be classified into natural and artificial scaffolds. Natural scaffolds are obtained by decellularizing natural materials. For example porcine and ovine arteries or veins have been successfully decellularized and re-cellularized with human cells (Teebken *et al.*, 2000; Zhao *et al.*, 2009). A specific use of natural

scaffolds is small diameter bypass surgery, whereas synthetic grafts are usually applied for larger arterial bypass surgery (Veith *et al.*, 1986; MacNeill *et al.*, 2002). Until now, these models have been studied in either pre-clinical approach on different level of clinical trials. Some of them are even used in surgery already (Langer & Vancanti, 1993). For example in the cardiovascular system, polymers such as Dacron or expanded polytetra-fluororthylene have been developed and widely used as large-diameter (more than 5 mm internal diameter) vascular grafts in bypass surgery already long time ago (Debakey *et al.*, 1964; Campbell *et al.*, 1975; Molina *et al.*, 1978).

Artificial scaffolds are synthetic materials such as a collagen matrix for blood vessels. These synthetic conduits display a convenient scaffold source due to their good mechanical properties and durability *in vitro* and *in vivo*. However, in the vascular system, their usefulness in small vessels (with a diameter less than 6 mm) is limited due to their lack of growth capacity *in vivo*, risk for infection, development of neointima, and potential disadvantage for calcium deposition and thrombus formation (Chard *et al.*, 1987; Cabver *et al.*, 1995). Although several methods were used to modify the grafts such as protein lining and polymer resurfacing and reduced thrombosis as well as neointimal hyperplasia was observed short term after modification. However, very little improvement of long-term arterial graft patency was found (Devine *et al.*, 2001).

On the above mentioned synthetic scaffolds, autologous vascular cells were subsequently seeded so that they can replace the damaged vessels. Mature endothelial cells were used to re-cellularize artificial scaffolds, which improved blood perfusion and vascular cell viability after transplantation (Mandrup *et al.*, 1997; Kawaguchi *et al.*, 1998). A large amount of these endothelial cells were required to ensure adequate vascularization of artificial arterial-like constructs. However, mature SMCs and ECs have limited proliferation potential and autologous cell isolation procedures are often highly invasive for the patient. On the other hand, the numbers of patients who suffered from cardiovascular diseases is increasing rapidly

simultaneously with a decreasing average age of disease onset in recent years. Therefore, it is necessary to look for an abundant source of mature endothelial and smooth muscle cells for vascular grafts to treat cardiovascular diseases (Mckee *et al.*, 2003; Poh *et al.*, 2005).

1.4 Stem Cells

Stem cells are regarded as promising cell sources for tissue engineering and replacement (Lund *et al.*, 2009). These cells are undifferentiated biological precursors that have two unique properties: self-renewal and the ability to differentiate into specialized cells under the appropriate conditions (McKay, 2000). According to the differentiation potential (from high to low), stem cells can be generally categorized: totipotent, pluripotent, multipotent, oligopotent, and unipotent. Totipotent cells can differentiate into every cell type including the embryonic and the extra-embryonic tissues, and therefore have the ability to develop a complete organism. Pluripotent cells can differentiate into the cells of all three germ layers, ectoderm, mesoderm and endoderm (McKay, 2000), but not to the extra-embryonic membranes and therefore cannot form a complete organism. Multipotent cells are able to differentiate into several cell lineages, usually these lineages belong to the same germ layer where the multipotent cells are derived from. Oligopotent cells can differentiate into only a few cell lineages that are closely related to their original layer. Unipotent cells can give rise to only one cell type and therefore are described as precursor cells or progenitors. The different stem cell types and their differentiation potential are shown in figure 7.

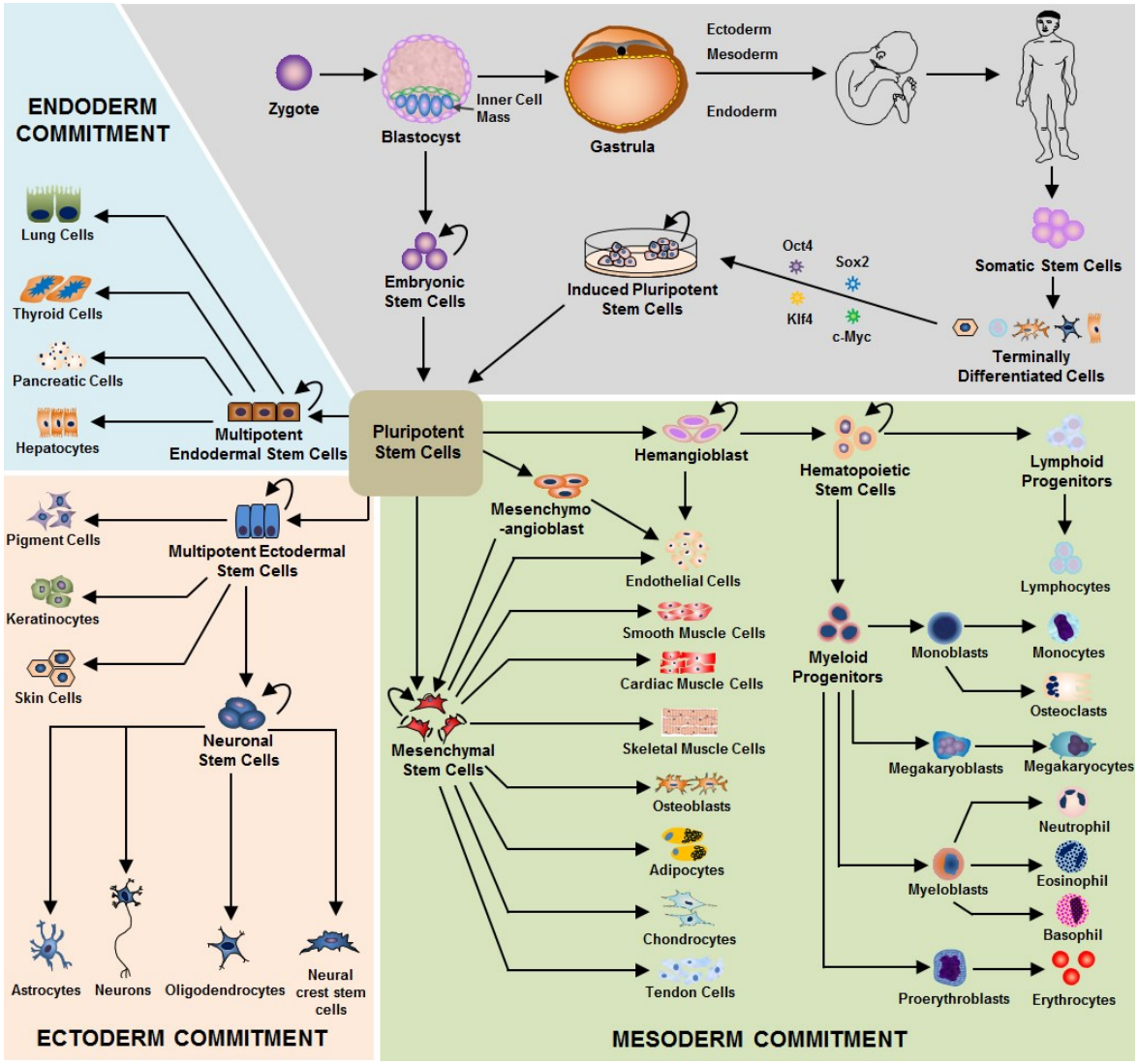


Figure 7. Stem Cell Types and Their Differentiation Potential.
Stem cells can be classified according to their potential into pluripotent (embryonic stem cells, induced pluripotent stem cells), multipotent (mesenchymal stem cell, hematopoietic stem cells, neuronal stem cells, and endodermal stem cells), and oligopotent (monoblast, proerythroblast). Pluripotent stem cells have the potential to differentiate into all cell lineages of the three germ layers. Multipotent stem cells have the capacity to commit towards several cell lineages that are originated from the same germ layer. Oligopotent and unipotent stem cells can differentiate into a few cell lineages, or even one type of cells respectively.

Since the pluripotent cells can differentiate towards cells from all germ layers, they provide great hope for cell-based therapies in Regenerative Medicine. The first discovered and well-studied pluripotent stem cells were embryonic stem (ES) cells

which are isolated from the inner cell mass of embryos in the blastocyst stage (Thomson *et al.*, 1998). However, the isolation and subsequent use of ES cells is strictly limited by law in most countries due to its ethical issues (Whittaker, 2005). In 2006, induced pluripotent stem cells (iPS) have been created and used as an alternative source of embryonic stem cells. The iPS cells were first generated by genetic reprogramming of differentiated somatic cells using four transcription factors: Oct4, Sox2, c-Myc, and Klf4 (Takahashi & Yamanaka, 2006; Takahashi *et al.*, 2007; Wernig *et al.*, 2007) and shortly afterwards another combination of four transcription factors: Oct4, Sox2, Lin28, and Nanog gave the same result (Yu *et al.*, 2007). The iPS cells show very similar characteristics to ES cells such as morphology, proliferation, surface antigens, pluripotent cell-specific genes, differentiation potential and telomerase activity, but with very less ethical concerns and iPS cells keep part of epigenetic profile of the original source. Thus, iPS cells are one of the most interesting research topics in Regenerative Medicine and the 2012 Nobel Physiology and Medicine Prize was awarded for this finding.

In 2009 reprogramming was successful with one factor, Oct4 only when neural stem cells were used instead of fibroblasts (Kim *et al.*, 2009) or several small chemical molecules were combined in the programing process (Zhu *et al.*, 2010; Li *et al.*, 2011). In the beginning, reprogramming strategies required the use of retroviral or adenoviral transfection of a multiprotein expression vector combined with the piggyback transposon/transposase system to deliver the stemness factor transgenes (Woltjen *et al.*, 2009). This procedure causes difficulties if iPS cells are considered for clinical applications. Due to the integration step and thus un-predictable altered gene regulation might leads to cancer formation. In addition both, ES and iPS cells are strongly restricted in clinical transplantation because they are prone to form teratomas, even if iPS cell are reprogrammed without using integrating vectors (Hentze *et al.*, 2009). Another limitation for this technique is that the reprogramming efficiency is very low (0.1%), especially when no integrating viral vectors were used to avoid the potential for insertion mutagenesis (Woltjen *et al.*,

2009). In 2013 Deng's group reported that while only applying seven chemical molecules insteading of transfection, fibroblasts can be reprogrammed into iPS cells (Hou *et al.*, 2013). In 2014 it was published that using mechanical force and low pH condition can reprogram lymphocytes into iPS with significantly increased efficiency (Obokata *et al.*, 2014a; Obokata *et al.*, 2014b). The development of iPS generation is summarized in figure 8.

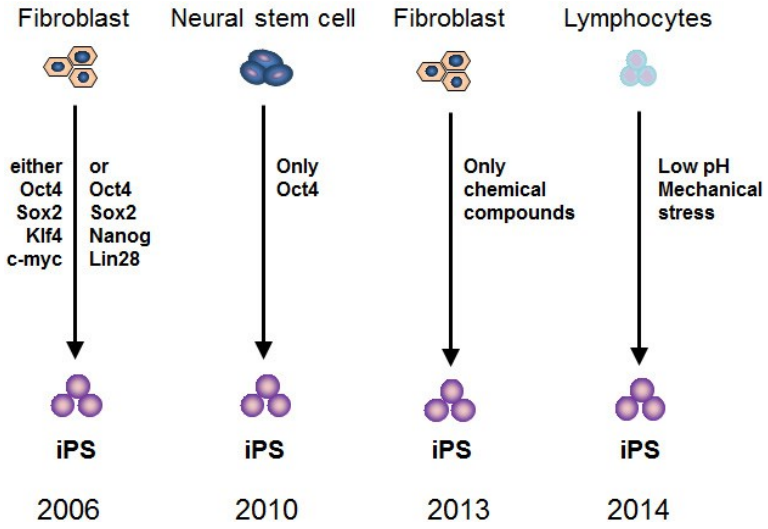


Figure 8. Development of Reprogrammed iPS Cells.
Induced pluripotent stem cells (iPSC) were developed by Shinya Yamanaka in 2006 by reprogramming mouse fibroblasts with the four transcription factors Oct4, Sox2, Klf4, and c-myc. Later another group showed it is also possible to reprogram the cells with another cluster of transcription factors Oct4, Sox2, Nanog and Lin28. In 2010 neural stem cells were reprogrammed *via* only one factor Oct4. In 2013 the reprograming technology was achieved by Deng's group without any transcription factor but instead with seven chemical compounds. Recently two Nature papers showed that particular environmental factors such as low pH and mechanical stress can reprogram the mouse lymphocytes to the pluripotent state.

1.4.1 Mesenchymal Stem Cells

Mesenchymal stem cells (MSCs) belong to the adult stem cells which are able to repair body cells and maintain the normal turnover of regenerative organs. MSCs can be obtained from bone marrow (Becker *et al.*, 1963; Cudkowicz *et al.*, 1964),

peripheral blood, umbilical cord, umbilical cord blood, skeleton muscle, placenta, and adipose tissue (Zuk *et al.*, 2002; Gimble & Guilak, 2003; Kern *et al.*, 2006).

MSCs are multipotent and able to differentiate towards a number of different cell lineages. For example MSCs can be successfully differentiated towards chondrocytes, osteoblasts or adipocytes (McKay, 2000; Zippel *et al.*, 2012) but MSCs are also able to differentiate towards several additional mesodermal cell lineages. For example MSCs can differentiate into skeleton muscle cells (Wakitani *et al.*, 1995; Ferrari *et al.*, 1998; Gronthos *et al.*, 2001), cardiac muscle cells (Planat-Benard *et al.*, 2004), and smooth muscle cells (Rodriguez *et al.*, 2006; Lee *et al.*, 2006; Heydarkhan-Hagvall *et al.*, 2008; Wang *et al.*, 2010). Interestingly, MSCs also show the capacity to differentiate into cells derived from other germ layers (see Fig. 9). For example, MSCs can differentiate into neuron-like cells *in vitro* (Scuteri *et al.*, 2011), and into astrocytes after implanting into the mouse brain *in vivo* (Kopen *et al.*, 1999). A successful differentiation towards islet-like cells was demonstrated for MSCs as well (Liu & Han, 2008), which provides a good insulin producing cell source and might be applied as diabetes therapy (Karnieli *et al.*, 2007; Limbert *et al.*, 2009). In addition, hepatocyte-like cells can also be generated from MSCs *in vitro* and *in vivo* (Seo *et al.*, 2005; Banas *et al.*, 2007; Pournasr *et al.*, 2011). In addition MSCs also pose the ability to differentiate into endothelial cells (Wosniza *et al.*, 2007; Konno *et al.*, 2010; Zhang *et al.*, 2011; Portalska *et al.*, 2013). This "trans-differentiation" phenomenon can be explained due to the fact that MSCs although mainly originated from mesoderm, also in parts are originated from the other two germ layers. Trans-differentiation, although therefore not applicable to mesenchymal stem cells is a widely used term often to doubt a specific differentiation since lineage commitment was thought not to be reversible until iPS cells came up (Slayton & Spangrude, 2004). Another explanation is that MSCs might not be a specific "pure" adult stem cells, but instead a diverse mixture of several specific progenitor cells (Tuan *et al.*, 2003). The different sources of MSCs and their multipotent differentiation potential is summarized and in figure 9.

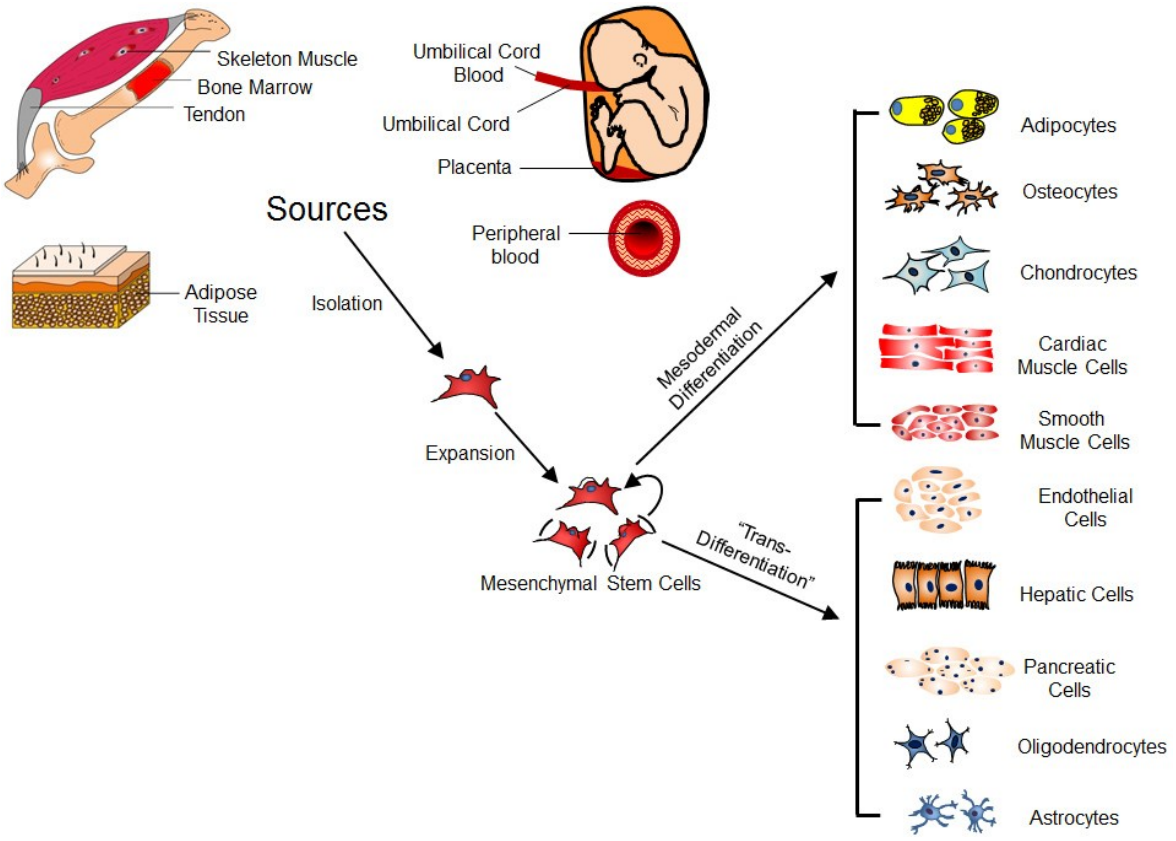


Figure 9. Sources and Differentiation Potential of Human Mesenchymal Stem Cells.
Human mesenchymal stem cells can be isolated from various tissues or organs such as umbilical cord, umbilical cord blood, placenta in embryonal tissue and peripheral blood, skeleton muscle, bone marrow, tendon, and adipose tissue in adult. The mesodermal differentiation potential of MSCs has been confirmed with the differentiation potential into adipocytes, osteoblasts, chondrocytes, skeleton muscle cells, smooth muscle cells, and cardiac cells. MSCs also show their potential to differentiate towards cells of other germ layers such as endothelial cells, pancreatic cells, astrocytes, oligodendrocytes, and hepatic cells.

To characterize MSCs, minimal criteria were suggested by the International Society for Cellular Therapy (ISCT) (Dominici *et al.*, 2006). MSCs should therefore:

i) Display plastic adherence

ii) Show a multipotent differentiation potential *in vitro* towards the adipogenic, osteogenic and chondrogenic cell lineage as demonstrated by specific staining.

iii) Express the mesenchymal markers CD73, CD90 and CD105, and be negative for markers CD34, CD45, CD14 or CD11b, CD79α or CD19 and HLA-DR.

Until now, adipose tissue derived mesenchymal stem cells (AT-MSCs) and bone marrow derived mesenchymal stem cells (BM-MSCs) are the two most frequently investigated MSCs. It has been reported that AT-MSCs express a similar pattern of mesenchymal cell markers (Zuk *et al.*, 2001; Lee *et al.*, 2004). But some surface antigen expressions differ between BM-MSCs and AT-MSCs: CD49d, CD54, CD34, and CD106. CD49d is expressed only in AT-MSC, and the expression of CD106 was only detected in BM-MSCs (Zuk *et al.*, 2002; De Ugarte *et al.*, 2003a). No remarkable difference was observed in their morphology or immune phenotype (De Ugarte *et al.*, 2003b), except Peng and colleagues reported that BM-MSCs are larger than AT-MSCs (Peng *et al.*, 2008). In addition AT-MSCs showed a higher proliferative activity than BM-MSCs (Lee *et al.*, 2004; Peng *et al.*, 2008; Ahmadian Kia *et al.*, 2010). This might be because cyclin B2, cell division cycle associated 8, and Ki-67 were higher expressed in AT-MSCs if compared to BM-MSCs and UCB-MSCs (Lee *et al.*, 2004; Peng *et al.*, 2008).

AT-MSCs can be obtained as a population of 5000 cells from one gram adipose tissue which is 100-500 times higher than the MSC numbers obtained from bone marrow (Kitagawa *et al.*, 2006; Ding *et al.*, 2011). Next to this cell populations, maximal life span, and multipotential of BM-MSCs decreases with the increase of donor's age, which are not found in AT-MSCs (Muslller *et al.*, 2001; Stenerup *et al.*, 2003). Further AT-MSCS are shown at least as immunosuppressive as BM-MSC *in vitro* (Puissant *et al.*, 2005). AT-MSCs were not capable of inducing CD^{8+} T cell mediated lysis, which was found for BM-MSCs. This indicates a less immunogenic profile of AT-MSCs compared to BM-MSCs (Roemeling-van Rhijn *et al.*, 2013).

In addition, AT-MSCs are especially attractive as autografts and allografts, since most of them are obtained from healthy donors and can be harvested in a reasonable amount (Oedayrajsingh-Varma *et al.*, 2006; Lin *et al.*, 2007). They cause less ethical issues because liposuction material is regarded as a waste product of plastic surgery (Zuk *et al.*, 2002). Their harvestings also have less invasive procedure compared to bone marrow (De Ugarte *et al.*, 2003).

The potency of AT-MSCs to differentiate towards three major mesodermal cell types, namely adipocytes, osteoblasts and chondrocytes *in vitro* (Pittenger *et al.*, 1999; Zuk *et al.*, 2002; Lee *et al.*, 2004) is also a difference if compared to umbilical cord blood or dental follicle derived MSCs, which are unable to differentiate into the adipogenic lineage (Kern *et al.*, 2006; Zippel *et al.*, 2012).

1.5 Stem Cells for Vascular Tissue Engineering

1.5.1 Stem Cells in Regenerative Medicine

Stem cells are widely considered and used to re-establish the function of damaged cells or tissues or to generate organs for tissue replacement (Daley, 2012). For example patients with injuries from accidents or degenerative diseases always require a large amount of mature cells which can be generated from stem cells with sufficient numbers and quality. In addition, stem cells can be utilized in drug development to develop test target treatment diseases with a genetic predisposition (Braam *et al.*, 2010; Davis *et al.*, 2011). For example the oldest, most known and widely used stem cell therapy is the transplantation of bone marrow hematopoietic stem cells to treat multiple myeloma or leukemia patients (Becker *et al.*, 1963; Cudkowicz *et al.*, 1964). Another clinical available stem cell therapy is using skin stem cells growing skin grafts for patients with severe burns of the body since 1970s (Burke *et al*., 1975). This therapy improve the wound healing and has less scars, however the new skin has no hair follicles, sweat glands and blood vessels which requires further regeneration (Gardien *et al*., 2014).

Several other stem cell applications are being studied in clinical trials such as spinal cord injury (Cummings *et al.*, 2005), reconstruction of large bone defects (Beyth *et al.*, 2011), osteo-integration of tooth implants (Volponi *et al.*, 2013), cardiac muscle regeneration (Ptaszek *et al.*, 2012), corneal disease (Daniels *et al*., 2006). In addition stem cells have also been considered as a supplemental source to treat

diabetes (El-Badri & Ghoneim, 2013), liver damages (Zhang & Wang, 2013), Parkinson's disease as well as Alzheimer's disease (Lindvall *et al.*, 2006; Singec *et al.*, 2007; Androutsellis-Theotokis *et al.*, 2009) and others in the future. The wide usage of stem cells is summarized in figure 10.

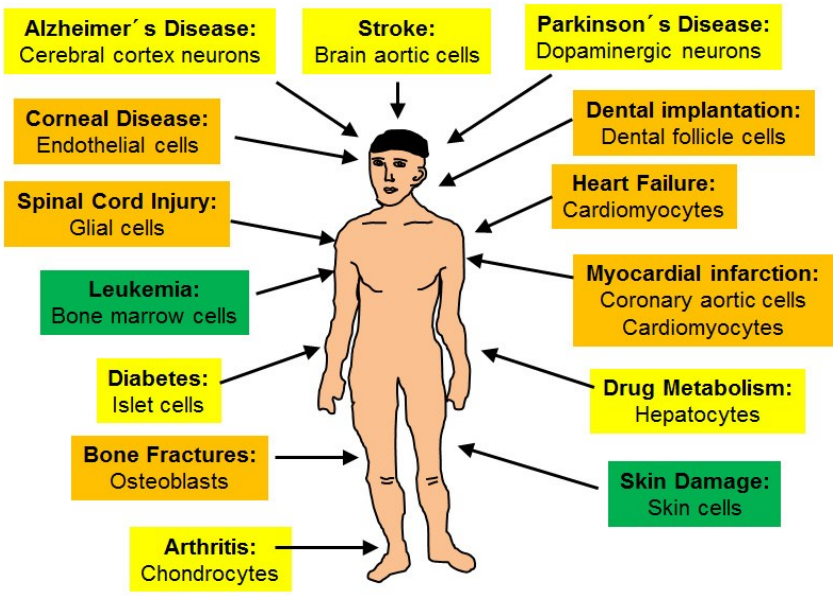


Figure 10. The Usage of Stem Cells in Regenerative Medicine.

The stem cell therapy has drawn an increasing attention in recent years due to their repair function capacities and the rising requirement of tissue replacement. The current established stem cell therapies are for leukemia and skin damage (marked in green). Different types of stem cells have been used to treat various diseases in different level of clinical trials such as heart failure, myocardial infarction, bone fractures, corneal disease, and spinal cord injury (marked in orange). In addition, stem cells are also considered for treatment of Alzheimer's disease, Parkinson's disease, and stroke so on in pre-clinical studies (marked in yellow).

Taken together, stem cell therapy has been already applied into the clinic and provides a promising way to achieve patient-specific therapies for various diseases in the future. However, basic research has to be performed carefully to exclude tumor formation and undesired differentiations, to reduce possible immune rejection, and optimize the long-term repair. In order to solve these problems, the differentiation processes with identification of the related key molecules and signaling pathways are prerequisite and need further detailed investigation.

1.5.2 Mesenchymal Stem Cells in Vascular Tissue Engineering

The interest of using MSCs for cardiovascular tissue engineering is increasing rapidly (Heydarkhan-Hagvall *et al.*, 2008; Zhang *et al.*, 2012), because they showed the potential to differentiate towards cardiac muscle cells (Planat-Benard *et al.*, 2004; Janeczek Portalska *et al.*, 2012; Mohanty *et al.*, 2013), smooth muscle cells (Lee *et al.*, 2006; Rodriguez *et al.*, 2006), and endothelial cells (Wosniza *et al.*, 2007; Portalska *et al.*, 2013). In addition, MSCs seem to be hypoimmunogenic and thus suitable for allogeneic transplantation (Niemeyer *et al.*, 2010). MSCs are also able to induce immunosuppression upon transplantation (Porada *et al.*, 2006; Huang *et al.*, 2008).

1.5.2.1 Endothelial Cell Differentiation of MSCs

Vascular endothelial cells play a crucial role in various vascular functions. Damage or dysfunction of ECs leads to serious cardiovascular diseases such as heart attack and stroke. Mesenchymal stem cells have been considered as a favourable source to obtain a large amount of ECs to replace the damaged ECs.

Differentiated endothelial cells are characterized with the major endothelial cell markers. For example vascular endothelial growth factor receptor 2 (VEGFR2) is one of the two major VEGF receptors and is regarded as an early stage marker during endothelial cell differentiation (Ferrara, 2004; Bai *et al.*, 2010). Later stage markers such as von Willebrand factor (vWF), platelet-endothelial cell adhesion molecule 1 (PECAM-1), endothelial nitric oxide synthases (eNOS) and vascular endothelial-cadherin (VE-cadherin) are used to identify mature differentiated endothelial cells (Huang *et al.*, 2008; Bai *et al.*, 2010). Because the change of mRNA expression and surface marker profile is not sufficient to prove the success of differentiation, endothelial cell functional tests such as three-dimensional capillary-like tube formation assay on matrigel or the up-take acetylated low-density

lipoproteins (LDLs) assay are used to further characterize the cells. (Huang *et al.*, 2008; Chen *et al.*, 2009).

The most used medium to induce endothelial cell differentiation contains normally VEGF and fibroblast growth factor (FGF). VEGF is the most common supplementary agent and enhances the endothelial cell differentiation significantly (Oswald *et al.*, 2004). FGF induces MSC differentiation towards ECs even without adding VEGF and promotes angiogenesis during the formation of large and mature blood vessels (Perets *et al.*, 2003). Other soluble factors such as endothelial growth factor (EGF) are also known to enhance the differentiation process. More data suggest that other factors could influence the EC differentiation as well. For example high cell density culture enhanced EC differentiation (Whyte *et al.*, 2011). The seeding cell number is approximately 10,000 cells/cm^2, which is 2800 cells/cm^2 in adipogenic and 1400 cells/cm^2 in osteogenic differentiation (Zippel *et al.*, 2012). Shear stress (Fischer *et al.*, 2009; Bai *et al.*, 2010) and mechanical stimuli (Maul *et al.*, 2011) also seem to be a successful way to induce EC differentiation. In addition elasticity and other parameters of scaffolds induced MSC commitment towards ECs when MSCs were seeded on elastic nanofiber hydrogels (Wingate *et al.*, 2012) or on three-dimensional matrices (Valarmathi *et al.*, 2009; Zhang *et al.*, 2009).

Several signaling pathways are involved in mediating endothelial cell differentiation. For example the Rho/MRTF-A signaling pathway acts as an important player in controlling VEGF-induced EC differentiation of human and rat BM-MSCs (Wang *et al.*, 2013). A sustained activation of p42 MAPK/ERK, but not p44 MAPK/ERK by VEGF mediates bone marrow stem cell differentiation towards endothelial cells in a time-dependent manner up to 14 days (Xu *et al.*, 2008). In addition, the inhibition of PI3K/Akt pathway blocks EC differentiation of AT-MSCs (Cao *et al.*, 2005). An overview of the above mentioned factors and signaling pathways involved in EC differentiation is summarized in figure 11.

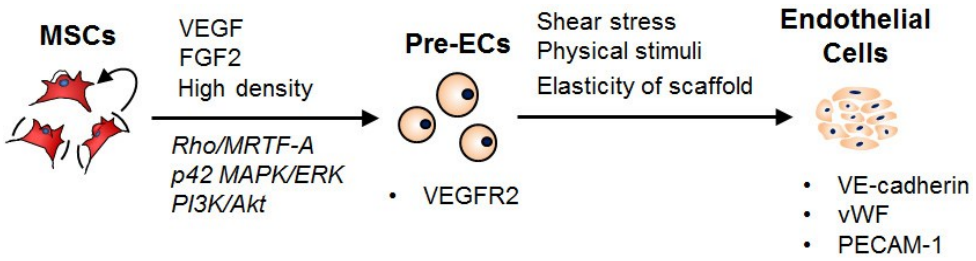


Figure 11. Differentiation of Endothelial Cells from MSCs.
Mesenchymal stem cells differentiate towards endothelial cells under certain conditions such as in the presence of VEGF and FGF2. Rho/MRTF-A, p42 MAPK/ERK, and PI3K/Akt improve the differentiation when they are activated. Endothelial precursor cells express the endothelial cell marker VEGFR2 in the early stage whereas the mature endothelial cells express the later stage endothelial cell markers eNOS, VE-cadherin, vWF, PECAM-1.

1.5.2.2 Smooth Muscle Cell Differentiation of MSCs

Vascular smooth muscle cells are an important player in mechanically supporting endothelium, essentially mediating angiogenesis, and controlling blood pressure (Owens *et al.*, 2004). Compared to the vascular vein or capillary artery, a successful construction of artificial engineered macrovascular blood vessel requires generating a functional SMC layer. Mature SMCs have the unique feature displaying a synthetic or a contractile phenotype under different conditions. The synthetic phenotype is similar to fibroblast morphology. SMCs are highly proliferative and secret ECM proteins such as collagen, elastin and proteoglycans (Harris *et al.*, 2009). In contrast, contractile SMCs demonstrate a spindle-shaped morphology with a prominent nucleus located in the center. These SMCs proliferate at an extremely low rate but are able to contract (Chan-Park *et al.*, 2009).

Until now, the success of MSC differentiating towards smooth muscle cells has been widely achieved. These differentiated SMCs are characterized on the molecular level with a series of smooth muscle cell markers including smooth muscle actin-α (SMA-α), transgelin-2 (SM22α), calponin, caldesmon, smoothelin, and smooth

muscle myosin heavy chain (SMMHC) (Solway *et al.*, 1995; Gong *et al.*, 2008). SMA-α and SM22α are usually regarded as early markers of smooth muscle cell commitment, whereas calponin, caldesmon and SMMHC are late markers (Ross *et al.*, 2006). The functional test of differentiated smooth muscle cells is reasonable after the proof of surface markers. A contraction assay in a collagen gel lattice after stimulation with agents such as potassium chloride or carbachol was used recently to identify differentiated SMC activity *in vitro* (Espagnolle *et al.*, 2014).

Several growth factors were used to induce a successful SMC differentiation (Vater *et al.*, 2011). Transforming growth factor beta (TGF-β) is the most potent inducer of both, early stage and the mature commitment process (Sinha *et al.*, 2004). Gong and colleagues showed that a concentration of 0.1–10 ng/mL TGF-β increased smooth muscle cell marker expressions such as calponin in a dose-dependent manner (Gong *et al.*, 2008). Heparin also enhanced SMA-α, SM22α and calponin expression during MSC commitment towards SMCs (Rodríguez *et al.*, 2005). However, the role of platelet-derived growth factor- ββ (PDGF-ββ) in SMC differentiation is discussed controversially. An example was found that PDGF-ββ appears to suppress expression of SMC markers such as SM-22α and SMMHC *in vitro* (Dandré & Owens, 2004). Other growth factors such as bone morphogenetic protein-4 (BMP-4) and ascorbic acid seem to influence the SMC differentiation as well (Narita *et al.*, 2008; Wang *et al.*, 2010). Next to this a close cell-to-cell contact is required for SMC differentiation (Galmiche *et al.*, 1993). For example a high cell density (approximately 5,000 cells/cm^2) culture of MSCs is more prone to differentiate towards SMCs (Wang *et al.*, 2006).

Several signaling pathways are involved in regulating the SMC differentiation. Myocardin and Smad2 cooperate and induce MSC differentiation towards SMCs in the presence of TGF-β (Wang *et al.*, 2012). TGF-β was also shown to induce SMC differentiation *via* the Notch ligand Jagged 1 (JAG1) and subsequent activation of SMAD3 and Rho kinase (Kurpinski *et al.*, 2010). Blocking the ERK/MAPK signaling pathway enhanced the differentiation of BM-MSCs into smooth muscle cells

(Tamama *et al.*, 2008). An overview of the above mentioned factors and signaling pathways regulating SMC differentiation is summarized in figure 12.

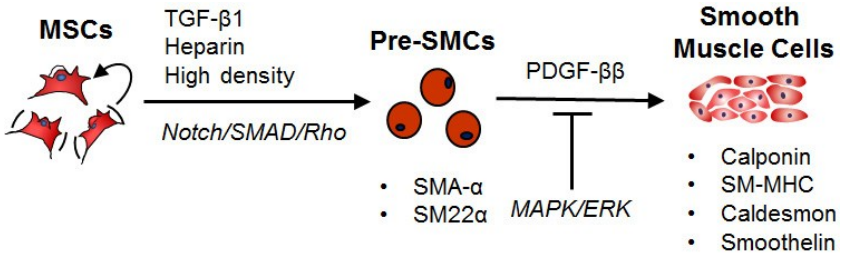


Figure 12. Differentiation of Smooth Muscle Cells from MSCs.
Mesenchymal stem cells differentiate under the influence of Notch signaling towards smooth muscle cells, whereas ERK/MAPK inhibits the differentiation. Smooth muscle precursor cells express early stage SMC markers such as SMA-α and SM-22α. The expression of mature SMC markers such as calponin, SM-MHC, caldesmon, smoothelin was found in late stage differentiated SMCs.

1.6 Aim of the Study

Cardiovascular deconditioning leads to dysfunction of cardiovascular system, from which astronauts are suffering during space missions. No effective drugs are available until now because the molecular underlying mechanism is mainly unclear. The aim of this study is:

→ to evaluate the differentiation potential of hMSCs towards ECs and SMCs.

→ to characterize the differentiated human ECs and SMCs.

→ to investigate P2 receptor function in regulating the differentiation processes.

→ to uncover the underlying signaling pathways during the differentiation process.

→ to examine changes in P2 signaling in ECs and SMCs under simulated microgravity.

→ to create endothelial and smooth muscle cell co-culture models for the study of cardiovascular diseases such as cardiovascular deconditioning.

→ to investigate the paracrine effect on molecular changes and cell behavior in ECs and SMCs under simulate microgravity.

2. MATERIALS AND METHODS

2.1 List of abbreviations

Abbreviation	Description
1g	Normal gravity
°C	Degree centigrade
AC	Adenylate cyclase
ADP	Adenosine diphosphate
ADPβS	Adenosine 5'-O-(2-thiodiphosphate)
Akt (=PKB)	Protein kinase B
AM	Adipogenic differentiation medium
AMPαS	Adenosine 5'-O-(thiomonophosphate)
Ap3A	Diadenosine triphosphate
Ap4A	Diadenosine tetraphosphate
Ap5A	Diadenosine pentaphosphate
APS	Ammonium persulfate
A3P5PS	Adenosine 3'-phosphate 5'-phosphosulphate
ATCC	American Type Culture Collection
ATP	Adenosine triphosphate
ATPγS	Adenosine 5'-O-(3-thiotriphosphate)
AT-MSC	Human adipose tissue-derived stem cell
BMP	Bone morphogenic protein
BM-MSC	Bone marrow mesenchymal stem cell
bp	Base pairs
BzATP	3'-Benzoylbenzoyl adenosine 5´-triphosphate
Ca^{2+}	Calcium ions
cAMP	3'-5'-cyclic adenosine monophosphate
CD	Cluster of differentiation
cDNA	Complementary deoxyribonucleic acid
CH3COOH	Acetic acid
C26H24N4O	Oil Red O
CM	Conditioned medium
cm^2	Centimetres 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
ESC	Embryonic stem cell

EtBr	Ethidium bromide
FACS	Fluorescence activated cell sorting
FCS	Fetal calf serum
GLUT4	Glucose transporter type 4
GSK-3	Glucogen synthase kinase 3
GTP	Guanosine 5'-triphosphate
HMEC-1	Human microvascular endothelial cells
IF	Immunofluorescence
IκB	Inhibitor of kappa B
IL	Interleukine
iPS	Induced pluripotent stem cells
IP3	Inositol 1,4,5-triphosphate
Ip5I	Diinosine pentaphosphate
ISCT	International Society for Cellular Therapy
isoPPADS	Pyridoxal-5-phosphate-6-azophenyl-2',5' disulphonic acid
KCl	Potassium chloride
$KHCO_3$	Potassium hydrogencarbonate
KH_2PO_4	Potassium dihydrogenphosphate
Klf4	Krüppel-like factor 4
LDL	Low density lipoprotein
MAPK	Mitogen-activated protein kinase
α,β-MeATP	α,β-Methylene-adenosine-5'-triphosphate
µg	Microgram (1×10^{-6} g)
µL	Microliter (1×10^{-6} L)
MG	Simulated microgravity
mL	Milliliter (1×10^{-3} L)
mM	Millimol per liter
mRNA	Messenger ribonucleic acid
MRS2159	Pyridoxal-5-phosphate-6-azophenyl-4'-carboxylate
MRS2500	(N)-Methanocarba-N6-methyl-2-iododeoxyadenosine-3',5'-bisphosphate
MRS2179	2'-Deoxy-N6-methyladenosine-3',5'-bisphosphate
MRS2279	(N)-Methanocarba-N6-methyl-2-chloro-2'-deoxyadenosine-3',5'-bisphosphate
MRS2365	(N)-Methanocarba-2-Methylthioadenosine-5'-diphosphate
MRS2500	(N)-Methanocarba-N6-methyl-2-iodo-2'-deoxyadenosine-3',5'-bisphosphate
MRS2578	1,4-di-(Phenylthioureido)-butane
MSC	Mesenchymal stem cell
NaCl	Sodium chloride
Na_2HPO_4	Disodium hydrogen phosphate
Na_2S2O_3	Sodium thiosulfate
NCEP-ATPIII	National Cholesterol Education Program - Adult Treatment Panel III
NH_4Cl	Ammonium chloride
NF023	8,8′-[Carbonylbis(imino-3,1-phenylene)]bis-(1,3,5-naphthalenetrisulfonic Acid)
NF279	8,8′-(Carbonylbis(imino-4,1-phenylenecarbonylimino-4,1-phenylenecarbonylimino))bis-1,3,5-naphthalenetrisulfonic Acid

NF-κB	Nuclear factor kappa B
Oct4	Octamer 4
ON	Osteonectin
PAI-1	Plasminogen activator inhibitor-1
PAPET-ATP	2-[2-(4-Aminophenyl)ethylthio]adenosine 5'-triphosphate
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
PPADS	Pyridoxal-5-phosphate-6-azophenyl-2',4'-disulphonic acid
PPARγ	Peroxisome proliferator-activated receptor γ
PPNDS	Pyridoxal-5-phosphate-6-(2'-naphthylazo-6-nitro-4',8'-disulphonate)
PTH	Parathyroid hormone
RB-2	Reactive blue 2
RNA	Ribonucleic acid
RT	Room temperature
RT-PCR	Reverse transcriptase polymerase chain reaction
SDS	Sodium dodecylsulfate
Sox2	SRY (sex determining region Y)-box 2
TEMED	N,N,N',N'-Tetramethylethylene-diamine
TGF-β	Transforming growth factor-β
TNP-ATP	2',3'-O-(2,4,6-Trinitrophenyl) adenosine triphosphate
TNFα	Tumour necrosis factor alpha
U	Units
UDP	Uridine 5'-diphosphate
UDP-glucose	Uridine diphosphate glucose
UDPβS	Uridine 5'-O-(2-thiodiphosphate)
Up3U	Diuridine triphosphate
UTP	Uridine 5'-triphosphate
UTPγS	Uridine 5'-O-(3-thiotriphosphate)
v/v	Volume per volume
WB	Western blot
WHO	World Health Organisation
w/v	Weight per volume
x g	Relative centrifugal force (RCF)

2.2 Materials

2.2.1 Equipment

Appliance	Supplier
Adapters for LightCycler® Capillaries	Roche, Mannheim, Germany
Camera, Canon Powershot A570IS	Canon, Tokyo, Japan
Centrifuge, Z 233 MK-2	Hermle, Gosheim, Germany
Centrifuge, Z 383 K	Hermle, Gosheim, Germany
CO_2-Incubator, MCO-18 AIC	Sanyo, Munich, Germany
Confocal Microscope, FW300	Olympus, Tokyo, Japan
FACS, CYTOMICS™ 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
LightCycler 1.5	Roche, Mannheim, Germany
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
Vortexer, Autovortex SA6	Stuart Scientific, Redhill, UK
Water Bath with Agitator, SWB 25	Thermo Fisher Scientific, Waltham, USA

2.2.2 Consumables

Consumable	Supplier
Biotinylated Protein Ladder	Cell Signaling TECHNOLOGY, Danvers, USA
Collagenase / Dispase	Biochrom AG, Berlin, Germany
DMEM	Biochrom AG, Berlin, Germany
dNTPs (2 mM; 100 mM)	Invitrogen, Carlsbad, USA
EGM-2 Medium	PromoCell, Heidelberg, Germany
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
Oregon Green 488 BAPTA-1/AM	Invitrogen Molecular Probes®, Carlsbad, USA
Human Phospho-Kinase Antibody Array (ARY003B)	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
Dream*Taq* 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
Type I Collagenase (6 U mg^{-1})	Biochrom AG, Berlin, Germany
Whatman Paper	VWR International Ltd, Luttersworth, 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). For the

clinorotation experiment, the cell culture slides were purchased from Nunc Thermo Fisher Scientific (Langenselbold, Germany).

2.2.3 Chemicals

Chemical	Supplier
2-Propanol	Roth, Karlsruhe, Germany
A-740003	Sigma-Aldrich, Steinheim, Germany
Acetic acid	Sigma-Aldrich, Steinheim, Germany
Acid fuchsine	Sigma-Aldrich, Steinheim, Germany
Acrylamide	Sigma-Aldrich, Steinheim, Germany
ADP	Sigma-Aldrich, Steinheim, Germany
Agarose	Sigma-Aldrich, Steinheim, Germany
Alizarin Red S	Sigma-Aldrich, Steinheim, Germany
Ampicillin	USB, Cleveland, USA
APS	Sigma-Aldrich, Steinheim, Germany
Apyrase	Sigma-Aldrich, Steinheim, Germany
Ascorbic-acid-2-phosphate	Sigma-Aldrich, St. Louis, USA
ATP	Sigma-Aldrich, Steinheim, Germany
β-Glycerophosphate	Sigma-Aldrich, St. Louis, USA
ß-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
DMSO	Roth, Karlsruhe, Germany
Eosin	Sigma-Aldrich, Steinheim, Germany
Ethanol	Merck KGaA, Darmstadt, Germany
Ethidium Bromide	Sigma, Deisenhofen, Germany
EDTA Disodium Salt	VWR International Ltd, Luttersworth, UK
$FeCl_3.6H_2O$	Sigma-Aldrich, Steinheim, Germany
Formaldehyde	Calbiochem, San Diego, USA
Glycerol	Roth, Karlsruhe, Germany
Heparin	Biochrom AG, Berlin, Germany
Hematoxylin	Sigma-Aldrich, Steinheim, Germany
HEPES	Sigma-Aldrich, Steinheim, Germany
Hydroxyurea	Sigma-Aldrich, Steinheim, Germany
Indomethacine	Sigma-Aldrich, Steinheim, Germany

Insulin	Sigma-Aldrich, Steinheim, Germany
I_2	Sigma-Aldrich, Steinheim, Germany
HCl	Roth, Karlsruhe, Germany
KCl	Roth, Karlsruhe, Germany
KH_2PO_4	Roth, Karlsruhe, Germany
DiI-labeled acetylated-low density lipoprotein (DiI-ac-LDL)	Biomedical Technologies Inc., Ward Hill, USA
2-MeSADP	Sigma-Aldrich, Steinheim, Germany
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_4Cl	Roth, Karlsruhe, Germany
$NaHCO_3$	Sigma-Aldrich, Steinheim, Germany
Na_2HPO_4	Roth, Karlsruhe, Germany
$Na_2S_2O_3$	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^{-1})	Biochrom AG, Berlin, Germany
PPADS	Sigma-Aldrich, Steinheim, Germany
Propidium Iodide	Sigma-Aldrich, Steinheim, Germany
RO-3	Tocris Bioscience, Bristol, UK
Rhodamine	Life Technologies, Carlsbad, USA
SDS	Amersham Biosciences, Bucks, UK
Suramin	Sigma-Aldrich, Steinheim, Germany
TEMED	Sigma-Aldrich, Steinheim, Germany
Transforming growth factor-β1 (TGF-β1)	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
UDP	Sigma-Aldrich, Steinheim, Germany
UDP-Glucose	Sigma-Aldrich, Steinheim, Germany
UTP	Sigma-Aldrich, Steinheim, Germany
Xylene Cyanol FF	Merck KGaA, Darmstadt, Germany

2.2.4 Antibodies

The primary antibodies for EC markers: eNOS (sc-8311, H-159) rabbit polyclonal Ig G, PECAM-1 (sc-1506, M-20) goat polyclonal IgG, VEGFR2 (sc-6251, A-3) mouse monoclonal IgG, VE-cadherin (sc-6458, C-19) goat polycloneal IgG SM22α (sc-51441, C-14) mouse monoclonal IgG, for SMC markers: Calponin (sc-28545, FL-297) rabbit polyclonal Ig G, SMA-α (sc-53015, CGA7) mouse monoclonal IgG, MYH-11 (sc-6956, G-4) mouse monoclonal IgG, MYOCD (sc-21561, M-16) goat polyclonal Ig G,; for P2 receptors: P2X1 (sc-25692, H-100) rabbit polyclonal Ig G, P2X5 (sc-25695, H-90) rabbit polyclonal IgG, P2X6 (sc-25697, H-81) rabbit polyclonal IgG, P2X7 (sc-31500, G-20) goat polyclonal IgG, P2Y1 (sc-15203, N-16) goat polyclonal IgG, P2Y2 (sc-15209, A-20) goat polyclonal IgG, P2Y4 (sc-20125, H-60) rabbit polyclonal IgG, P2Y6 (sc-16215, N-16) goat polyclonal IgG, P2Y11 (sc-98600, H-300) rabbit polyclonal IgG, P2Y14 (sc-48191, K-12) goat polycloneal IgG; for other proteins: Akt1 (sc-1618, C-20) rabbit polyclonal Ig G, p-Akt1/2/3 (sc-7985-R, Ser 473) rabbit polyclonal Ig G, β-actin (sc-47778, C4) mouse monoclonal IgG1, ERK (sc-94, K-23) rabbit polyclonal Ig G, p-ERK (sc-7383, E-4) mouse monoclonal IgG1, GAPDH (sc-20357, V-18) goat polyclonal IgG, PKC (sc-10800, H-300) rabbit polyclonal Ig G were purchased from Santa Cruz Biotechnology, Santa Cruz, USA.
Primary antibody for Phospho-p38 MAPK (Thr180/Tyr182, #9215) rabbit mAb was purchased from Cell Signaling TECHNOLOGY, Danvers, USA.
Primary antibodies for FACS: CD14-PC5, CD34-PC7, CD44-FITC, CD45-ECD, CD90-PC5, CD105-PE were purchased from Beckman Coulter, Marseille, France.
The secondary antibodies for HRP: Anti-goat (WB), Anti-mouse (WB), Anti-rabbit (WB) were purchased from Cell Signaling TECHNOLOGY, Danvers, USA; for FITC: anti-rabbit, anti-goat, anti-mouse were purchased from Southern Biotech, Birmingham, USA.
The secondary antibodies for FACS: IgG1(Mouse)-ECD, IgG1(Mouse)-FITC, IgG1(Mouse)-PC5, IgG1(Mouse)-PC7, IgG1(Mouse)-PE were purchased from Beckman Coulter, Marseille, France.

2.2.5 Oligonucleotides

Table 2. Primer Sequences.
Primer sequences for P2 receptor genes examined in human, mouse and rat cells, and CD73, CD90, CD105, vimentin, β-actin, the osteogenic markers BMP2, ON, as well as the adipogenic markers LDL, PPARγin humans were used according to previous study of the laboratory (Zippel *et al.*, 2012). P2 primers for bovine cells, the endothelial cell markers VEGFR2, eNOS, vWF, PECAM-1, VE-cadherin, the smooth muscle markers SMA-α, SM22α, calponin, MYH-11, and GAPDH for the respective

information are given below. P2X4 for human, mouse and rat system was used for bovine cells as well. Oligos were purchased from Metabion, Martinsried, Germany

Name	Forward Primer (5'→3') Reverse Promer (5'→3')	Product Size [bp]	Ta [°C]	$MgCl_2$ [mM]
P2X1	TCT AYG AGA AGG GCT ACC AGA CC TCC ACC TCC ACG GGG CAC CAG	361	60	2.5
P2X2	CCA TCA TCA CCA ARG TCA AGG TTG GGG TAG TGG ATG CTG TT	391	53	2.5
P2X3	GCC GCT GCG TSA ACT ACA GGT GGG RAT GAT GTT GAA CT	560	52	2.5
P2X5	GTC ATC GCC AAG AAC AAG AAG GTG CAG TCG GAA GAT GGG GCA GTA G	639	60	2
P2X6	GTT CTT CTT GGT GAC CAA CTT CC CGG ATY CCA TAG AGC TTG AGC A	678	58	2
P2X7	GGT TGT AAA AAG GGA TGG WTG G ACA GCA CTT GCA MCA GGG ATA A	723	55	3
P2Y1	CTT CCT GGG CAA CAG CGT GGC CAG CAC CAA GGG GAC ACA GAA CAT	502	61	3
P2Y2	TGT GCC GCC TCA AGA CCT GGA A GAA GGA GTA GTA GAG GGT GCG G	638	60	1.5
P2Y4	GAK TTC AAG TTC ATC CTG CTG TGA GYC CAT AGC ARA CAA GAG T	574	52	2
P2Y6	CGG GCY GCC TGG CTA GTG TG TTG GTG ATG TGR AAA GGC AGG AAG C	354	61	2
P2Y11	AGC GTC ATC TTC ATC ACC TG CAT GTA GAG TAG MGG GTG GA	621	52	2
P2Y12	ATC GCT ACC AGA ARA CCA CCA GGC CGG GCA AAA TGG AAW GGA ACA AAA CA	406	60	2.5
P2Y13	TGA GCA ACA AGG AAG CAA CAC CAT C ACA CAA AGA AGA CAG CCA CGA CAA C	258	60	3
P2Y14	ATG TAC GTC AGC ATY GTG TTC TT TAG GGG ATT CTG GCA ATR TGG T	455	56	2
VEGFR2	GAG AGG TGC TGC TYM GAT TT GGA AGG AAC TCT CAT TAG GA	649	52	1.5

Table 2 continued				
Name	**Forward Primer (5'→3')** **Reverse Promer (5'→3')**	**Product Size [bp]**	**Ta [°C]**	**$MgCl_2$ [mM]**
eNOS	AAG AGT TCT GGG GGM TCA TC AAG AGT TCT GGG GGM TCA TC	527	55	2
PECAM-1	GGT RAT AGC CCC RGT GGA TGA TTG GCC TTG GCT TTC CTC AG	411	59	2.5
VE-cadherin	CTG CAT CCT CAC CAT CAC AGT CTC GTA GCC GTA GAT GTG CAG	384	61	1
calponin	RCA GAT GGG CAG CAA CAA GG ATT TAT TGT GCT CCA GTG AAR TAG AA	443	51	3.5
SMA-α	CAG GGC TGT TTT CCC ATC CCA TCT CCA GAG TCC AGC A	393	51	1.5
SM22α	CAT GGC CAA CAA GGG TCC CAG TTG GGA TCT CCA CGG	453	53	2
MYH-11	GGG CAG AGC AAA ATC TTC TT GTT TCT TGA TCT TGG CCT CAG	658	51	2
GAPDH	CGT ATT GGG CGC CTG GTC ACC GCC AGT GAG CTT CCC GTT CAG C	653	61	2.5

2.2.6 Buffers, Solutions and Media

Table 3. Buffers, Solutions and Medium

Name	Reagents
Acidified Water	5 mL acetic acid to 1 L of distilled water
Adipogenic Differentiation Medium	Stem cell culture medium with 1 µM dexamethasone, 1 µM insulin, and 200 µM indomethacin
1% Aqueous Silver Nitrate Solution	1g silver nitrate dissolved in 100 mL distilled water
Alizarin Red S Solution	4 x 10^{-2} M Alizarin Red S; pH 4.3
Cell Line Culture Medium	DMEM with Phenol Red and stable 2 mM L-analyl-L-glutamine; 10% (v/v) FCS; 100 U penicillin; 100 µg mL^{-1} streptomycin
Decellularization Solution I	Sterile 0.1% trypsin with 0.02% ethylene diamine tetraacetic acid (EDTA) in PBS without Ca^{2+}, Mg^{2+}
Decellularization Solution II	Sterile RNase (20 µg/mL) and DNase (200 µg/mL) in PBS without Ca^{2+} and Mg^{2+}.

Table 3 continued	
Name	**Reagents**
DMEM Medium	DMEM with Phenol Red and stable 2 mM L-analyl-L-glutamine; 10% (v/v) FCS; 100 U penicillin; 100 µg 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
Endothelial Cell Differentiation Medium (EGM-2)	Endothelial cell growth medium 2 (EGM-2) with 2% FCS, VEGF, bFGF, IGF, and EGF
Eosin Solution	2.5 mg mL^{-1} Eosin, 0.1% glacial acetic acid
Erythrolysis Buffer "Winter"	1 x 10^{-4} M EDTA (pH 8.0), 0.154 M NH_4Cl, 1 x 10^{-2} M $KHCO_3$
Freezing Medium	DMEM, 20% (v/v) FCS, 10% (v/v) DMSO
Hematoxylin Solution	1.0 g mL^{-1} Hematoxylin, 50.0 g mL^{-1} [$KAl(SO_4)_2$ x 12 H_2O], 0.1 g mL^{-1} $NaIO_3$, 50.0 g mL^{-1} chloral hydrate, 1.0 g mL^{-1} citric acid
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 ß-mercaptoethanol
6x Loading Dye	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 10^{-3} M $C_{26}H_{24}N_4O$, 0.1 M C_3H_8O
Osteogenic Differentiation Medium	Stem Cell culture medium, 0.1 µM dexamethasone, 10 mM ß-glycerophosphate, 50 µM ascorbic acid
PBS, 10x	1.37 M NaCl, 0.015 M KH_2PO_4, 0.081 M Na_2HPO_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
Smooth Muscle Cell Differentiation Medium (SMDM)	Stem cell culture medium with 2 % FCS, 5 ng/mL PDGF-ββ, 5 ng/mL TGF-β1 and 30 µg/mL heparin
Stem Cell Culture Medium	DMEM; 10% (v/v) FCS; 100 U penicillin; 100 µg mL^{-1} 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

Table 3 continued	
Name	**Reagents**
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	0.25 M TRIS (hydroxymethyl) amino ethane, 1.92 M Glycine
Transfer Buffer, 1x	10% (v/v) 10x Transfer buffer, 10% (v/v) Methanol
van Gieson's Solution	0.5 g Acid fuchsine, 500 mL Saturated aqueous picric acid, 2.5 mL Concentrated HCl
Verhoeff's Solution	Solution A: 2% hematoxylin in 95% ethanol Solution B: 12.4 g $FeCl_3.6H_2O$ 495 mL Water 5 mL Concentrated HCl Solution C: 2% I_2 in 4% aqueous potassium iodide Working solution: Solution A 30 mL Solution B 20 mL Solution C 10 mL The working solution is made before using and used only once. The stock solutions (A, B, C) are stable for at least a year
Weigert's Iron-Hematoxylin Solution	Solution A: 5 g Hematoxylin 500 mL 95% Ethanol Solution B: 5.8 g $FeCl_3.6H_2O$ 495 mL Water 5 mL Concentrated HCl Working solution: Mix equal volumes of solution A and solution B. The mixture should be made just before using

Table 4. List of Gels

Gels used for electrophoresis are listed below.

Name	Reagents
Agarose Gel (1-2%)	1-2 g agarose in 100 mL 1x TBE
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

Table 5. List of Cell Lines

Cell lines utilized for experiments are given below. The HMEC-1 cell line was provided by Prof. Dulak (Jagiellonian University, Poland). The osteosarcoma cell lines MG-63 and SaOS-2 were provided by Dr. Herten (Heinrich Heine University of Duesseldorf, Germany). The adenocacima cell line MDA-MB-231 was provided by Prof. Luparallo (University of Palermo, Italy).

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

2.3 Methods

2.3.1 Isolation of Primary Cells

2.3.1.1 Stem Cell Isolation

Human mesenchymal stem cells (hMSCs) were isolated from human lipoaspirates of different aged female donors. The usage of this study was approved by the ethics commission of the University of Bonn (see chapter 2.5). The isolation procedure was performed according to a previous publication of the 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. 10 mg mL^{-1} type I collagenase (7.2 x 10^{-1} mL per 100 mL fat tissue) was added and incubated for 60 min at 37°C with agitation to release cells from the surrounding tissue. Upper phase was later centrifuged at 200 x g for 10 min at RT and the cell pellet was collected carefully. The pellet was subsequently treated with 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 at 200 x g for 10 min at RT, seeded in appropriate numbers with stem cell culture medium (see table 3) and incubated in a humidified atmosphere at 37°C with 5% CO_2. Mesenchymal stem cells were selected from e.g. fibroblasts due to their adherence to plastic. The cells were washed with 1x PBS to remove non-adherent cells after 24 hours and then supplied with fresh stem cell culture medium.

2.3.1.2 Endothelial and Smooth Muscle Cell Isolation

Primary endothelial and smooth muscle cells were isolated from cow. Fresh bovine aortas were bought from a slaughter house in Bonn Beuel, 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. All cutted aortas were placed into 1x PBS to avoid drying.

The isolation method of endothelial cells was modified and performed as described (Schwartz *et al.*, 1978). The cutted aorta was washed with 1x PBS and carefully transferred into cell culture dishes coated with type I collagenase (10 mg mL^{-1} in 1x PBS) the inner layer (endothelium) attached to the collagenase and incubated for 60 min at 37 °C. The endothelial cells were slightly scraped with a cell scraper and put onto gelatin (in 1x PBS (1% v/v)) coated culture plates. DMEM medium was added to the freshly crapped cells and incubated at 37 °C, 5% CO_2 under humidified conditions. After 24 hours, medium was exchanged to remove the cell debris.

The isolation method of smooth muscle cells was used as described (Stepp *et al.*, 1986). In brief, the cut aorta was washed with PBS and carefully scraped on a cell scraper to get rid of the endothelial cells. The outer layer was stripped off by using two forceps (one forceps to hold intima and media layer and the other one to pull off the adventitia). The media layer was subsequently cut into small pieces of approximately 2 mm x 2 mm sections with microdissecting scissors. The small pieces were distributed equally in a Petri dish and placed upside down into incubator for 3 hours to allow it to dry so that they are strongly adherent to the dish. An appropriate amount of fresh DMEM medium (2-3 mm above the tissue pieces) was added and the cells were incubated at 37 °C with 5% CO_2 in a humidified atmosphere. After about 7 to 10 days, the smooth muscle cells started to migrate from the tissue and proliferate on the surface of the culture dish.

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 and negative controls. They were cultured in culture line culture medium (see table 3). The medium was exchanged once a week.

2.3.2.2 Primary Cell Culture

Primary cells used in this study were mesenchymal stem cells derived from human adipose tissue; endothelial and smooth muscle cells isolated from bovine aorta.

i) Stem Cell Culture

Human MSCs derived from liposuction materials were grown in culture medium in a humidified atmosphere with 5% CO_2 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 hemocytometer, 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 donor gender, age and passage number.

ii) Primary Endothelial Cell Culture

Primary endothelial cells derived from bovine aorta were cultured in DMEM culture medium (see table 3) in a humidified atmosphere with 5% CO_2 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 hemocytometer. ECs were seeded in a ratio from 1:5 to 1:10 and frozen at -150°C in freezing medium marked with date of isolation and passage number.

iii) Primary Smooth Muscle Cell Culture

Primary SMCs cells derived from bovine aorta were cultured in DMEM culture medium (see table 3) in a humidified atmosphere with 5% CO_2 at 37°C. The medium for the SMCs was changed two times a week. SMC passaging and frozen method as described for EC in chapter 2.3.2.2 ii).

2.3.3 Fluorescent Activated Cell Sorting

The isolated MSCs were characterized by using FACS analysis with the MSC positive markers CD44, CD90 and CD105 and negative markers CD14, CD45 and CD34. MSCs were trypsinized, centrifuged at 200 x g for 5 min and counted. 1 x 10^6 cells were resuspended in 1 mL 0.1% PBSB and passed through a 100 µM cell strainer to achieve a single cell solution. 100.000 cells (100 µL of the cell solution) were incubated in the dark with either the isotype control (20 µL IgG1-FITC and IgG1-PE and 10 µL of the tandem conjugates IgG1-ECD, IgG1-PC5 and IgG1-PC7) or the antibodies CD44-FITC (20 µL), CD105-PE (20 µL) and CD90-PC5 (10 µL) as positive mesenchymal cell markers and CD14-PC5 (10 µL), CD34-PC7 (10 µL) and CD45-ECD (10 µL) as negative markers for 20 min. Cells were washed with 2 mL 0.1 % (w/v) PBSB, centrifuged at 200 x g for 5 min and resuspended in 1 mL 0.1% (w/v) PBSB. The cytometer settings and cell gates were adjusted to the isotype control, followed by measurement of the stem cell markers using the same conditions.

2.3.4 Stem Cell Differentiation

To confirm the multilineage differentiation potential of MSCs and to obtain differentiated human smooth muscle and endothelial cells, the MSCs were first induced towards osteoblasts, adipocytes, and after confirmation the MSCs were differentiated towards smooth muscle and endothelial cells. All differentiation experiments were performed with cell passage No. 2-4.

2.3.4.1 Adipogenic Differentiation

For the adipogenic differentiation, MSCs were seeded in a density of 2.8 x 10^3 cells per cm^2 in DMEM stem cell medium (see table 3). After one day of culture, the

medium was changed to adipogenic differentiation medium that contained 1 µM dexamethasone, 1 µM insulin and 200 µM indomethacin. Cells were grown in adipogenic differentiation medium for 28 days at 37°C with 5% CO_2 under humidified conditions. The 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.

2.3.4.2 Osteogenic Differentiation

For osteogenic induction, MSCs were seeded in a density of 1.4 x 10^3 cells per cm^2 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 was prepared according to Lee and colleagues (Lee *et al.*, 2004), but DMEM was used instead of α-MEM. 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.

2.3.4.3 Endothelial Cell Differentiation

For endothelial cell differentiation, MSCs were seeded in a density of 10,000 cells per cm^2 in culture medium. After one day of culture, medium was exchanged to endothelial cell differentiation medium (EGM-2) (see table 3). The medium was exchanged twice a week. MSCs were grown in endothelial cell differentiation medium for 14 days in a humidified atmosphere as described in chapter 2.3.4.1. As negative control, cells were cultivated in stem cell culture medium without the induction agents.

2.3.4.4 Smooth Muscle Cell Differentiation

For smooth muscle cell differentiation, MSCs were seeded in a density of 5,000 cells per cm^2 in culture medium. After one day of culture, medium was exchanged to smooth muscle cell differentiation medium (SMDM) (see table 3). The medium was exchanged twice a week. MSCs were grown in smooth muscle cell differentiation medium for 14 days in a humidified atmosphere as described in chapter 2.3.4.1. As negative control, cells were cultivated in stem cell culture medium without the induction agents.

2.3.5 Cell Specific Staining

2.3.5.1 Immunofluorescent Staining

Approximately 80% confluent cells were washed twice with PBS and fixed with 4% (v/v) paraformaldehyde for 15 min at RT. After rinsed with 1x PBS twice, cells were incubated with each of following primary antibodies: P2X7, P2Y1, P2Y2, P2Y11, VEGFR2, MYH-11, MYOCD antibodies (in a ratio of 1:100), eNOS, VE-cadherin, PECAM-1 (in a ratio of 1:200), calponin (in a ratio of 1:300), in the antibody dilution buffer overnight. After washing with 1x PBS for three times, cells were incubated with FITC labeled anti-rabbit, goat or 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 (1:10000 in 1x PBS) for 5 min. After subsequent washing with PBS, images were taken with a fluorescent microscopy in Rheinbach (Zeiss, Germany) or a confocal microscopy in Palermo (Olympus, Japan) respectively.

2.3.5.2 Oil Red O Lipid Staining

After adipogenic differentiation, the differentiated adipocytes were visualized by lipid

droplet formation *via* Oil Red O staining. The medium was removed. After rinsing with PBS, cells were fixed with a 10% formaldehyde solution for 90 min at 37°C in a humidified atmosphere. Afterwards cells were washed with 1x PBS for three times and then incubated with a 4.284 x 10^{-3} M Oil Red O staining solution (see table 3) at 37°C. After 30 min incubation, the staining solution was carefully removed. Pictures were taken immediately because the lipid-droplets are prone to rupture after exposure to air for long time. MSCs cultured in parallel in standard culture medium were set and stained as a negative control.

2.3.5.3 Alizarin Red S Staining

After osteogenic differentiation, the osteogenic differentiated cells were visualized by staining extracellular calcium deposits with Alizarin Red S staining (Scherberich, 2007). The medium was removed and cells were fixed with a 4% (v/v) formaldehyde solution for 5 min at 37°C. The cells were later washed with 1x PBS and incubated with 4 x 10^{-2} 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 set and stained as a negative control. The calcium deposits in the ECM were evident as red. Representative images of stained cells were obtained with a microscopy (Carl Zeiss, Germany).

2.3.5.4 von Kossa Staining

After osteogenic differentiation, the osteogenic differentiated cells were visualized by staining the phosphate *via* von Kossa staining. The medium was removed and cells were fixed with a 4% (v/v) formaldehyde solution for 20 min. The cells were rinsed with distilled water and incubated with 5% (w/v) silver nitrate solution (see table 3) in the absence of strong light for 30 min. The cells were washed with distilled water and subsequently washed with 5% sodium thiosulfate to remove excess silver

salts. After 3-5 minutes, the cells were washed with distilled water. MSCs cultured in parallel in standard culture medium were set and stained as a negative control. The calcium deposits were evident as dark brown or black deposits. Representative images of stained cells were obtained with a microscopy (Carl Zeiss, Germany).

2.3.6 LDL Up-take

Cells were incubated with 10 µg/mL DiI-labeled acetylated-low density lipoprotein (DiI-ac-LDL) for 4 hours at 37°C and investigated with an immunofluorescent microscope (Carl Zeiss, Germany) at a wavelength of 565 nm. After staining with LDL, cells were fixed with 4% formalaldehyde for 15 min and subsequently incubated with DAPI (1:10000 in PBS) and rinsed with 1x PBS. Images from two channels (wavelength: 565 nm for LDL; 358 nm for DAPI) were taken with an immunofluorescent microscopy (Carl Zeiss, Germany).

2.3.7 Matrigel Tube Formation Assay

GFR-Matrigel (100 µL per well of 10 mg/mL) was added into a 96-well plate at 4 °C and incubated for 1 hour at 37 °C to solidify. MSCs were seeded at a density of 5 x $10^4/cm^2$. After a 3-day incubation period, the formation of tube-like structures was examined with a phase-contrast microscope (Carl Zeiss, Germany). For the agonist, antagonist and apyrase experiments, the ligands and apyrase were added on the first day of differentiation.

2.3.8 Decellularization of Bovine Artery and Human Varicose Vein

Fresh bovine aortic arteries were obtained from a slaughter house. During the transportation, arteries were kept in ice-cold 1x PBS. Residual and connective tissue was carefully removed with the scissors. Arteries were washed with 1x PBS until

there was no blood residual left. Artery was subsequently cut latitudinally into sections with 3 cm for arteries and 1cm for veins. Some sections were fixed immediately as the control, whereas the sections for decellularization were transferred into 50 mL falcon tube and incubated in sterile Solution I (see table 3) for 48 hours in a waterbath. Then the sections were incubated with Solution II (see table 3) for 6 hours and after thus treated in Solution I for another 24 hours. All steps were conducted at 37°C under continuous shaking. Finally, sections were washed with 1x PBS and ready for embedment. If sections were not used immediately, they were stored in 1x PBS at 4°C.

For human varicose vein, the preparation steps were similar except the incubation for Solution I was only 24 hours in the first round, and incubation with Solution II was set of 4 hours.

2.3.9 Tissue Fixation, Processing and Embedment

Decellularized artery or vein sections were fixed in 4% formaldehyde overnight. After rinsing three times with tap water for 30 min each, sections were treated with 50%, 70%, 90%, and pure ethanol for 2 hours, respectively and incubated with pure ethanol for another 2 hours. Sections were treated twice with pure xylene for 2 hours and subsequently with 1:1 xylene/paraffin overnight. Dehydrated sections were incubated twice with paraplast for 3 hours. Sections were embedded with paraffin. Solidified sections were later cut into slides with the thickness of 15 µm using a manual microtome (Leica Biosystems, Germany) to prepare for histological staining.

2.3.10 Histological Staining

Three histological staining were performed to analyze and compare the decellularized arteries and veins with nor decellularized controls.

2.3.10.1 Hematoxylin and Eosin Staining

Solidified slides were dewaxed and hydrated in the following incubation with pure xylene, pure xylene, 96% ethanol, 70% ethanol, 50% ethanol and distilled water for 4 min, 4 min, 4 min, 4 min, 4 min and 6 min respectively. Section slides were stained with Hematoxylin Solution (see table 3) for 30 min. The staining was judged using an invert light microscope (hund, Germany) until it reached in the appropriate intensity. After rinsing with tap water for approximately 10 min until the sections turned blue, slides were stained with Eosin Solution (see table 3) for 1 min. After washing with demineralized water for a few seconds, slides were dehydrated with 70% ethanol, 96% ethanol, 100% ethanol, xylene, xylene for 40 sec, 4 min, 4 min, 4 min and 4 min respectively. Slides were covered with mounting medium and a coverslip. Cell nuclei will stain blue (hematoxylin), cell plasma will stain pink (eosin). Images are taken using a microscope (Carl Zeiss, Germany).

2.3.10.2 van Gieson Staining

Solidified slides were dewaxed and hydrated in the following incubation with pure xylene, pure xylene, 96% ethanol, 70% ethanol, 50% ethanol and distilled water for 5 min, 5 min, 5 min, 5 min, 5 min and 6 min, respectively. Slides were stained with Weigert's hematoxylin working solution (see table 3) for 10 min. After rinsing with tap water, slides were immediately judged using an invert light microscope (hund, Germany) to ensure that nuclei were stained selectively. Slides were subsequently stained with van Gieson's solution (see table 3) for 5 min. After washing in acidified water for a few seconds, slides were dehydrated with 70% ethanol, 96% ethanol, 100% ethanol, xylene, and xylene for 40 sec, 4 min, 4 min, 4 min and 4 min, respectively. Slides were covered with mounting medium and a coverslip. Cell nuclei will stain black, collagen will stain red and cytoplasm will stain yellow. Images were taken using a microscope (Carl Zeiss, Germany).

2.3.10.3 Verhoeff's Staining

Solidified slides were dewaxed and hydrated as described in 2.3.10.2. Section slides were stained with working solution of Verhoeff's staining (see table 3) for 45 min. After rinsing with tap water, slides were investigated using an invert light microscope (hund, Germany) to ensure that elastic laminae were already stained in vessel walls. After washing with distilled water for a few seconds, slides were dehydrated as described in 2.3.10.2. Slides were covered with mounting medium and a coverslip. Cell nuclei and elastin fibers were stained black, which could be confirmed using a microscope (Carl Zeiss, Germany).

2.3.11 Functional Analysis with Agonists and Antagonists

Human MSCs were cultured in smooth muscle, endothelial cell differentiation medium or control medium supplemented with the nucleotides ATP (100 µM), UTP (100 µM), ADP (100 µM), UDP (100 µM), UDP-glucose (100 µM), the P2 receptor universal antagonists suramin (100 µM). The specific concentrations were used according to the previous study in the laboratory (Zippel *et al.*, 2012). Not for all P2 receptors selective artificial agonists and antagonists have been synthesized until now (Jacobson *et al.*, 2012). The highly selective and potent P2X3 antagonist RO-3 (used in the following concentration 1 µM, 5 µM, 20 µM, 50 µM), the P2X7 antagonist A-740003 (1 µM, 5 µM, 20 µM, 50µM), the P2Y1 agonist MRS2365 (0.1 µM, 1 µM, 5 µM, 50 µM), and the P2Y1 antagonist MRS2500 (0.1 µM, 1 µM, 5 µM, 50 µM) were used to specifically activate or inhibit the respective target P2 receptor subtypes. Half of the medium was replaced by fresh medium (supplemented with twice the concentration of nucleotides or ligands) twice a week. After 14 days of differentiation, cells were stained with the EC marker eNOS, or SMC marker calponin (see chapter 2.3.5.1), respectively. Pictures were taken using a fluorescent microscope (Carl Zeiss, Germany). In case of endothelial cell differentiation, a

matrigel experiment was also set (see in chapter 2.3.7) to evaluate P2 receptor ligand effect on tube-formation after the differentiation.

2.3.12 Apyrase Nucleotide Cleavage

Human MSCs were cultured in smooth muscle or endothelial cell differentiation medium or control medium supplemented with 5 U/mL apyrase. Half of the medium was replaced by fresh medium (supplemented with double concentration of apyrase) two times a week. After 14 days of differentiation, cells were stained with eNOS and calponin (see chapter 2.3.5.1), respectively and pictures were taken using a fluorescent microscopy (Carl Zeiss, Germany). A Matrigel experiment was set as described in chapter 2.3.7 to evaluate the influence of extragerous nucleotides on tube-formation after the differentiation.

2.3.13 RNA Isolation and Reverse Transcription

RNA was extracted with innuSOLV RNA Reagent. 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 DNAs were synthesized from 2.0 µg total RNA using reverse transcriptase and an oligo-dT primer.

2.3.14 Polymerase Chain Reaction and Agarose Gel Electrophoresis

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, a primer-dependent magnesium chloride concentration (see table 2), 0.2 mM dNTPs and 1.25 U Dream*Taq* polymerase 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 microgravity and differentiation experiments, respectively. The RT-PCR product was evaluated with agarose gel (1-2 %) electrophoresis at 12 V / cm for 30 min in 1x TBE buffer. Agarose gels included 4 µl ethidium bromide solution (10 mg mL^{-1}) per 100 mL. A 100 bp DNA ladder was utilized to confirm the product size.

2.3.15 Protein Isolation

Cells were washed with ice cold 1x PBS and scrapped in PBS with a cell scraper. Afterwards, the cell pellet was collected by centrifuging for 5 min at 500 x 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 x g for 5 min at RT to remove cellular debris. The protein lysates were stored at -80°C.

2.3.16 Western Blotting

The protein lysates were diluted 1:2 in Laemmli buffer (Bio-Rad), boiled for 5 min and separated by electrophoresis with SDS-polyacrylamide gel. A prestained 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 100 µg protein samples were added for each lane) separation was run at 30 mA for approximately 1.5 hour.

The protein was semi-dry transferred to a PVDF membrane for 2 h at a maximum

of 25 V. The membrane was blocked in 1x TBST containing 5% (w/v) nonfat dry milk for 60 min. Afterwards the membrane was incubated with one of the following antibodies: P2X1, P2X6, P2X7, P2Y2, P2Y4, P2Y6, P2Y14, eNOS, VEGFR2, SMA-α, MYH-11, SM22α antibodies (in a ratio of 1:300), P2X5, P2Y1, P2Y11, VE-cadherin, PECAM-1 (in a ratio of 1:500), calponin (in a ratio of 1:1000),or the β-actin and GAPDH antibody (in a ratio of 1:3000) 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 against mouse, rabbit or goat for 60 min in 1x TBST containing 5% (w/v) nonfat dry milk at RT (all secondary antibodies for P2 receptors and EC, SMC markers in a ratio of 1:3000 and for β-actin and GAPDH in a ratio of 1:5000). The membrane was washed in 1x TBST for three times for 10 min. Pictures were taken after 3 min of incubation with the Immobilon Western Chemiluminescent HRP Substrate. The density of the bands was analyzed by using Image J software (NIH). As positive controls, protein extracts from different cell lines were used as described in each experiment in details (see chapter 2.3.4).

2.3.17 Phosphorylation Kinase Array

The different P2 underlying signaling pathways were evaluated measuring the phosphorylated levels of 46 kinases of the human phosphor-kinase array (R&D System). After 14 days of smooth muscle and endothelial cell differentiation, protein samples of not differentiated and differentiated cells were collected separately and 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 46 kinase phosphorylation sites were analyzed using Image J software (NIH).

2.3.18 Treatment of EC and SMC with a Clinostat

The fast-rotating 2-D clinostat used in this study was originally developed by the Institute of Aerospace Medicine, German Aerospace Center (DLR) (see Fig. 13A). It has 6 parallel horizontal axes, each for fixation for up to 4 slide flasks. ECs and SMCs were seeded at a density of 10,000 cells/cm^2 onto 9 cm^2 cell culture slide flasks. When they reached a confluence level of 60%-70%, the culture flasks were filled up completely with DMEM medium. To avoid shear stress and thus the induction of respective metabolic changes in signal transduction pathways e.g. apoptosis, air bubbles were removed carefully. The flasks were inserted on the clinostat and rotated at 60 rpm for 24h in the CO_2 incubator at 37 °C. Controls were also filled with medium and placed simultaneously under normal gravity (1g).

Cells from the whole flask were first used to analyze the P2 receptor expression pattern that altered subtypes could be distinguished from unaffected. Later, according to the clinostat principle, only cells exposed to defined *g*-forces were taken for further analysis. This means that only cells from the middle of the flask were taken (see Fig. 13B). In defined: under a constant speed of 60 rpm the maximal residual acceleration at an area of 6 mm amounts to ≤ 0.0121g, providing a high quality of simulated microgravity (Eiermann *et al.*, 2013). Thus, only cells within this 6mm area were isolated to evaluate altered P2 subtypes for both gene and protein expression in details. To maintain the cells accurately and consistently in the center, a special chamber consisting of two cover plates, attached to a bottom plate (developed by DLR) was used to allow slide insertion without wiping off the cell layer. A corresponding cell scraper was used to scratch the cells from the specific 6 mm width area in the center (Fig. 13C).

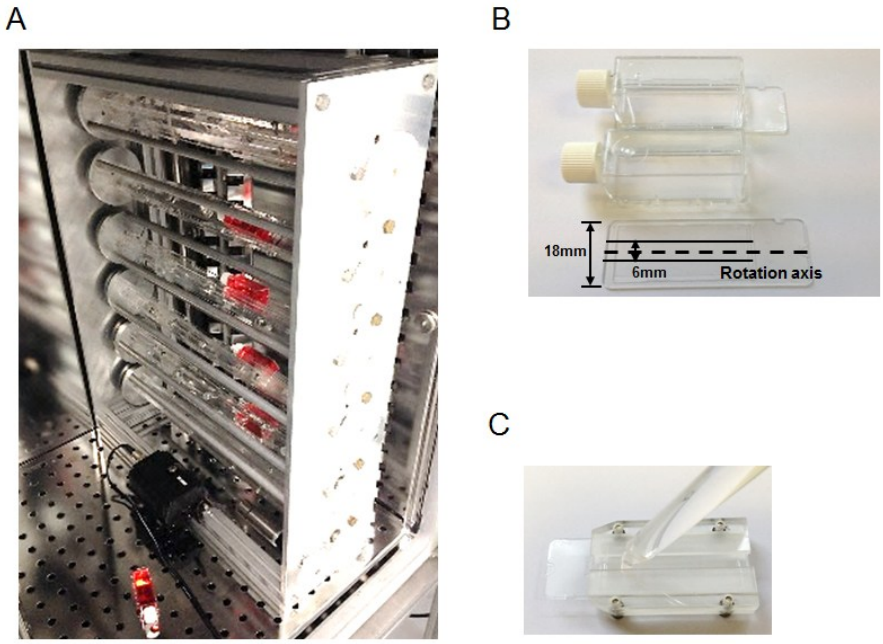


Figure 13. Cell Culture in a Clinostat to Simulate Microgravity Conditions. The clinostat (A) was used to simulate the microgravity environment by rotating cells. Only cells grown in the 6 mm area in the middle of culture slide (B) received the optimal simulated microgravity and were thus harvested using a special cell scraper (C).

2.3.19 Conditioned Medium

To evaluate a possible paracrine effect on P2 receptor expression, ECs and SMCs were seeded in a density of 2500/cm^2. After approximately 2-3 days, cell growth medium was collected when cells reached a confluence of 80% - 90%. The conditioned medium (CM) was composed out of cell growth medium and normal DMEM medium in a ratio of 1:2 on respective cell type. The SMC-conditioned medium was subsequently fully added into the culture slide with ECs and set of a 24h clinorotation as a group of (EC MG+CM). The ECs in normal gravity group (EC 1g) and in clinorotation but filled with normal DMEM medium (EC MG) were set simultaneously. The similar experiments were set for SMCs with normal gravity (SMC 1g), clinorotation (SMC MG), and clinorotation filled with EC-conditioned

medium (SMC MG+CM).

To investigate a possible paracrine effect on cell proliferation and migration, cell growth medium were collected from cells cultured 24h in normal gravity and cultured 24h in the clinorotation respectively. The used conditioned medium (CM) was composed out of cell growth medium and normal DMEM medium in a ratio of 1:2 on respective cell type. The ECs were subsequently treated with normal DMEM medium, SMC-conditioned medium from normal gravity (CM SMC+1g) and clinorotation (CM SMC+MG) separately for proliferation or migration assays. Similar experiments were set and performed with SMCs as well. All experiments were performed with samples from three cows.

2.3.20 Proliferation Assay

For the proliferation assay 20,000 ECs were seeded separately in each well of 12-well plates. ECs were grown in DMEM medium, in a SMC-conditioned medium in normal gravity (SMC CM+1g) and in a SMC-conditioned medium in simulated microgravity (SMC CM+MG) (see chapter 2.3.19 for conditioned medium details). ECs incubated with the respective medium were subsequently obtained after 24h and 48h incubation and numbers were calculated. Similarly experiments were set for SMCs: SMCs were cultured in DMEM medium, in EC-conditioned medium in normal gravity (EC CM+1g), and EC-conditioned medium after simulated microgravity (EC CM+MG) for 24h and 48h. Cell number was counted.

2.3.21 Wound Assay

For the wound assay ECs and SMCs (10,000/cm^2) were seeded and grown to 80% - 90% confluence. A straight scratch injury was made using a sterile 1 mL pipette tip on 6-well plates. The ECs were incubated for 24h at 37°C in a CO_2 incubator with normal DMEM medium, SMC-conditioned medium in normal gravity (SMC CM+1g)

and SMC-conditioned medium in simulated microgravity (SMC CM+MG). On the other hands, SMCs were cultured in DMEM medium, EC-conditioned medium in normal gravity (EC CM+1g) and EC-conditioned medium in simulated microgravity (EC CM+MG). Hydroxyurea (5 mM) was added to inhibit cell proliferation. Images were taken using a phase contrast microscope (Carl Zeiss, Germany). The numbers of migrated cells in three individual areas were calculated and quantified using Image J software (NIH).

2.4 Statistical Analysis

Statistical analysis was applied for the experiments using the Microsoft Office program Excel 2010 and SPSS 12.0. Data are shown as means ± standard deviation. Experiments were repeated at least three times for three donors, which is given as n = number of experiments. The probability (p) value was calculated using LSH test to assess differences between two groups. Levels of significance were labeled as follows (* $p \leq 0.05$), (** $p \leq 0.01$) and (*** $p \leq 0.001$). Significance was given with the appropriate number of asterisks or in numbers.

2.5 Ethics

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: “Gewinnnung 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 on informed consent.

3. RESULTS

3.1 Isolation and Characterization of Human MSCs from Adipose Tissue

3.1.1 Isolation of Human Adipose Tissue Derived MSCs

Stem cells were isolated from human adipose tissue from different aged patients. For the investigation of P2 receptors, cells of three female (20, 33 and 44 years old) donors were used.

The liposuction aspirates were collected in a sterile plastic collection bag during surgery (Fig. 14A). Human MSCs were isolated *via* phase separation and collagenase digestion (Fig. 14B), as described in section 2.3.1.1. The enrichment of hMSCs was achieved due to their plastic adherence (Dominici *et al.*, 2006). In order to remove contaminating cell debris and non-adherent red blood cells, cells were washed extensively with 1x PBS after 24 hours. The characteristic fibroblast-like morphology was shown in figure.14C for the isolated hMSCs.

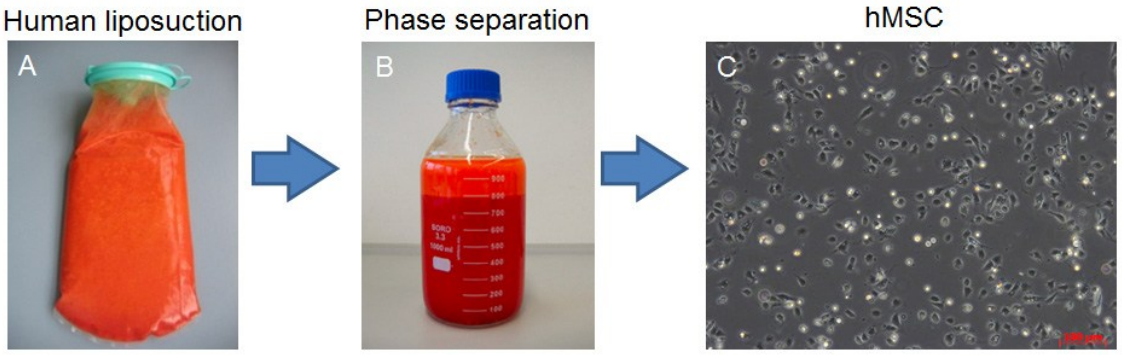


Figure 14. Stem Cell Source and Isolation.
Human MSCs were isolated from liposuction material (A) by phase separation with PBS and subsequent collagenase digestion (B). MSCs are adherent to plastic cell culture dishes. The fibroblast-like morphology was observed (C).

3.1.2 Characterization of Human Adipose Tissue Derived MSCs

The isolated hMSCs were evaluated if they meet the minimal criteria suggested by the International Society for Cellular Therapy (Dominici *et al.*, 2006), which are plastic adherence, the multipotent differentiation potential into their respective mesenchymal lineages (osteo-, adipo- and chondrogenic), as well as the expression of the mesenchymal cell markers CD73, CD90 and CD105 and the lack of the negative markers such as CD14, CD34 and CD45. To do so, a FACS analysis was performed. In addition, the expression of another mesenchymal marker CD44 was also examined.

FACS data from the previous publication of the laboratory (Zippel *et al.*, 2012) showed the isolated cells expressed the mesenchymal cell markers CD44 (97.2%), CD90 (97.6%) and CD105 (91.6%) and as expected, these cells were negative for CD14 (1.2%) and CD45 (6.1%) (Fig. 15). CD34, a common marker for hematopoietic stem cells and endothelial progenitor cells (EPCs), was found here to be presented on a subpopulation (56.3%) of isolated hMSCs (shown as Fig. 15).

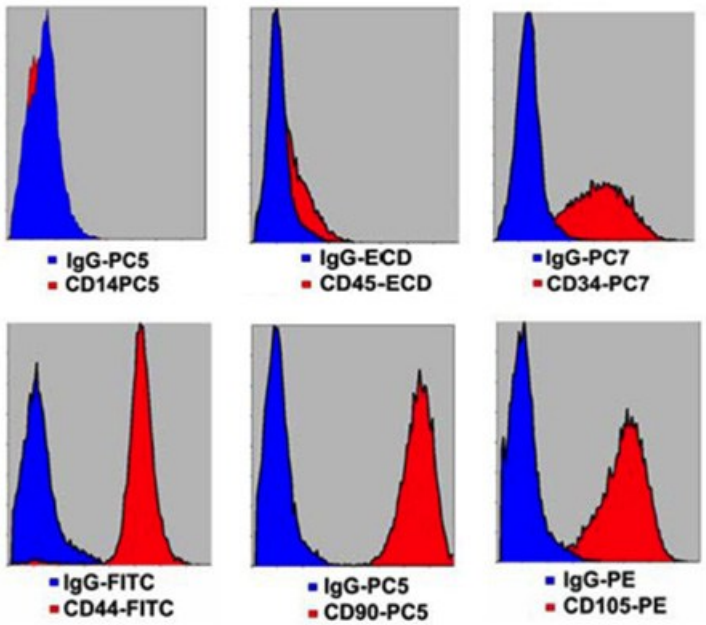


Figure 15. Characterization of MSC Surface Markers.
hMSCs are detected by FACS analysis for the hematopoietic cell markers CD14, CD45, and CD34, for the mesenchymal markers CD44 CD90 and CD105. The data shown are representative for three examined donors.

In addition to FACS analysis, hMSCs were investigated for the gene expression of mesenchymal cell markers using semiquantitative RT-PCR. The gene expression of the mesenchymal markers CD73, CD90, and CD105 was present in the MSCs of the three chosen donors. β-actin served as an internal control (Fig. 16).

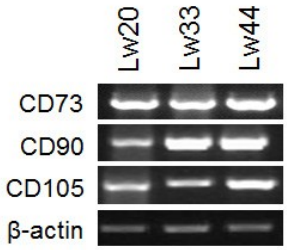


Figure 16. Gene Expression of Mesenchymal Markers.
The RT-PCR showed gene expression of the mesenchymal markers CD73, CD90, and CD105 in isolated MSCs for three representative donors (Lw20, Lw33, Lw44). β-actin served as internal control.

Since mesenchymal stem cells and fibroblasts display a similar morphology, stem cells were tested for the gene expression of the fibroblast marker vimentin. Vimentin was not expressed in the isolated hMSCs, which exclude a possible contamination with fibroblasts (Fig. 17).

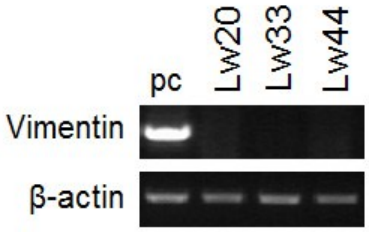


Figure 17. Exclusion of a Fibroblast Contamination.
In order to exclude the possible contamination of MSCs with fibroblasts, the gene expression of the fibroblast marker vimentin was tested in three representative donors (Lw20, Lw33, Lw44). pc, positive control, the cell line NIH-3T3. β-actin served as internal control.

In addition to the expression of mesenchymal markers, MSCs were further characterized by mesodermal differentiation towards the osteogenic and adipogenic

lineages. Human MSCs were differentiated towards osteoblasts as described (see 2.3.4.2). The osteogenic differentiation was identified using Alizarin Red S staining of calcium deposits (Fig. 18A) and von Kossa staining (Fig. 18B) of phosphates. Further analysis with RT-PCR showed an enhanced gene expression of the osteogenic markers bone morphogenetic protein 2 (BMP2) and osteonectin (ON) (Fig. 18C) in the osteogenic differentiated MSCs if compared to un-differentiated MSCs.

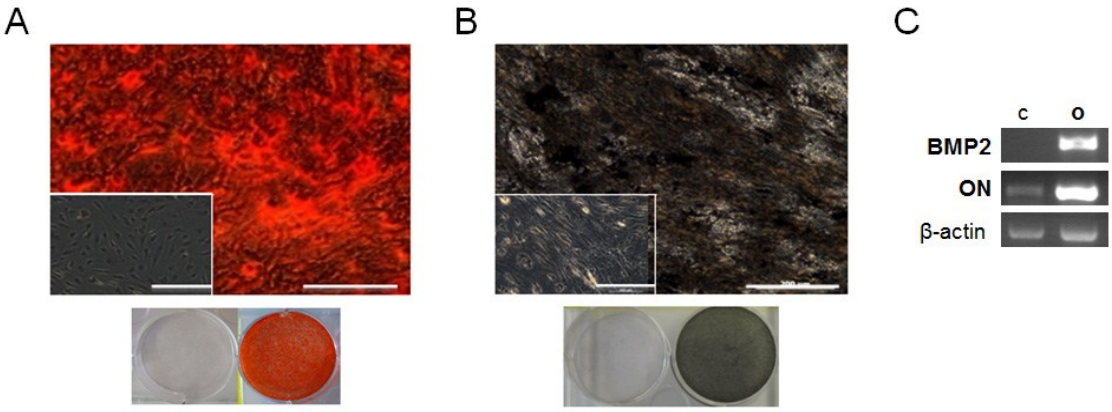


Figure 18. Osteogenic Differentiation of hMSCs.
Mineralization typical for osteogenic differentiation was examined by Alizarin Red S staining of Ca^{2+} deposits (A) and von Kossa staining of phosphates (B). RT-PCR showed an increased gene expression of osteogenic markers BMP2 and ON in differentiated MSCs compared to un-differentiated MSCs (C). β-actin served as internal control. All these pictures are representative for one donor out of three. c, control un-differentiated cells; o, osteogenic differentiated cells. The bar in the A and B is 200 µm.

Adipogenic differentiation was performed as described (see 2.3.4.1) visualized by Oil Red O staining of the lipid droplets (Fig. 19A). Subsequent RT-PCR experiments showed an up-regulation of the adipogenic markers lipoprotein lipase (LPL) and peroxisome proliferator activated receptor gamma (PPARγ) (Fig. 19B).

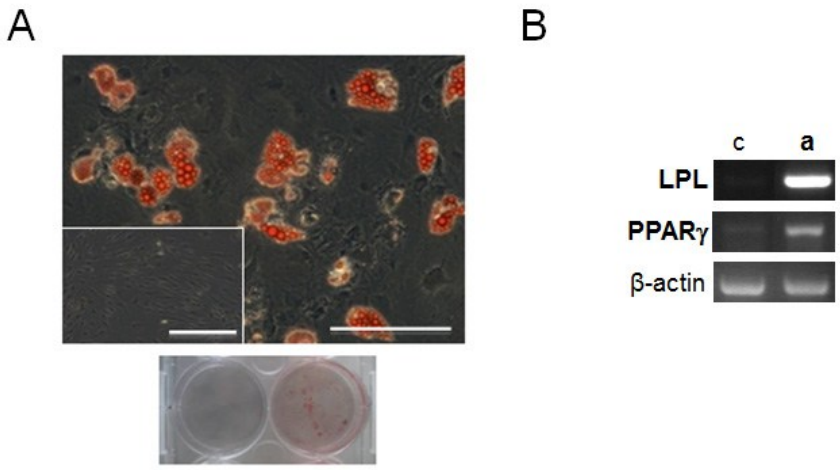


Figure 19. Adipogenic Differentiation of hMSCs.
Human MSCs were induced towards adipocytes in the presence of dexamethasone, insulin and indomethacin for 4 weeks. Adipogenic differentiation was evaluated by Oil Red O staining of un-differentiated and adipogenic differentiated MSCs (A). RT-PCR showed an up-regulation of the adipogenic markers LPL and PPARγ in differentiated if compared to un-differentiated MSCs (B). β-actin served as internal control. All pictures are representative for one donor out of three. c, control un-differentiated cells; a, adipogenic differentiated cells. The bar in A is 200 µm.

3.2 Establishment of Smooth Muscle and Endothelial Cell Differentiation

3.2.1 Smooth Muscle Cell Differentiation of hMSCs

After 14 days of smooth muscle cell differentiation (see 2.3.4.4), the differentiated cells were firstly characterized by investigating the gene expression of the smooth muscle cell specific markers SMA-α, SM22α, calponin and comparing them to the level in un-differentiated MSCs by RT-PCR (Fig. 20A). In addition, Western blot confirmed the found up-regulation on the protein level (Fig. 20B). Immunofluorescent staining also showed that towards smooth muscle cell differentiated MSCs positively expressed calponin (Fig. 20C).

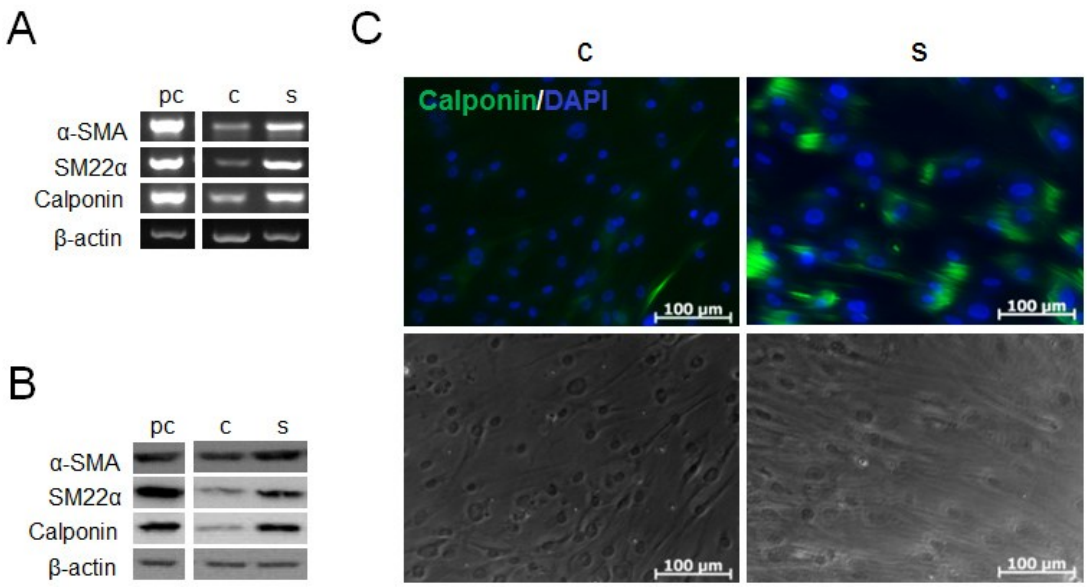


Figure 20. Smooth Muscle Cell Differentiation Capacity of hMSCs.
An up-regulation of smooth muscle cell specific markers SMA-α, SM22α, and calponin was found in smooth muscle differentiated cells on both gene (A) and protein (B) level. Differentiated cells showed positive immunofluorescent staining for calponin (C). β-actin served as internal control. Smooth muscle cells from bovine aorta were used as positive control to identify the smooth muscle cell markers. pc, positive control; c, un-differentiated MSCs; s, smooth muscle differentiated MSCs.

3.2.2 Endothelial Cell Differentiation of hMSCs

After 14 days endothelial cell differentiation (see 2.3.4.3), the differentiated cells were first characterized by evaluating the gene expression of the endothelial cell specific markers VEGFR2, eNOS and PECAM-1 (Fig. 21A), which was subsequently followed by investigating the protein level by Western blot (Fig. 21B) and the immunofluorescent staining for eNOS (Fig. 21C). To evaluate whether the differentiated endothelial cells obtained the typical function of primary endothelial cells, two endothelial cell functional experiments were performed. The differentiated endothelial cells sprouted more tubes (Fig. 21D) and took up LDL (Fig. 21E) if compared to the un-differentiated MSCs in a Matrigel assay.

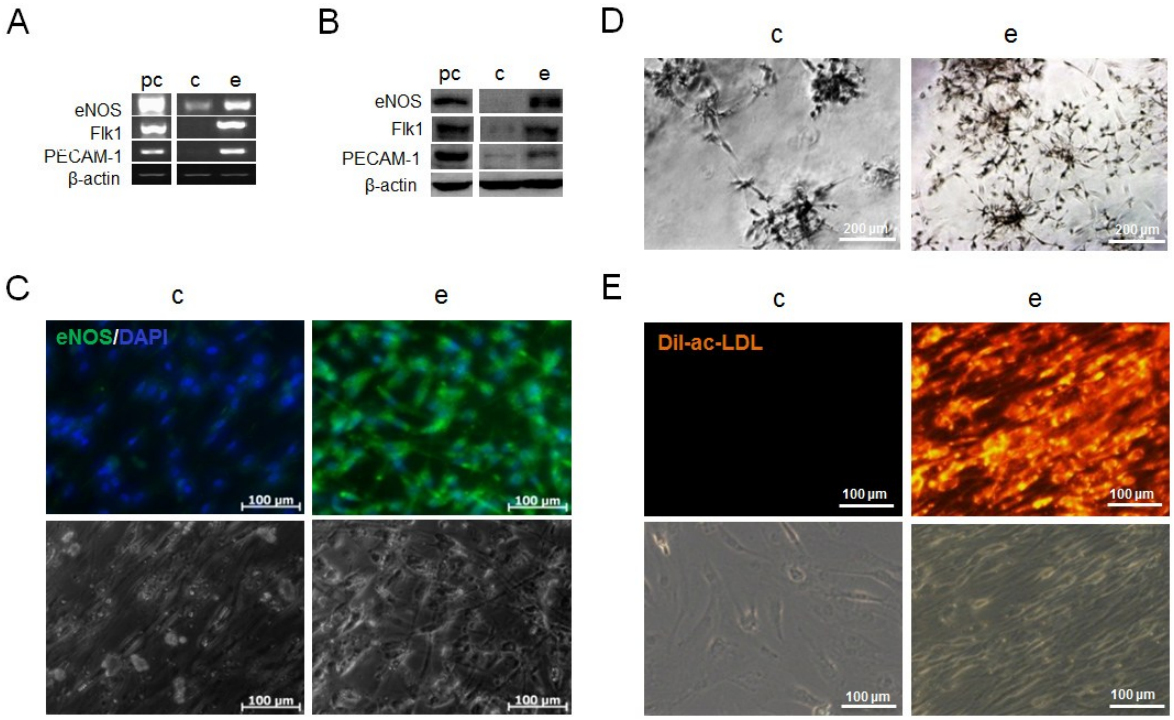


Figure 21. Endothelial Cell Differentiation Capacity of hMSCs.
MSC differentiated towards endothelial cells expressed the endothelial cell markers VEGFR2, eNOS and PECAM-1 by RT-PCR (A) and Western blot (B) if compared to un-differentiated MSCs. Immunofluorescent staining of the stem cells displayed that the differentiated cells positively expressed eNOS (C). The differentiated cells showed formed more tubes in Matrigel (D) and uptake of DiI-ac-LDL (E) than un-differentiated cells. β-actin served as internal control. pc, positive control HMEC-1; c, un-differentiated cells; e, endothelial differentiated cells.

3.3 The Role of P2 Receptors in Smooth Muscle and Endothelial Cell Differentiation

3.3.1 P2 Receptor Regulation in Smooth Muscle Cell Differentiation

3.3.1.1 P2 Receptor Expression in SMC Differentiation

To investigate the role of P2 receptors in smooth muscle cell differentiation, hMSCs were induced towards the smooth muscle cell lineage and the gene expression of

all known P2 receptors was analyzed and compared between un-differentiated and differentiated cells. After 14 days of smooth muscle cell differentiation, the expression of P2X1, P2Y4 and P2Y14 was increased, whereas P2X3, P2X7 and P2Y1 were down-regulated as shown by RT-PCR (Fig. 22A). Next to the analysis of the gene level, Western blot experiments confirmed the similar changes of P2X3, P2X7, P2Y1, and P2Y14 on the protein level in smooth muscle cell differentiated MSCs compared to un-differentiated MSCs (Fig. 22B). The results of three donors were normalized with β-actin and subsequently quantified. The changes in P2X3, P2X7, P2Y1, and P2Y14 protein expression were found to be significant.

3.3.1.2 Effect of Natural Ligands on SMC Differentiation

Although P2 receptors showed a different expression pattern after smooth muscle cell differentiation, it is more important to identify whether these receptors have a functional role, thus, can directly influence the lineage commitment by their agonists or antagonists.

The natural P2 receptor agonists ATP, UTP, ADP, UDP, UDP-glucose as well as the universal P2 receptor antagonist suramin were tested for their functional effect on smooth muscle cell differentiation. No significant apoptosis signals were observed during the differentiation process. The administration of UDP-glucose significantly enhanced the expression of calponin. This is in line with the up-regulation of P2Y14, which suggest P2Y14 was functionally involved in smooth muscle cell differentiation. On the other hand, the adding of ATP and ADP reduced the calponin expression and this effect was compensated *via* additional supplementation of suramin. This indicated that P2Y1, P2X3, or P2X7 might be functional responsible during the differentiation process.

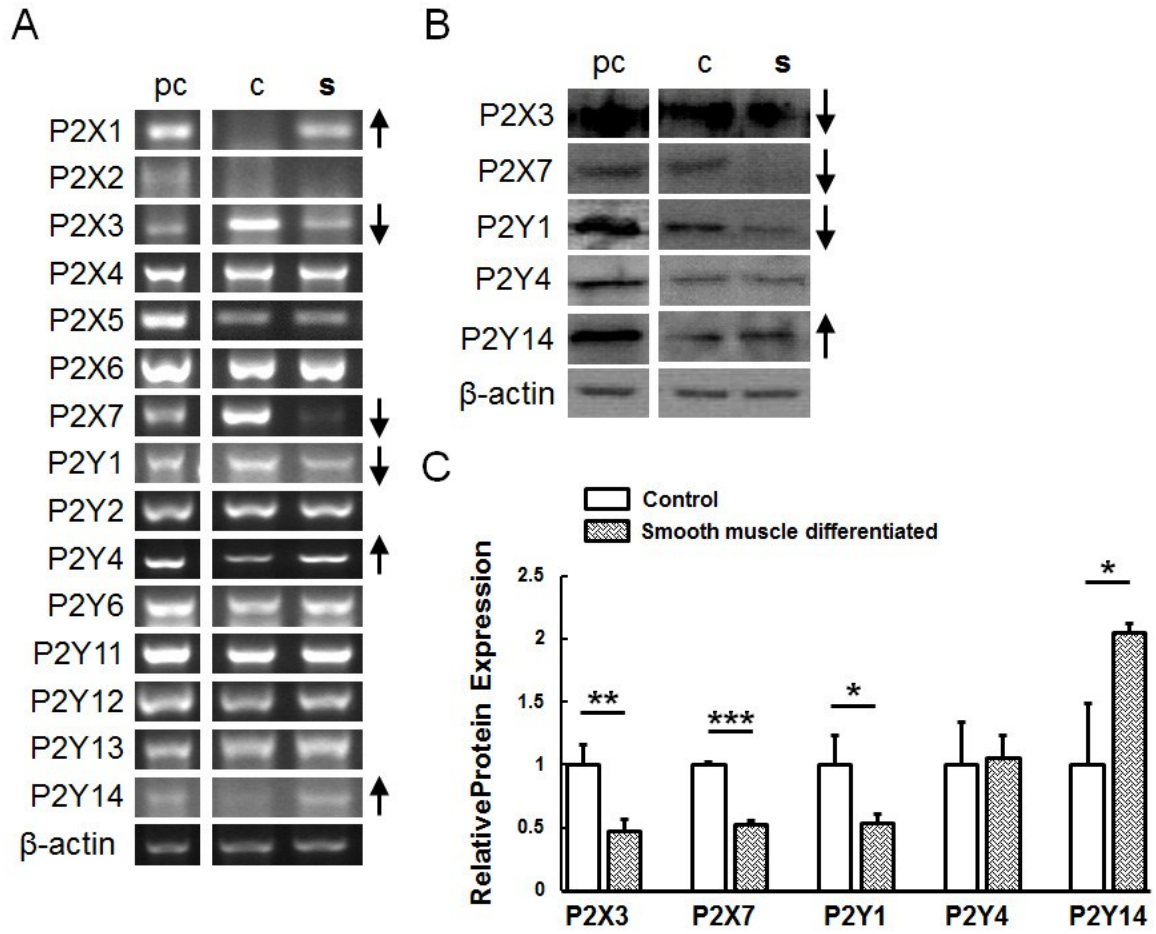


Figure 22. P2 Receptor Expression during Smooth Muscle Cell Differentiation. P2 receptor gene expression was first analyzed by RT-PCR in smooth muscle cell differentiated MSCs and compared to un-differentiated MSCs (A). The altered P2 receptor subtypes were subsequently confirmed in protein expression by Western blot (B). β-actin served as internal control. The graph (C) represents the quantification of the Western blot bands normalized against β-actin in undifferentiated and differentiated cells. Since there is no single cell type that expresses all P2 receptor subtypes, different positive controls were used here: HMEC-1 for P2X3, P2X4, P2X5, P2X7, P2Y1, P2Y2, P2Y4, P2Y11; MG-63 for P2X6, P2Y6 and mouse brain for P2X1, P2X2, P2Y12, P2Y13, P2Y14. The β-actin of the positive control is only given for HMEC-1 as representative for all three positive controls. c, control, un-differentiated cells; s, towards smooth muscle cell differentiated MSCs. ↑ indicates an up-regulation of the P2 receptor subtype during differentiation, and ↓ indicates a down-regulation. The data shown are representative for one donor out of three.

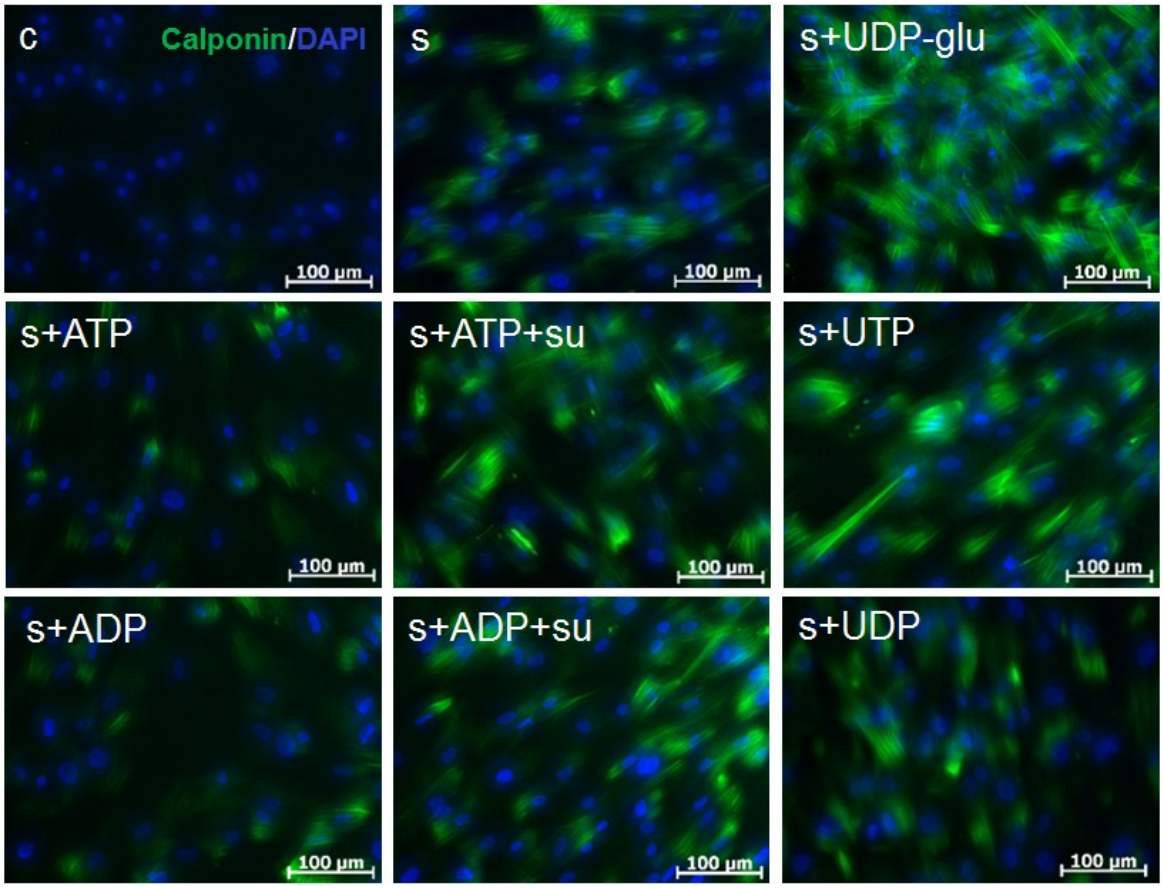


Figure 23. Influence of Natural P2 Receptor Ligands on SMC Differentiation. During 14 days of smooth muscle cell differentiation, the following concentration of ATP (100 µM), ADP (100 µM), UTP (100 µM), UDP (100 µM), UDP-glucose (100 µM), and suramin (100 µM) were added into the respective differentiation medium. Half of the medium was exchanged with double the amount of agonists or antagonists twice a week. Towards smooth muscle cell differentiated MSCs were evaluated by immunofluorescent staining with calponin. UDP-glucose enhanced the calponin expression whereas ATP and ADP decreased the expression. Suramin compensated the effect of ATP and ADP. The pictures shown are representative for 1 donor out of 3. All measurements were performed in triplicate.

3.3.1.3 Influence of Artificial Ligands on SMC Differentiation

To investigate which specific P2 receptor subtype is functionally involved and even more predominant during the differentiation process, several highly potent and selective artificial P2 receptor agonists and antagonists: MRS2365 (P2Y1 agonist),

RO-3 (P2X3 antagonist), A-740003 (P2X7 antagonist), as well as MRS2500 (P2Y1 antagonist) were administered during the 14 days smooth muscle cell differentiation. Different concentrations of these artificial ligands were tested to find the appropriate concentration for each ligand and examine whether their impact was in a dose-dependent manner. A series of concentrations (1, 5, 20, 50 μM) of RO-3 was added during the differentiation, but RO-3 seemed to have no significant effect on the calponin expression (Fig. 24). This suggested that P2X3 might be not functional in the differentiation process. A similar set of different concentrations for A-740003 was used as well. The concentration of 5 μM seemed to significantly enhance calponin expression (Fig. 24), which indicated that P2X7 plays a functional role in smooth muscle cell differentiation. P2Y1 was down-regulated when hMSCs were induced towards smooth muscle cells but was up-regulated when hMSCs was differentiated towards endothelial cells (see 3.2.1 and 3.2.2). This suggests that P2Y1 might be the key subtype in controlling these lineage commitments. Therefore, a highly potent and selective agonist MRS2365 (0.1, 1, 5, 50 μM) and antagonist MRS2500 (0.1, 1, 5, 50 μM) of P2Y1 was used to examine its functional role. A concentration of 1 μM MRS2500 already significantly enhanced calponin expression while 1 μM MRS2365 inhibited the differentiation process (Fig. 25).

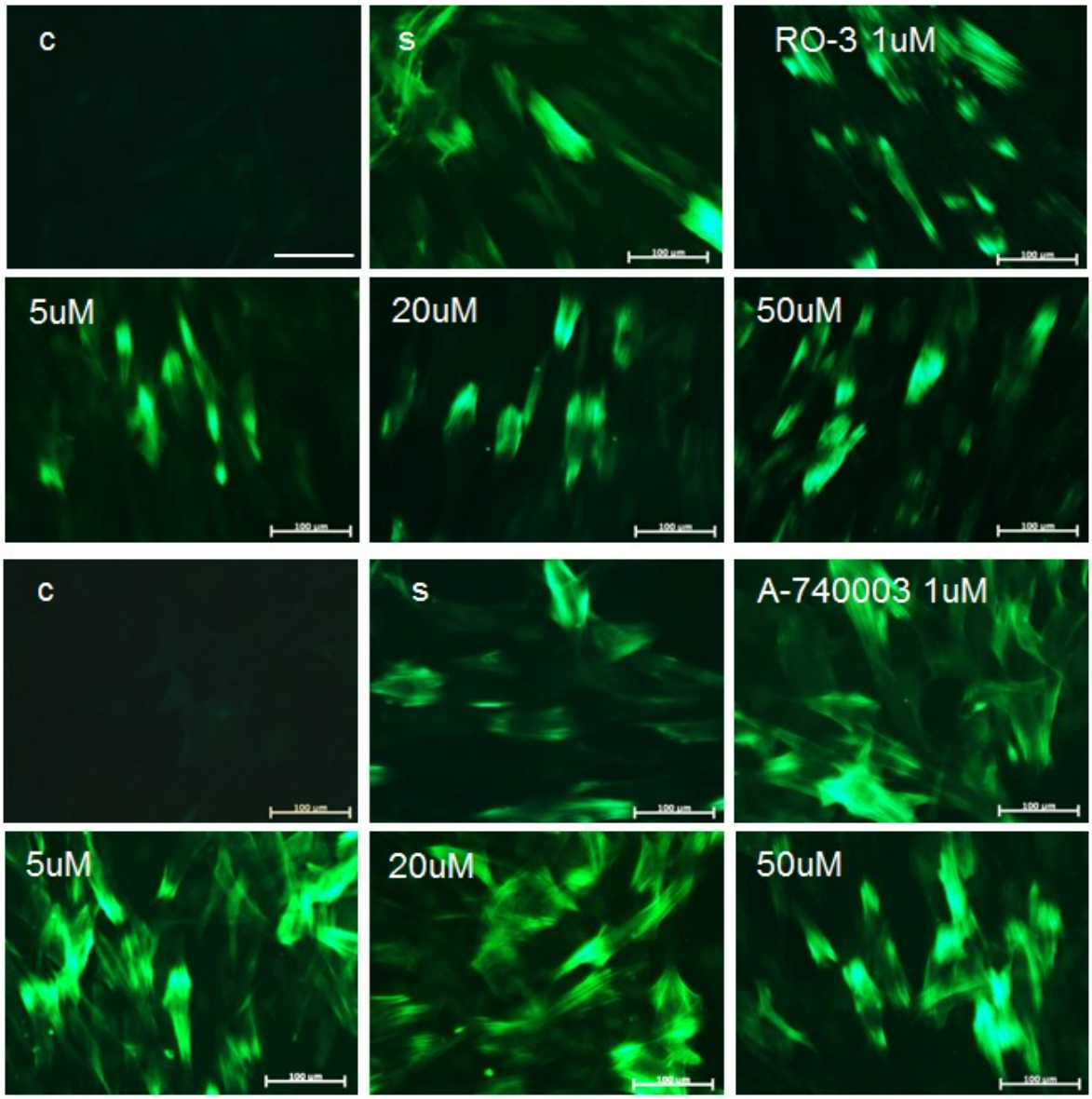


Figure 24. Effect of Artificial P2X3 and P2X7 Antagonists on SMC Differentiation.

During 14 days of smooth muscle cell differentiation, various concentrations of RO-3 (1, 5, 20, and 50 μM) and A-740003 (1, 5, 20 and 50 μM) were added to the differentiation medium. Half of the medium was exchanged with double amount of ligands twice a week. Smooth muscle differentiated cells were evaluated by immunofluorescent staining with calponin. The concentration of 1 μM P2X7 artificial antagonist A-740003 enhanced the calponin expression. The P2X3 antagonist RO-3 seemed to have no effect. The pictures shown are representative for one donor out of three. All measurements were performed in triplicates. The bar in all pictures is 100 μm.

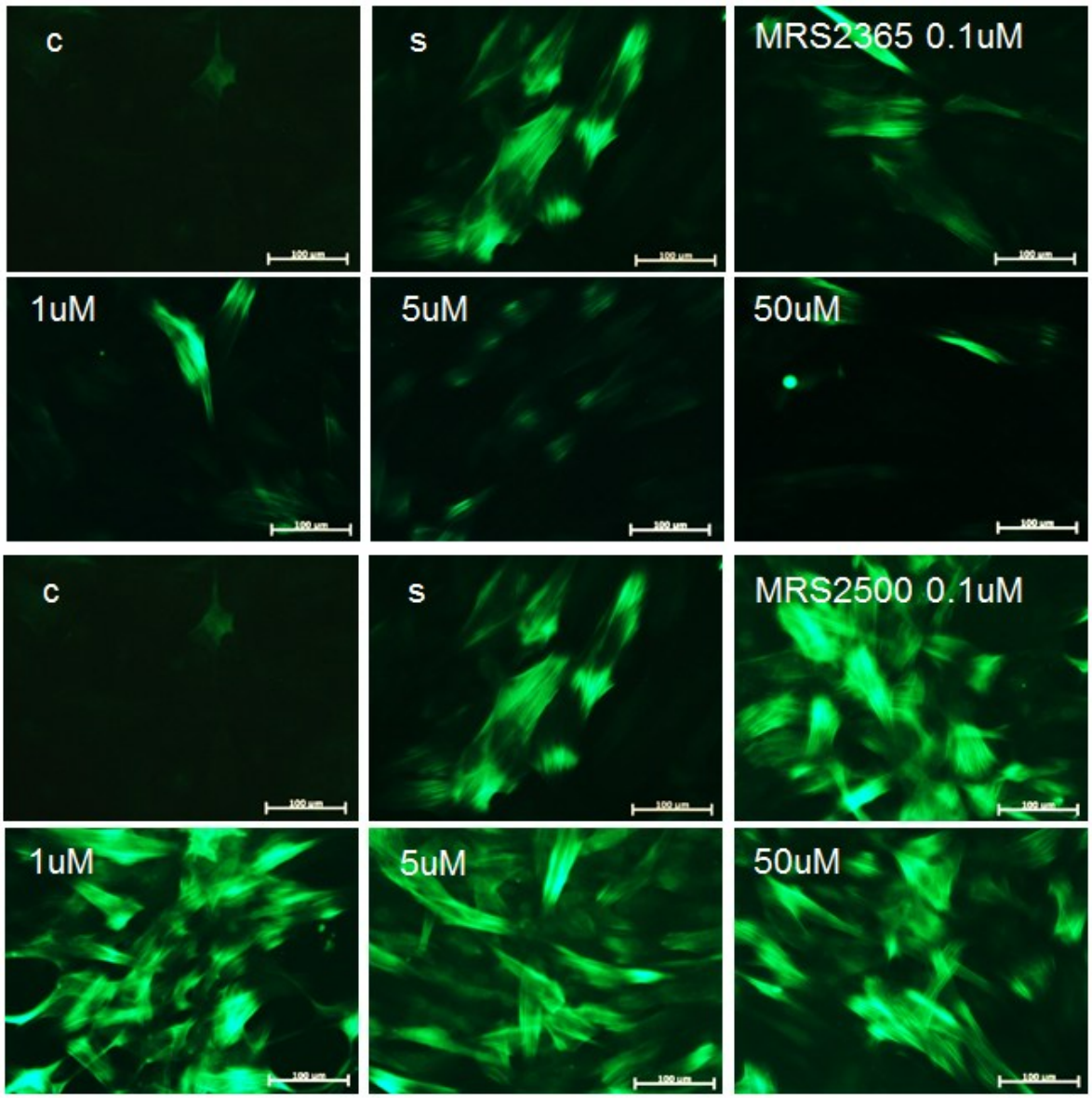


Figure 25. Effect of Artificial P2Y1 Agonist and Antagonist on SMC Differentiation.

During 14 days of smooth muscle cell differentiation, different concentrations of the P2Y1 agonist MRS2365 (0.1, 1, 5, 50 µM), and antagonist MRS2500 (0.1, 1, 5, 50 µM) were added to the differentiation medium. Half of the medium was exchanged with double amount of agonist or antagonist twice a week. Smooth muscle differentiated cells were evaluated by immunofluorescent staining with calponin. A concentration of 0.1µM MRS2500 enhanced the calponin expression, 0.1µM MRS 2365 inhibited the expression. The pictures shown are representative for one donor out of three. All measurements were performed in triplicates. The bar in all pictures is 100 µm.

3.3.1.4 Effect of Endogenous Nucleotide Release on SMC Differentiation

In order to evaluate whether endogenous nucleotides influence smooth muscle cell differentiation, apyrase was added into differentiation medium. Apyrase is a diphosphohydrolase that hydrolyzes extracellular nucleotides. Apyrase administration of 5 U/mL enhanced the calponin expression significantly (Fig. 26). This finding suggests that the release of endogenous nucleotides from hMSCs had a pivotal influence in the regulation of smooth muscle cell differentiation.

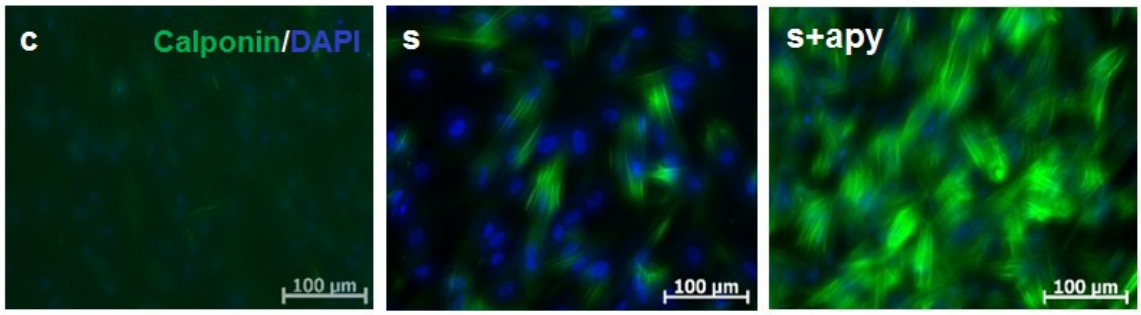


Figure 26. Effect of Endogenous Nucleotide Release on SMC Differentiation. A concentration of 5 U/mL apyrase was added to the differentiation medium. Half of the medium was exchanged with double amount of apyrase two times a week. Endogenous nucleotide released during the differentiation process inhibited smooth muscle cell differentiation. c, control, un-differentiated cells; s, towards smooth muscle cell differentiated MSCs, s+apy, towards smooth muscle cell differentiated MSCs supplemented with apyrese. The pictures shown are representative for one donor out of three. All measurements were performed in triplicates.

3.3.1.5 P2 Receptor Underlying Signaling Pathways in SMC Differentiation

To investigate the P2 receptor underlying signaling pathways during the differentiation process, a protein kinase activity assay was used to evaluate the phosphorylation level of 46 kinases. After 14 days of smooth muscle cell differentiation, Akt, p53, p70, p27, PLC, paxillin and c-jun phosphorylation was activated while AMPKα1 was inhibited (Fig. 27A). The phosphorylation spots were subsequently analyzed with Image J software for quantification. The above mentioned changes were found to be significant (Fig. 27B). The increase of Akt (Fig.

27C) phosphorylation level was further confirmed *via* Western blot.

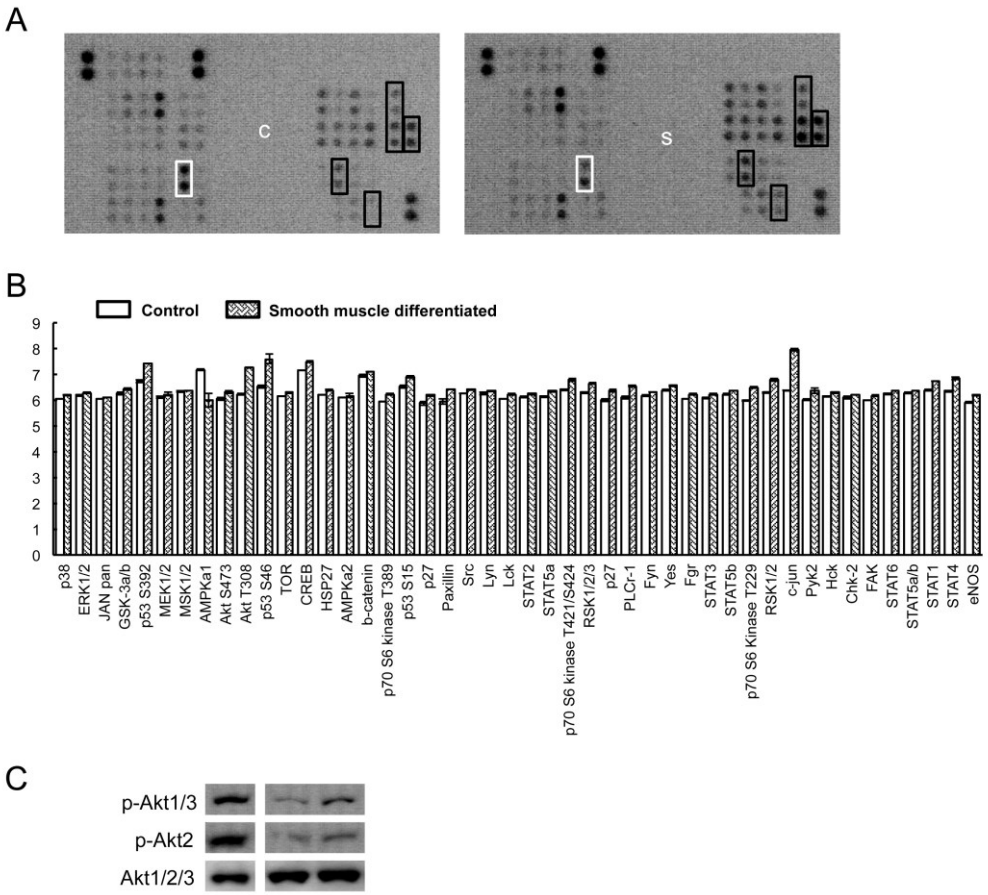


Figure 27. P2 Receptor Underlying Signaling Pathways in SMC Differentiation. A human phospho-kinase array was performed to evaluate 46 kinase phosphorylation sites after smooth muscle cell differentiation. The black rectangles indicate spots with stronger phosphorylation after the differentiation (A), while white rectangles suggest spots with weaker phosphorylation after differentiation. Akt, p53, paxillin and c-jun were activated while AMPKα1 was inhibited after smooth muscle differentiation significantly (B). In addition, Western blot analysis confirmed that Akt was activated in smooth muscle cell differentiation (C).

3.3.2 P2 Receptor Regulation in Endothelial Cell Differentiation

3.3.2.1 P2 Receptor Expression in EC Differentiation

Human MSCs were differentiated towards endothelial cells and the gene expression

in un-differentiated and differentiated cells was compared for all known P2 receptor subtypes. After 14 days endothelial cell differentiation, seven receptor subtypes showed an up- or down-regulation: the expression of P2X5, P2Y1, P2Y2, P2Y4, P2Y11, and P2Y14 was increased, whereas P2Y6 was down-regulated as shown by RT-PCR (Fig. 28A).

In addition to gene expression analysis, Western blot experiments were performed and the above mentioned gene alteration of P2X5, P2Y1, P2Y2, P2Y4, P2Y11 and P2Y14 in towards endothelial cell differentiated MSCs compared to un-differentiated MSCs was confirmed (Fig. 28B). The Western blot results shown are one representative donor out of three. The bands from all three donors were normalized with β-actin and later analyzed for quantification and the significance was evaluated (Fig. 28C). The alterations of P2X5, P2Y1, P2Y2, P2Y4, P2Y6, P2Y11, P2Y14 on the protein level were found to be significant.

3.3.2.2 Effect of Ligands on EC Differentiation

To investigate whether P2 receptor ligands have a direct impact on endothelial cell differentiation, differentiated medium was supplemented with the P2 receptor natural ligands ATP, ADP, UTP, UDP and UDP-glucose and universal antagonist suramin. ATP, ADP, or UDP-glucose significantly enhanced the eNOS expression, however UDP reduced the expression (Fig. 29). In addition, P2Y1 was up-regulated in endothelial cell differentiation, whereas it was down-regulated in smooth muscle cell differentiation (see 3.3.1.1). This suggests P2Y1 might play a key role in controlling these lineage commitments. In line with data from previous experiments, 1 μM of the P2Y1 artificial agonist MRS2365 and antagonist MRS2500 were administered during endothelial cell differentiation. MRS2365 significantly increased the eNOS expression and MRS2500 inhibited it (Fig. 29).

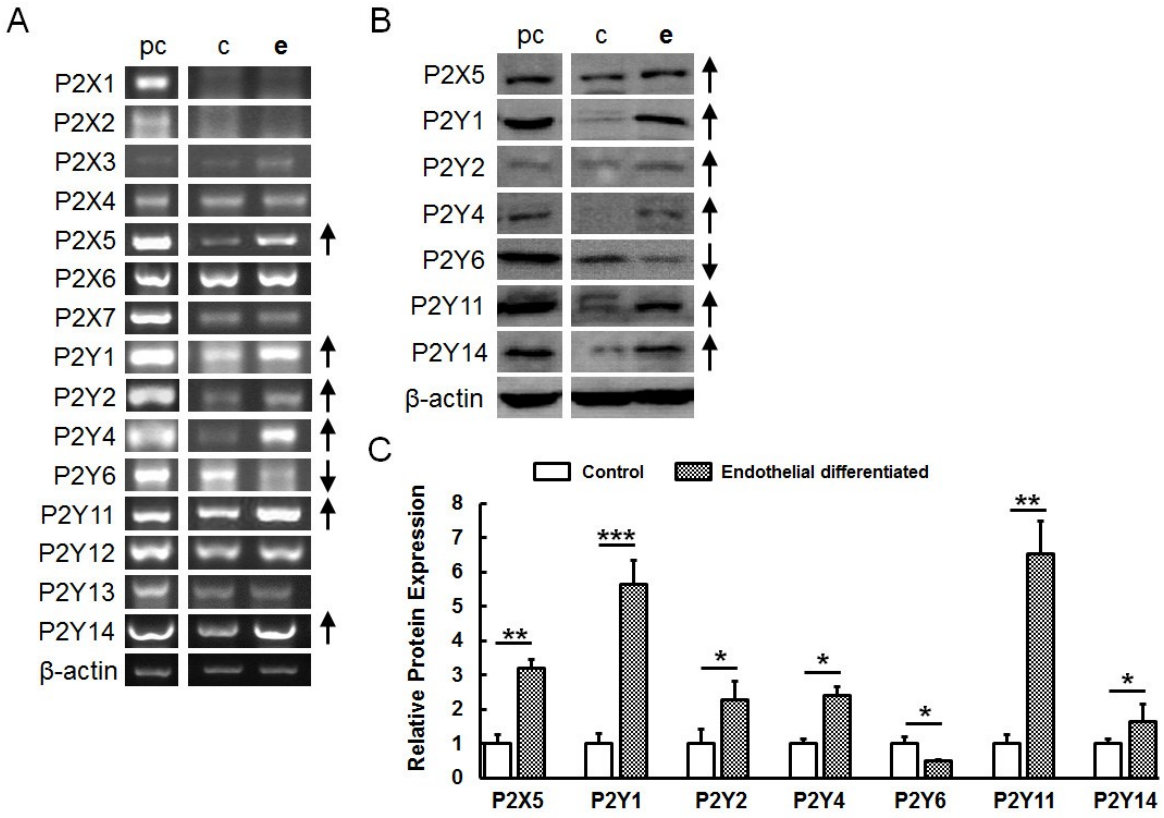


Figure 28. P2 Receptor Expression in Endothelial Cell Differentiation.
RT-PCR was performed with P2 receptor subtypes on the gene level after endothelial cell differentiation (A). Western blot data confirmed the changed gene profile (B). β-actin served as internal control. The positive control was set as described for smooth muscle cell differentiation (see 3.3.1). Graph (C) represents the quantification of the Western blot bands normalized with β-actin in un-differentiated and differentiated cells. Since there is no single cell type that expresses all P2 receptor subtypes, different positive controls were used here: HMEC-1 for P2X3, P2X4, P2X5, P2X7, P2Y1, P2Y2, P2Y4, P2Y11; MG-63 for P2X6, P2Y6 and mouse brain for P2X1, P2X2, P2Y12, P2Y13, P2Y14. The β-actin of the positive control is only given for HMEC-1 as representative for all three positive controls. c, un-differentiated MSCs; e, towards endothelial cell differentiated MSCs. ↑ indicates an up-regulation of the P2 receptor subtype during differentiation, and ↓ indicates a down-regulation. The data shown are representative for one donor out of three.

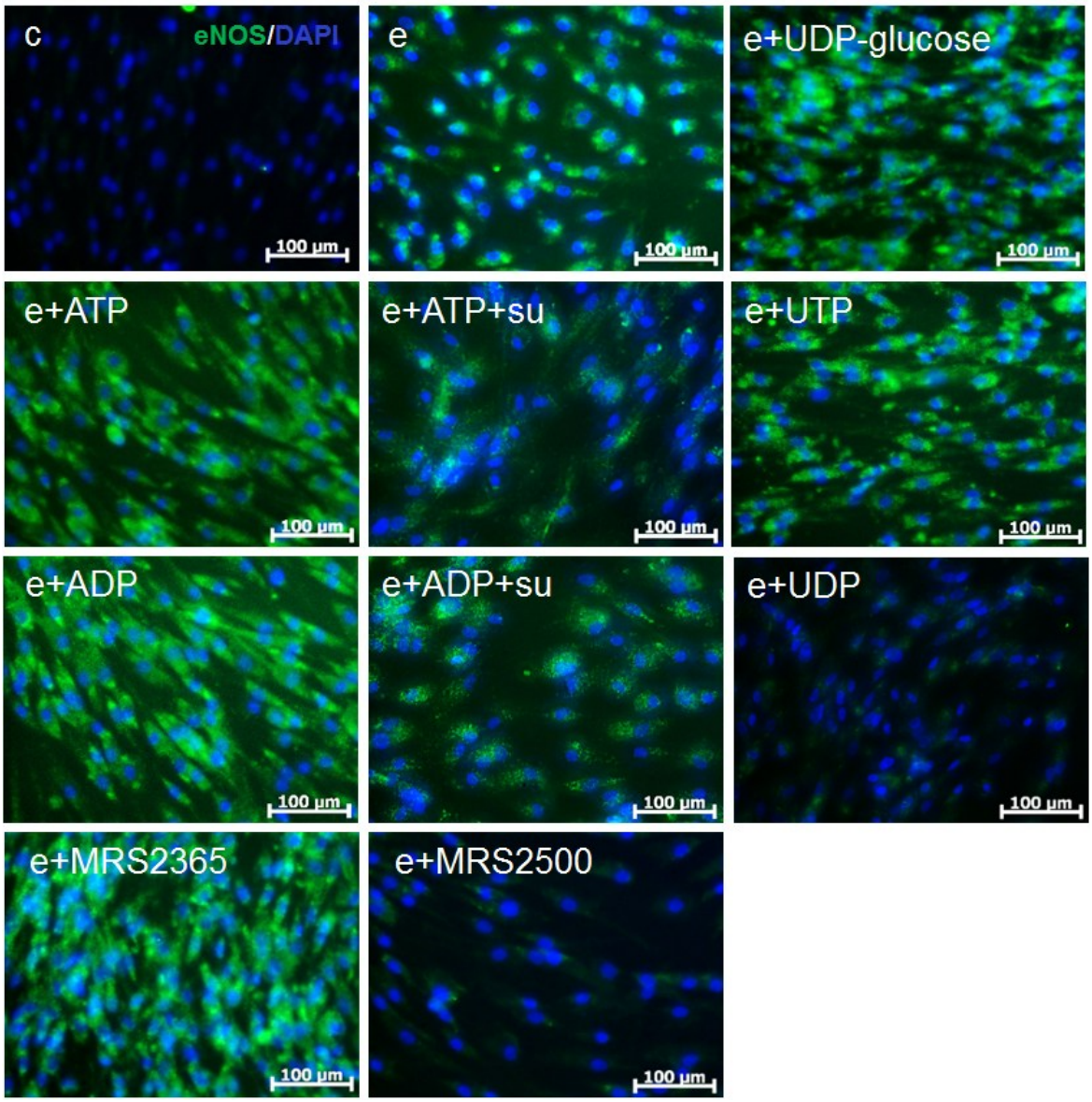


Figure 29. Influence of P2 Receptor Ligands on EC Differentiation.
During the endothelial cell differentiation, certain concentrations of ATP (100 µM), ADP (100 µM), UDP (100 µM), UDP-glucose (100 µM), suramin (100 µM), MRS2365 (1 µM) or MRS2500 (1 µM) were added to the differentiation medium. Half of the medium was exchanged with double the amount of agonists or antagonists twice a week. Endothelial differentiated cells were evaluated by fluorescent staining with eNOS. ATP, UDP-glucose, and ADP significantly enhanced the eNOS staining while UDP reduced eNOS expression. c, control un-differentiated MSCs, e, towards endothelial cell differentiated MSCs, e+su, towards endothelial cell differentiated MSCs supplemented with suramin. The P2Y1 agonist MRS2365 enhanced the endothelial differentiation while its antagonist MRS2500 inhibited it. The pictures shown are representative for one donor out of three. All measurements were performed in triplicate.

3.3.2.3 Effect of Endogenous Nucleotide Release on EC Differentiation

The application of 5 U/mL apyrase to the endothelial cell differentiation medium decreased eNOS expression (Fig. 30). This finding suggests that the release of endogenous nucleotides from hMSC during differentiation acts as a pivotal factor in regulating endothelial cell differentiation.

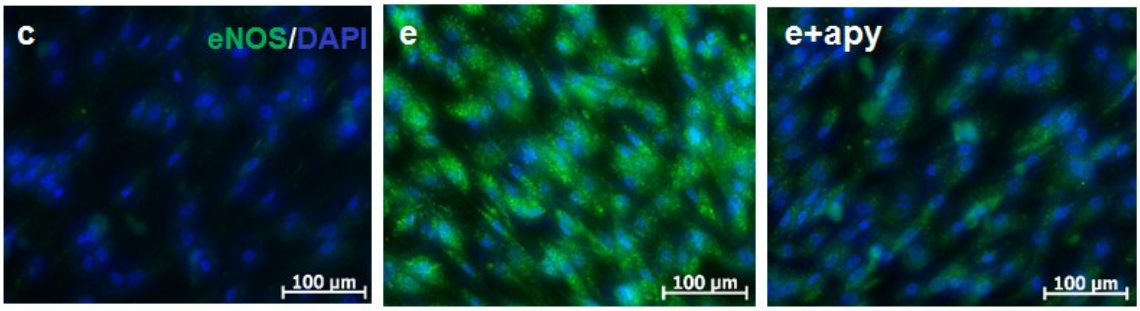


Figure 30. Influence of Endogenous Nucleotide Release on EC Differentiation. The concentration of 5 U/mL apyrase was added to differentiating medium. Half of the medium was exchanged with double amount of apyrase two times a week. Endogenous nucleotides release enhanced eNOS expression. c, control, un-differentiated cells; e, towards endothelial cell differentiated MSCs, e+apy, towards endothelial cell differentiated MSCs supplemented with apyrese. The pictures shown are representative for one donor out of three. All measurements were performed in triplicates.

3.3.2.4 Effect of Ligands on EC Differentiation Evaluated with Matrigel Assay

To further identify the P2 receptor function during endothelial cell differentiation, a Matrigel tube formation assay was performed with the administration of different ligands. Since Matrigel is stable in a cell incubator for maximal 7 days, the differentiation medium was added with ligands and apyrase on day 1 and picures were taken on day 3. In line with the eNOS experiment, ATP, ADP, and UDP-glucose increased the sprouting of tubes whereas UTP seemed to have no effect (Fig. 31). The administration of apyrase inhibited the tube formation (Fig. 31).

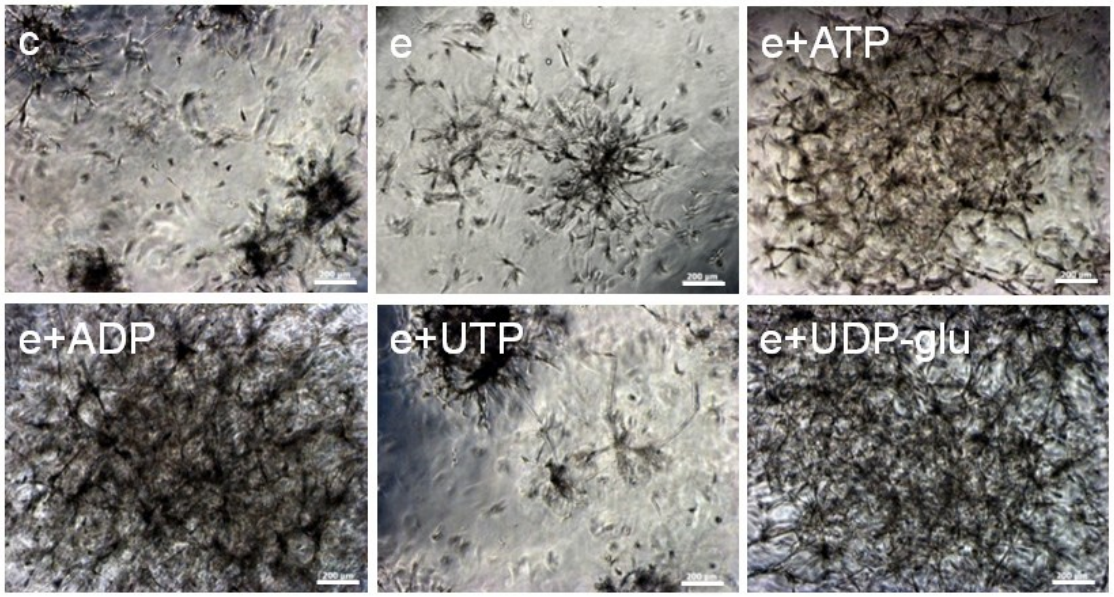


Figure 31. Effect of P2 Receptor Ligands on EC Differentiation Evaluated by Tube Formation.
Endothelial differentiated cells were evaluated by tube formation using Matrigel. ATP, UDP-glucose, and ADP significantly enhanced the tube formation while UTP reduced. Endogenous nucleotides released during the differentiation increased the tube formation. The pictures shown are representative for one donor out of three. All measurements were performed in triplicate. The bar in all pictures is 200 µm.

3.3.2.5 P2 Receptor Underlying Signaling Pathways in EC Differentiation

In order to examine P2 receptor underlying signaling pathways during the endothelial cell differentiation, a protein kinase activity assay was performed to evaluate the phosphorylation level of 46 kinases. After 14 days of endothelial cell differentiation, ERK1/2, β-catenin, p70, RSK, paxillin, STAT1 and c-jun were inhibited while CREB and HSP27 were activated (Fig. 32A). All phosphorylation spots were normalized and later quantified against the control. The above mentioned alterations were found to be significant after statistical analysis (Fig. 32B). The increase of p38 and ERK1/2 (Fig. 32C) phosphorylation levels were subsequently confirmed *via* Western blot.

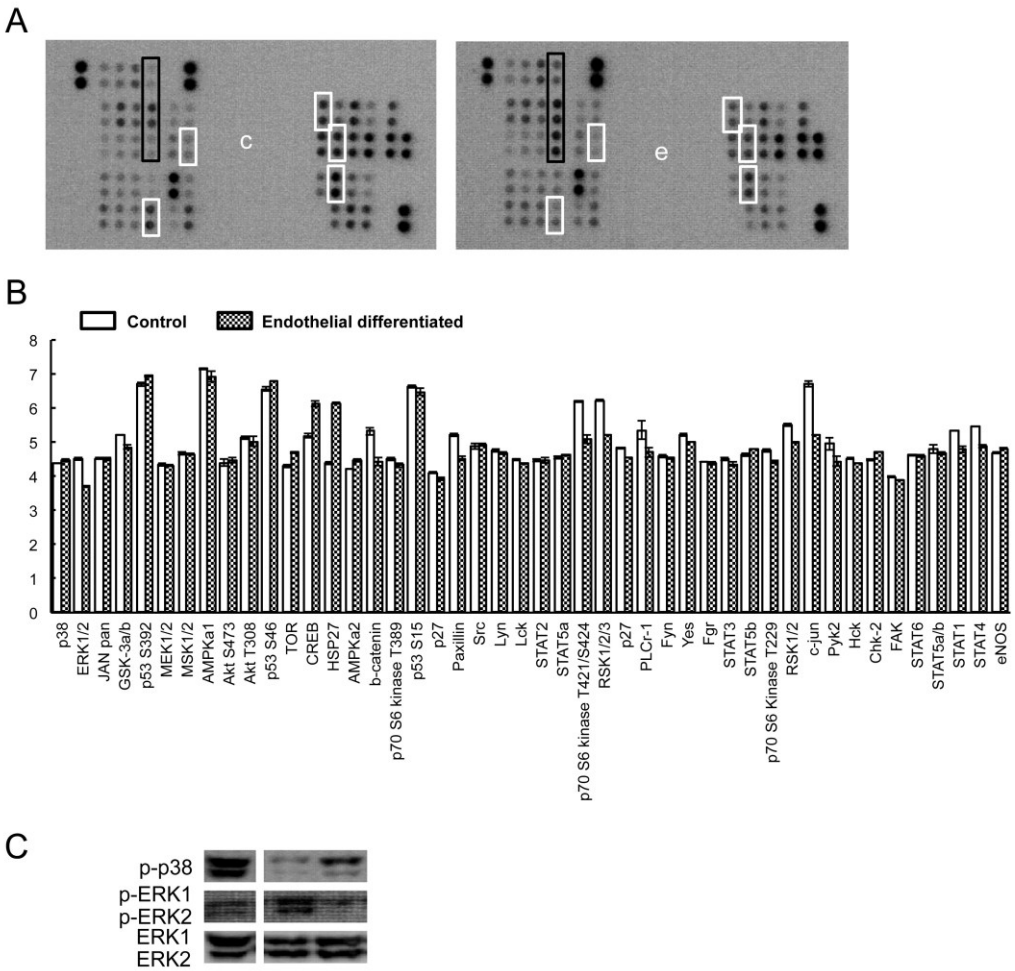


Figure 32. P2 Receptor Underlying Signaling Pathways in EC Differentiation. 46 kinase phosphorylation sites were evaluated using a human phospho-kinase array after endothelial cell differentiation. The black rectangles indicate spots with stronger phosphorylations after differentiation, while white rectangles indicate spots with weaker phosphorylations after differentiation, respectively (A). ERK1/2, β-catenin, STAT1, RSK1/2 and c-jun were inhibited while CREB and HSP27 were activated after endothelial differentiation (B). In addition, a phosphorylation Western blot analysis confirmed that p38 was activated while ERK1/2 was inhibited in endothelial cell differentiation (C).

3.4 Isolation and Characterization of Endothelial and Smooth Muscle Cells

As control cells for the differentiated human endothelial and smooth muscle cells, bovine endothelial and smooth muscle cells were used due to lack of regular access

to a human source for ethical reasons. In addition a comparison of all sources for the blood vessel model was to be performed.

3.4.1 Isolation of Bovine Endothelial and Smooth Muscle Cells

Primary endothelial cells (ECs) were isolated from bovine aorta (Fig. 33A) using collagenase digestion (Fig. 33B). Smooth muscle cells (SMCs) were obtained using a migration method (Fig. 33C) as described. Primary ECs and SMCs were isolated from three cows. The isolated endothelial and smooth muscle cells showed their typical morphology such as brick-shape for endothelial cells (Fig. 33D) and spindle-shape for smooth muscle cells (Fig. 33E). The following experiments were performed using cells between the passage two-four.

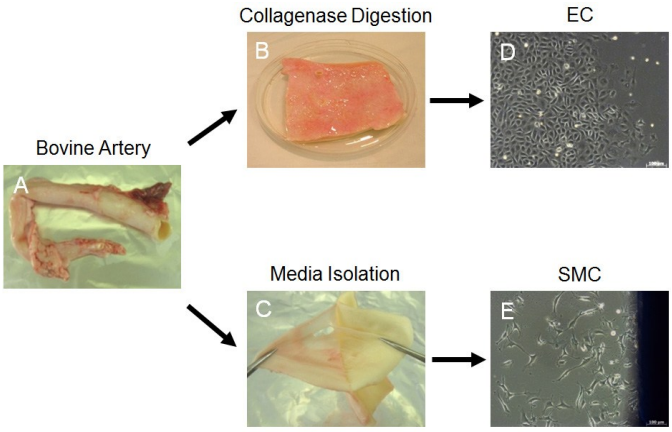


Figure 33. Isolation of Bovine Aortic Endothelial and Smooth Muscle Cells. Bovine aorta (A) was obtained from a slaughter house. After digesting with collagenase (B), endothelial cells were scratched from intima (D). The media layer (C) was cut into small pieces, smooth muscle cells migrated and proliferated from the tissue to the cell culture plate surface (E).

3.4.2 Characterization of Bovine Endothelial and Smooth Muscle Cells

To confirm the character of the isolated aortic endothelial and smooth muscle cells, several endothelial and smooth muscle cell markers were used to examine the cells on both, gene and protein level. The isolated ECs showed positive gene expression

of endothelial cell markers such as VEGFR2, VE-cadherin, and PECAM-1; whereas SMCs positively expressed smooth muscle cell markers for instance SMA-α, calponin and MYH-11 (Fig. 34A). Western blot experiments were performed to confirm the gene expression data on the protein level. ECs positively expressed VEGFR2, VE-cadherin, and PECAM-1, while SMCs were positive for calponin, SMA-α and MYH-11 protein expression (Fig. 34B). Importantly both, gene and protein data showed that ECs were negative for SMC markers except a weak band found in calponin. SMCs were negative for the three tested endothelial cell markers. These results indicate that isolated ECs and SMCs were without major cross contaminations. The immunofluorescent staining data further confirmed the results from the RT-PCR and Western blot analysis (Fig. 34C, D). In addition, isolated ECs also showed the typical endothelial activity by up-taking LDL (Fig. 34E).

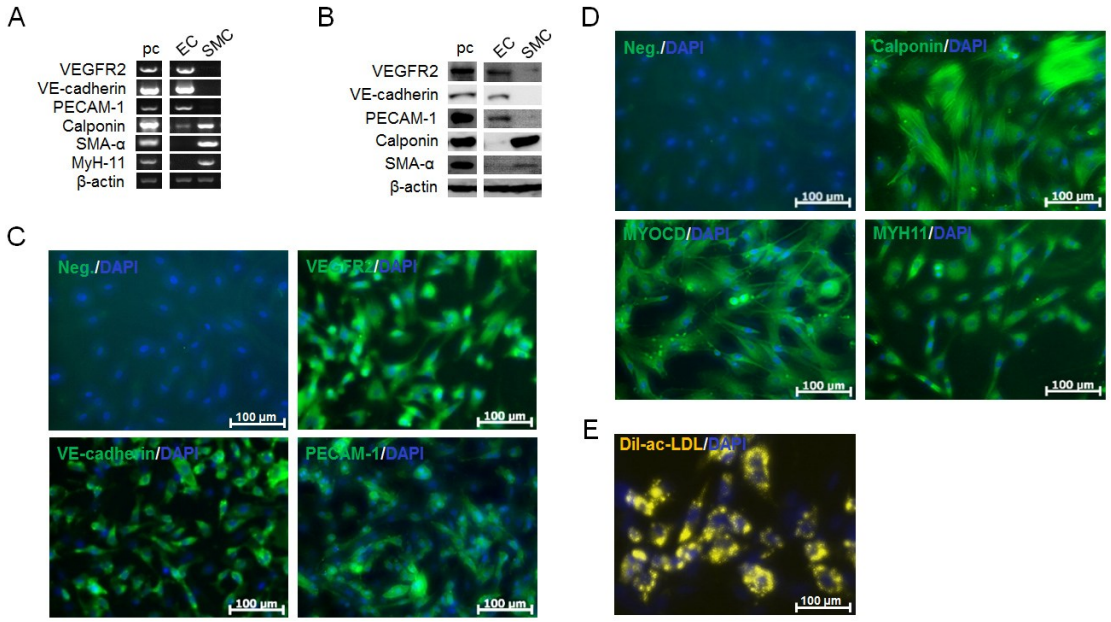


Figure 34. Characterization of Bovine Endothelial and Smooth Muscle Cells. The isolated cells were verified with the EC specific markers VEGFR2, VE-cadherin, PECAM-1 and the SMC specific markers calponin, SMA-α, MYH-11 by RT-PCR (A) and Western blot (B). GAPDH served as internal control. The cell lines HMEC-1 and C2C12 were used as positive controls. The endothelial (C) and smooth muscle cells (D) were also identified with the above-mentioned markers *via* immunofluorescent staining. The isolated endothelial cells were further examined for the typical endothelial activity of LDL up-take (E). All above pictures are representative of one cow sample out of three.

3.5 P2 Receptor Expression in ECs and SMCs after Simulated Microgravity

3.5.1 P2 Receptor Expression in ECs after 24h Simulated Microgravity

All fifteen P2 receptors were analyzed for their gene expression by RT-PCR. In the first experiment, RNA was collected in one set of clinorotation experiments from the whole culture flask. All P2 receptors were expressed in ECs with the exception of P2X3 and P2Y6. Next to this P2X5, P2Y4, P2Y11 and P2Y14 were up-regulated, while P2X7, P2Y1 and P2Y4 were down-regulated on the gene expression level in ECs under 24h simulated microgravity condition (MG) induced by clinorotation if compared to ECs under normal gravity (1g) (Fig. 35).

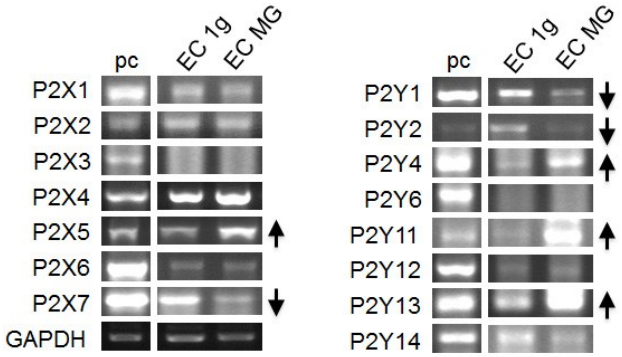


Figure 35. P2 Receptor Expression in ECs under Simulated Microgravity.
ECs of the whole surface of the flasks were isolated for RT-PCR. P2X5, P2Y4, P2Y11, P2Y13 were up-regulated and P2X7, P2Y1 and P2Y2 were down-regulated in the ECs after 24h under simulated microgravity condition in the clinostat.

In a further set of clinorotation experiments, the SMC-conditioned medium collected under normal gravity condition (see 2.3.19) was added to ECs. For this experiment, only cells grown in the 6 mm diameter area of the center (see 2.3.18) were taken to isolate RNA and protein. RT-PCR data showed that although the expression of P2X7 and P2Y1 was decreased after clinorotation, P2X7 in ECs showed an increase on the gene level when cultured in SMC-conditioned medium. P2Y11 protein expression in ECs was up-regulated and further increased also on the SMC conditioned medium compared to P2X7 (Fig. 36A). Western blot and

immunofluorescence confirmed the change of P2X7 on protein level (Fig. 36B, C).

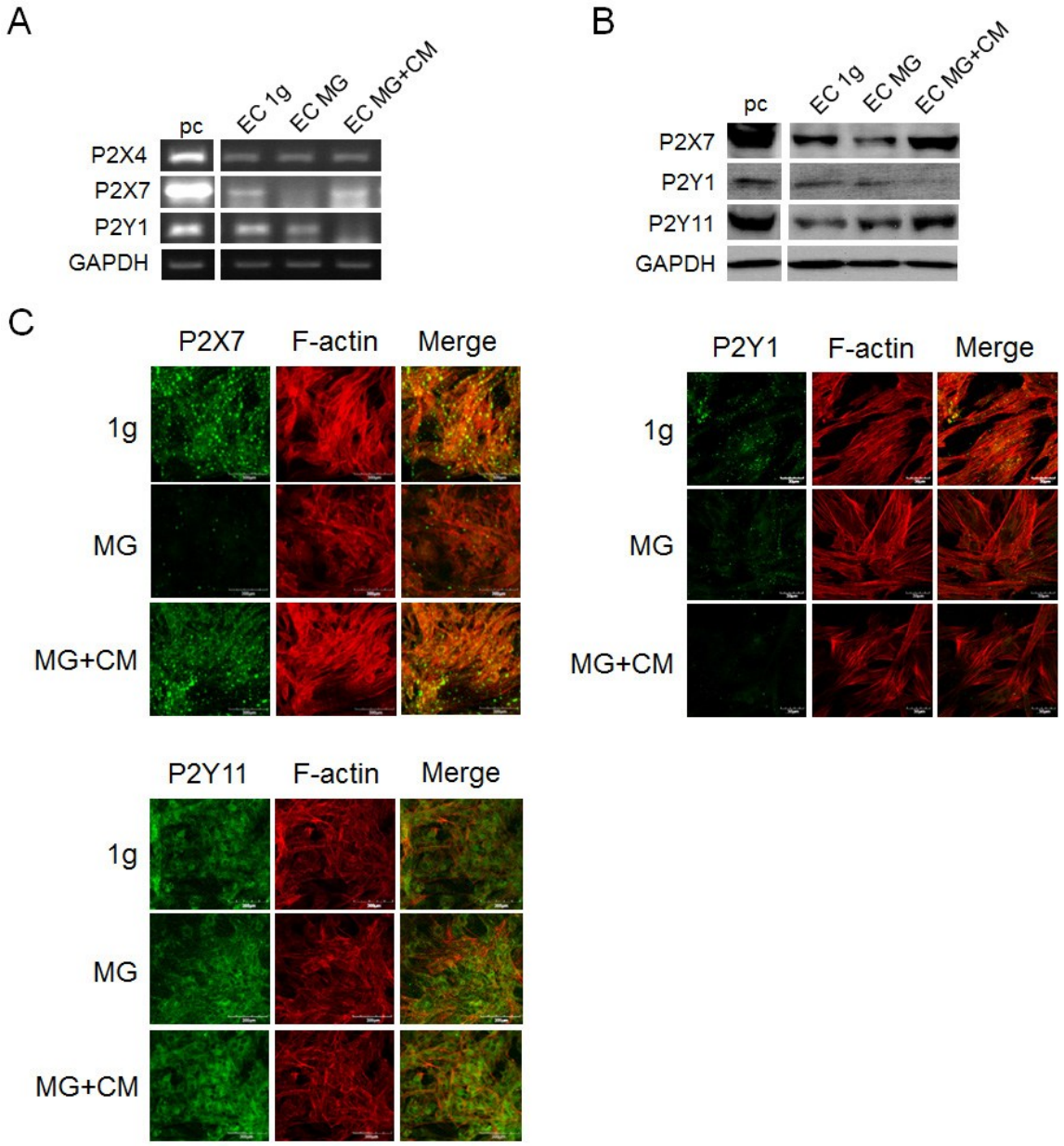


Figure 36. Paracrine Effect on P2 Receptor Expression in ECs under Simulated Microgravity.

Only ECs grown within 6 mm of the center had the optimal simulated microgravity condition and were therefore isolated to investigate P2 receptor alterations on the RNA (A) and protein (B) level after 24h simulated microgravity with and without the conditioned medium from SMCs grown in normal gravity. P2X7 was down-regulated in ECs after 24h simulated microgravity but the conditioned medium compensated this effect. However, there was no effect on the alteration of P2Y1 and P2Y11 after simulated microgravity condition due to the conditioned medium. Fluorescent staining confirmed the change of P2X7, P2Y1 and P2Y2 (C).

3.5.2 P2 Receptor Expression in SMC after 24h Clinorotation

Identical operational steps were undertaken to investigate SMCs under simulated microgravity (see 2.3.18). After 24h clinorotation, RT-PCR showed an increased gene expression of P2X4, P2X7 and P2Y2 whereas P2X2, P2Y1 and P2Y14 were down-regulated in SMCs under simulated microgravity condition (MG) if compared to the SMCs under normal gravity (1g) (Fig. 37).

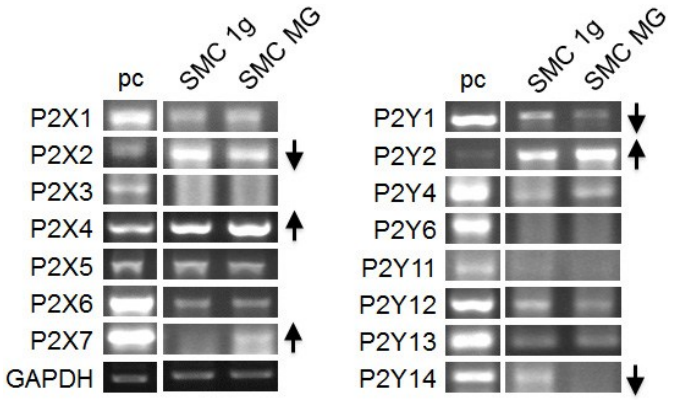


Figure 37. P2 Receptor Expression in SMCs under Simulated Microgravity.
The P2 receptor expression in smooth muscle cells after 24h in normal and simulated microgravity was evaluated. All cells on the surface of flasks were isolated for RT-PCR. P2X4, P2X7, P2Y2 were up-regulated, P2X2, P2Y1, P2Y12 and P2Y14 were down-regulated in the SMCs after 24h clinorotation.

After adding EC-conditioned medium (see 2.3.19) within clinostat experiment, clinorotation induced an up-regulation of P2X7 gene expression in SMCs as revealed by RT-PCR (Fig. 38A). Interestingly, P2X7 showed a decreased gene expression after adding EC-conditioned medium compared to its increase without EC-conditioned medium under 24h clinorotation. P2Y1 was up-regulated in SMCs under simulated microgravity, however conditioned medium showed no effect on its expression. Gene level alterations of P2X7 and P2Y2 were confirmed on the protein level by Western blot or fluorescent staining, however, P2Y1 showed an increasing protein expression in simulated microgravity and with EC-conditioned medium (Fig. 38B, C).

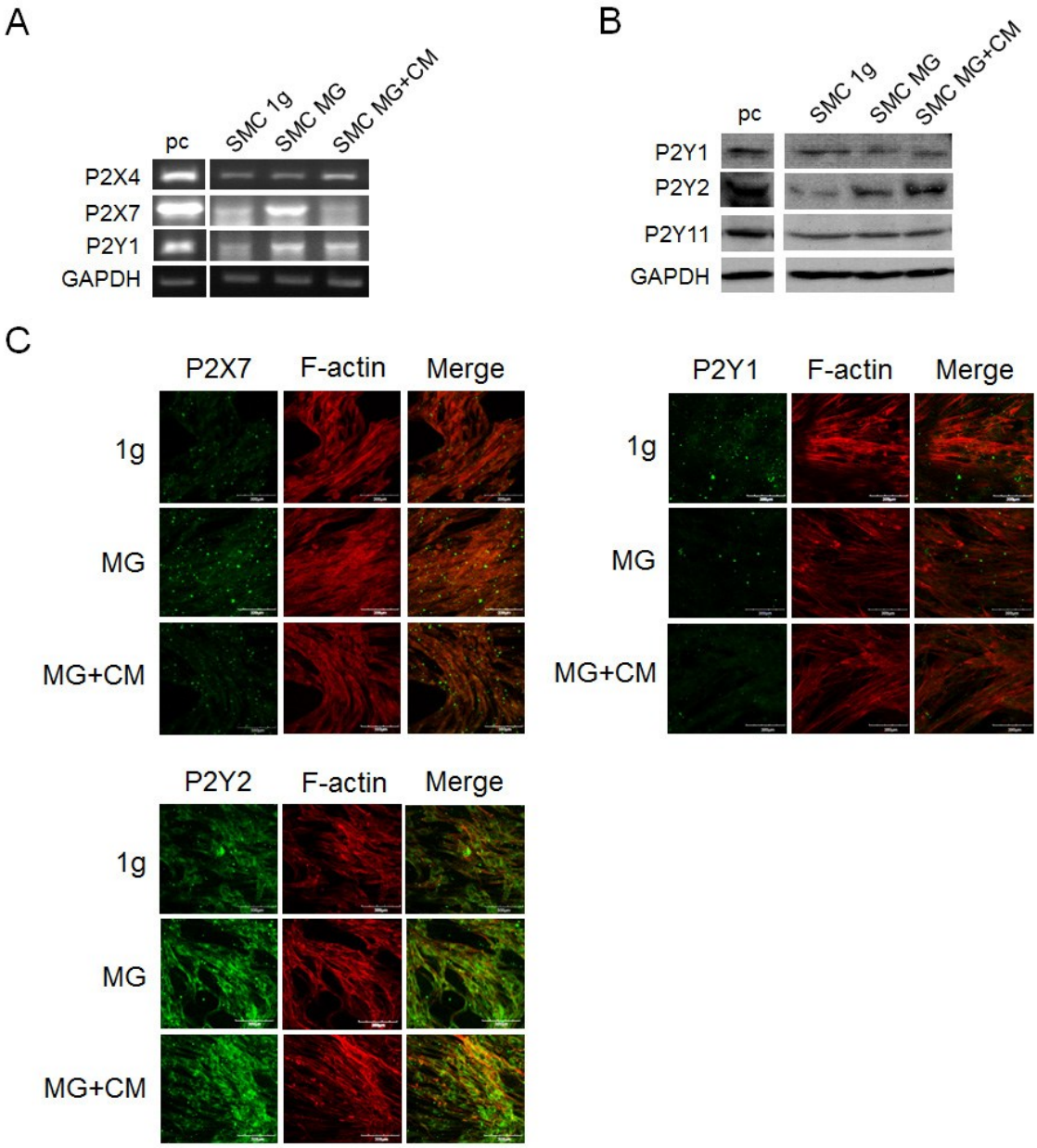


Figure 38. Paracrine Effect on P2 Receptor Expression in SMCs under Simulated Microgravity.

Only SMCs grown within 6 mm of the center had the optimal simulated microgravity condition and were thus isolated to confirm P2 receptor alteration on both RNA (A) and protein (B) level after 24h simulate microgravity with and without the conditioned medium derived from EC growth under normal gravity (1g). P2X7 was up-regulated in SMCs in simulated microgravity while conditioned medium could compensate this effect. However this effect was not observed on P2Y1 and P2Y11. Fluorescent staining confirmed the change of P2X7, P2Y1 and P2Y2 (C).

3.6 The Paracrine Effect on ECs and SMCs in Simulated Microgravity

3.6.1 Proliferation and Migration of ECs with SMC Conditioned Medium

Conditioned medium from SMCs collected after 24h normal gravity and simulated microgravity (see 2.3.19) was used to culture ECs evaluating the paracrine influence of SMCs on EC proliferation and migration. SMC-conditioned medium from normal gravity (SMC CM+1g) did not have a significant influence on EC proliferation after 24h, but caused a decrease of EC numbers after 48h. SMC-conditioned medium collected after simulated microgravity (SMC CM+MG) inhibited EC proliferation significantly after both, 24h and 48h, respectively (Fig. 39). Figure 39 is a representative example one of three cows. The numbers of proliferating ECs from the three individual cows are given in table 6.

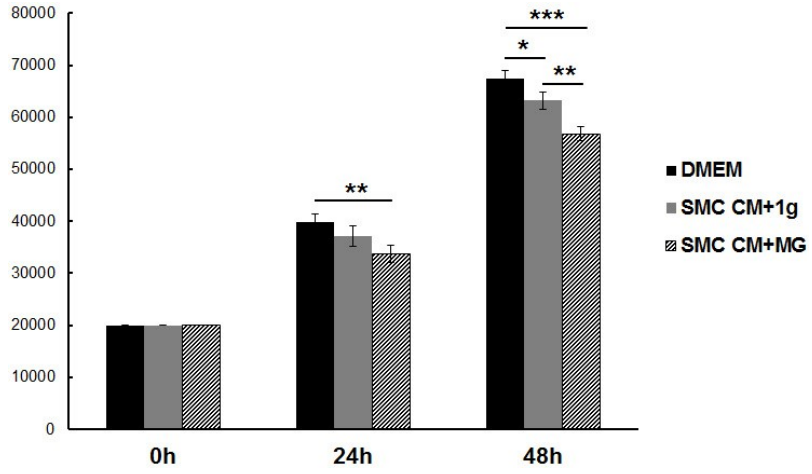


Figure 39. Effect of SMC-Conditioned Medium on EC Proliferation.
To evaluate the EC proliferation, normal DMEM medium, and conditioned medium of SMCs after normal gravity (SMC CM+1g) and simulated microgravity (SMC CM+MG), was added for a 24h and 48h culture period. The conditioned medium from SMCs after normal gravity showed a decrease of EC numbers after 48h. SMC-conditioned medium under simulated microgravity inhibited EC proliferation already after 24h and 48h significantly.

Table 6. Number of ECs after Incubation with SMC-Conditioned Medium

	Medium	0h	24h	48h
EC (Cow 1)	DMEM	20000	39938 ± 1452	67500 ± 1369
	SMC CM+1g	20000	37125 ± 2054	63187 ± 1663
	SMC CM+MG	20000	33750 ± 1591	56812 ± 1281
EC (Cow 2)	DMEM	20000	40469 ± 1041	87500 ± 3536
	SMC CM+1g	20000	39047 ± 2585	82500 ± 2041
	SMC CM+MG	20000	32266 ± 1094	63125 ± 4270
EC (Cow 3)	DMEM	20000	37708 ± 1301	70208 ± 2818
	SMC CM+1g	20000	36875 ± 3903	63542 ± 955
	SMC CM+MG	20000	23958 ± 955	41458 ± 2009

To mimic a wound in the endothelium a straight scratch through the cells was set. The SMC-conditioned medium cultured under normal gravity (SMC CM+1g) and simulated microgravity (SMC CM+MG) condition was added to study EC migration capacity. The conditioned medium from MG enhanced EC migration after 24h, and even more significantly after 48h in the presence of hydroxyurea (Fig. 40A, B). Figure 40 is a representative of example one of three cows. The numbers of migrating ECs from the three individual cows are given in the table 7.

Table 7. Number of Migrated ECs Incubation with SMC-Conditioned Medium

(ECs) Cow 1		(ECs) Cow 2		(ECs) Cow 3	
Medium	**24h**	**Medium**	**24h**	**Medium**	**24h**
DMEM	94 ± 5	DMEM	98 ± 9	DMEM	86 ± 5
SMC CM+1g	113 ± 8	SMC CM+1g	112 ± 9	EC CM+1g	109 ± 4
SMC CM+MG	163 ± 13	SMC CM+MG	151 ± 20	SMC CM+MG	138 ± 17

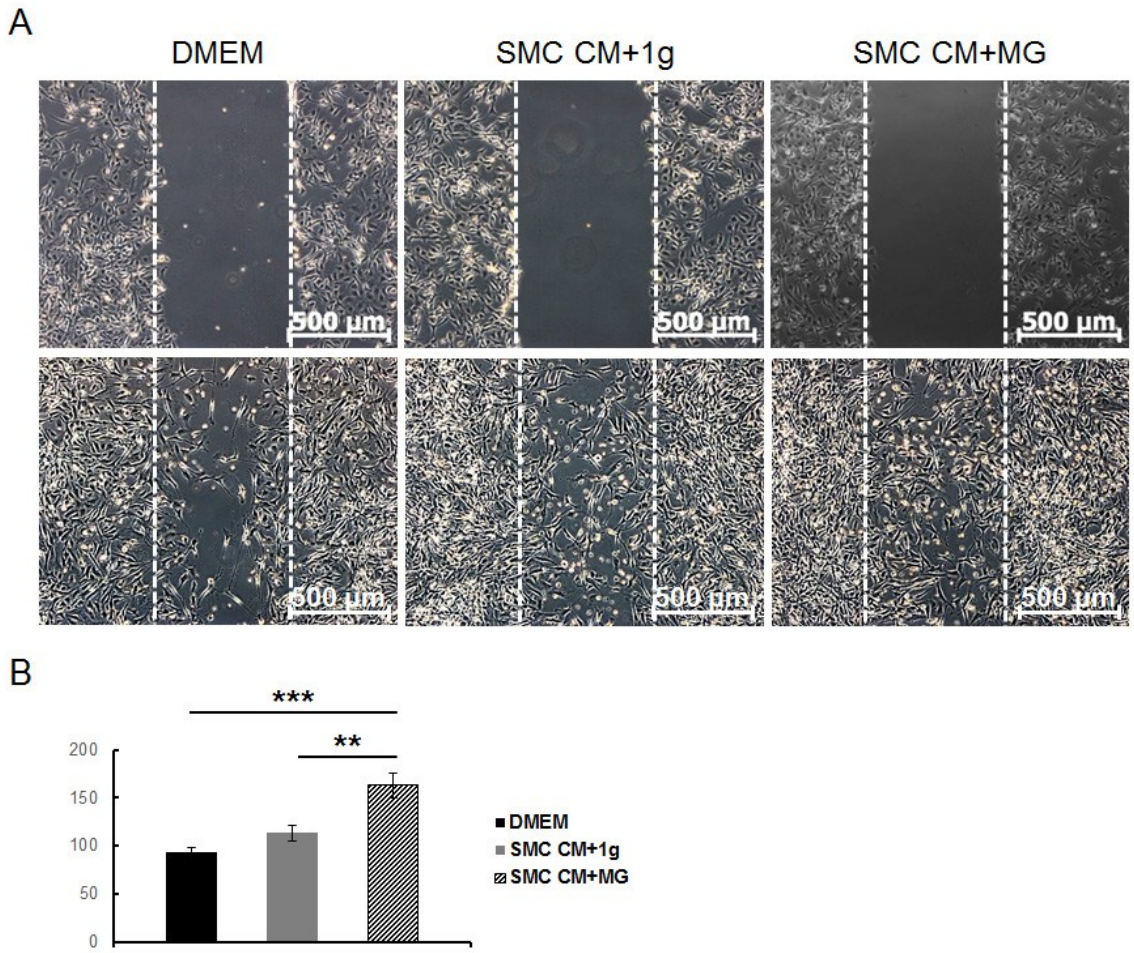


Figure 40. Effect of SMC-Conditioned Medium on EC Migration.
To evaluate EC migration, ECs were scratched and normal DMEM medium, SMC-conditioned medium under normal gravity and under simulated microgravity was added for a 24h culture period (A). Hydroxyurea was added to inhibit EC proliferation. Conditioned medium from SMCs under simulated microgravity (SMC CM+MG) enhanced EC migration significantly if compared to the normal DMEM medium and SMC-conditioned medium under normal gravity (B).

3.6.2 Proliferation and Migration of SMCs with EC Conditioned Medium

Experiments with SMCs were performed in a comparable manner as described for ECs (see 2.3.18 and 3.7.1). The conditioned medium collected from EC grown under normal gravity (EC CM+1g) reduced the proliferation of SMCs. However, the conditioned medium collected from EC grown under simulated microgravity

condition (EC CM+MG) compensated this effect (Fig. 41). Figure 46 is a representative of example one of three cows. The numbers of proliferating SMCs from the three individual cows are given in the table 8.

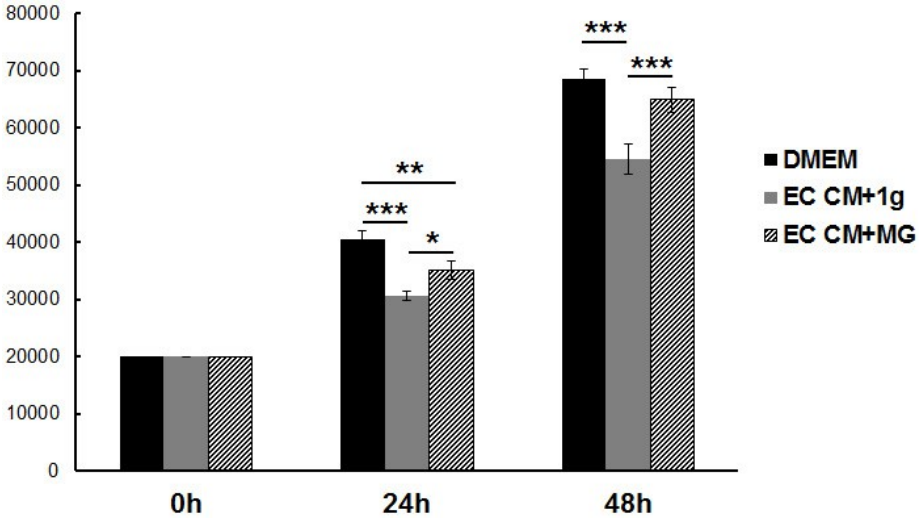


Figure 41. Effect of EC-Conditioned Medium on SMC Proliferation.
To investigate the SMC proliferation, normal DMEM medium, and EC-conditioned medium under normal gravity (EC CM+1g) and simulated microgravity (EC CM+MG), was added and SMCs were subsequently incubated for 24h and 48h. Conditioned medium from EC grown under normal gravity (EC CM+1g) significantly inhibited SMC proliferation after 24h and 48h incubation and conditioned medium from EC grown under simulated microgravity (EC CM+MG) led to a significant decrease of SMC numbers after 24h, which is not obvious after 48h.

Table 8. Number of Migrated SMCs after Incubation with EC-Conditioned Medium.

	Medium	0h	24h	48h
SMC (Cow 1)	DMEM	20000	40500 ± 1472	68625 ± 1652
	EC CM+1g	20000	30625 ± 854	54625 ± 2689
	EC CM+MG	20000	35125 ± 1548	65000 ± 2160
SMC (Cow 2)	DMEM	20000	49594 ± 986	142625 ± 5441
	EC CM+1g	20000	40594 ± 1161	94500 ± 4041
	EC CM+MG	20000	47344 ± 1120	122500 ± 5152
SMC (Cow 3)	DMEM	20000	41500 ± 3000	102083 ± 5052
	EC CM+1g	20000	28167 ± 2566	54583 ± 1909
	EC CM+MG	20000	38167 ± 2021	87917 ± 2602

Conditioned medium under simulated microgravity induced SMC migration after 48h but inhibited it after 24h (Fig. 42A, B). Figure 42 is a representative of example one of three individual cows. The numbers of migrating SMCs from the three individual cows are given in the table 9.

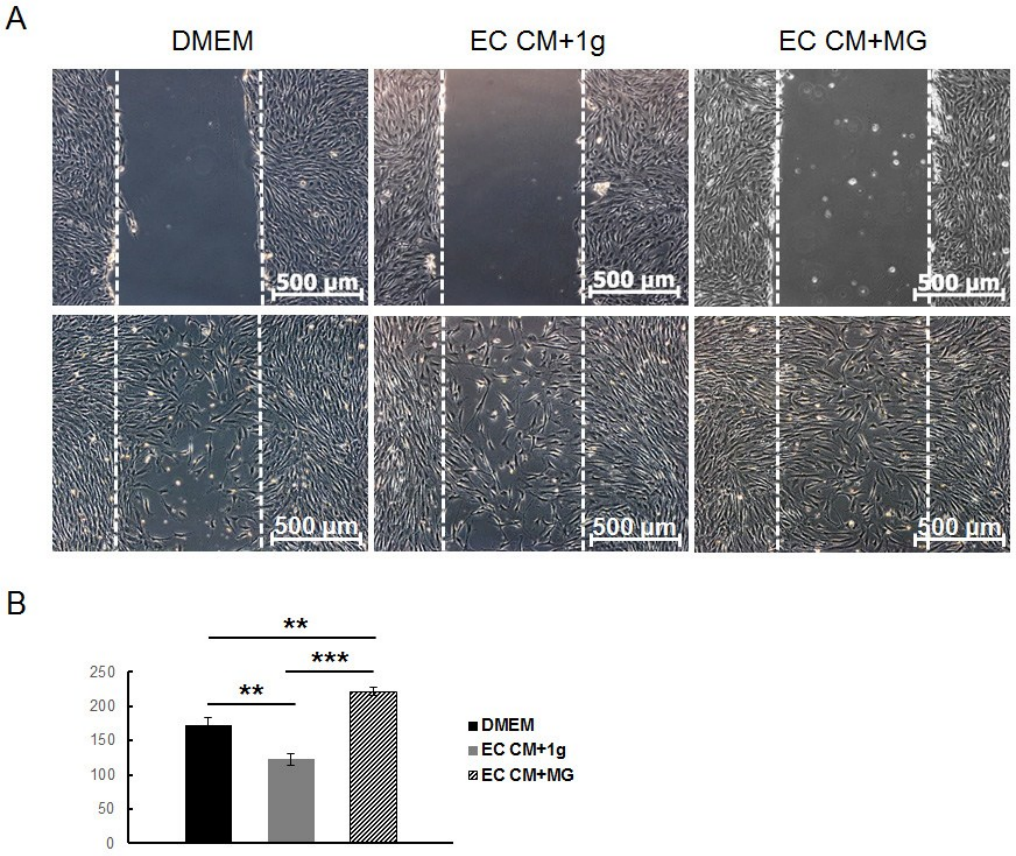


Figure 42. Effect of EC-Conditioned Medium on SMC Migration.
To investigate SMC migration, SMCs were scratched and incubated with normal DMEM medium, EC-conditioned medium under normal gravity and under simulated microgravity for 24h (A). EC-conditioned medium under normal gravity (EC CM+1g) inhibited the SMC migration significantly. Whereas EC-conditioned medium under simulated microgravity (EC CM+MG) enhanced migrated SMC numbers (B).

Table 9 Number of Migrated SMCs Incubated with EC-Conditioned Medium.

(SMCs) Cow 1		(SMCs) Cow 2		(SMCs) Cow 3	
Medium	**24h**	**Medium**	**24h**	**Medium**	**24h**
DMEM	173 ± 10	DMEM	169 ± 7	DMEM	147 ± 11
EC CM+1g	122 ± 9	EC CM+1g	129 ± 5	EC CM+1g	113 ± 6
EC CM+MG	221 ± 6	EC CM+MG	208 ± 15	EC CM+MG	188 ± 15

3.7 Construction of Endothelial and Smooth Muscle Cell Co-Culture Model

3.7.1 Cell Seeding on an Artificial Collagen Scaffold

A collagen matrix with a diameter of 5 mm was a gift from Matricel GmbH (Fig. 43A). The 3D structure of the scaffold was visible using a digital microscope (Keyence, Germany) (Fig. 43B). A number of 10,000 bovine aortic ECs were seed on the scaffold. After 24h adhesion, cell morphology was observed in 3D (see red area in Fig. 43C). To confirm the success of the seeding procedure, cell nuclei were stained with DAPI. The cells grew on the surface of scaffold (see Fig. 43D), which indicated that the collagen matrix was a suitable material for cell seeding and growing.

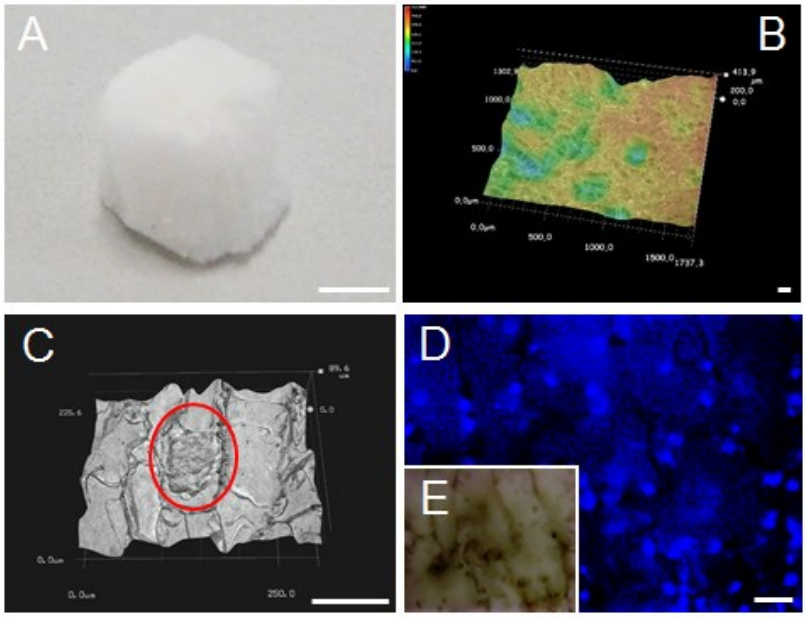
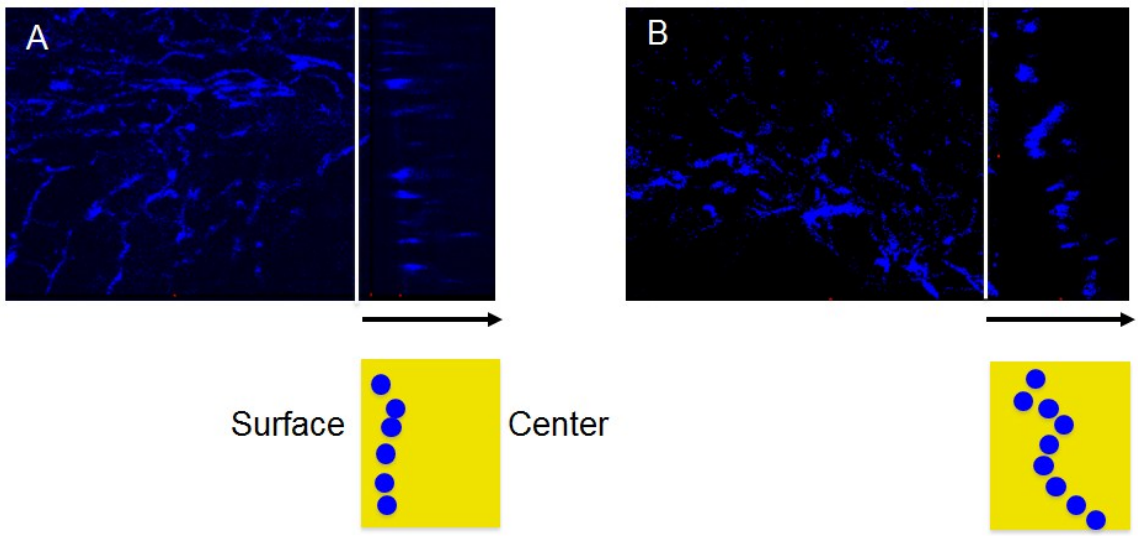


Figure 43. Cell Growth on an Artificial Collagen Scaffold
The artificial collagen scaffold was provided by Matricel GmbH (A). The 3D matrix structure was observed by a digital microscope. After drop-on seeding, the ECs from bovine aorta grew on the surface of the scaffold. ECs were observed on the surface of scaffold with DAPI staining (D) and bright field light (E). The bar in picture A is 2mm, in picture B, C, D is 100 µm.

3.7.2 Growth Behavior of ECs and SMCs on a Collagen Scaffold

Endothelial cells grow as monolayer on the surface of the intima, whereas smooth muscle cells are located inside of the media layer also coupled collagen and elastin fibers. In order to identify the growing behavior of isolated ECs and SMCs and further to create a model with two types of cells, ECs and SMCs were seeded with drop-on method separately. After 24h incubation, the cells were stained with DAPI and the confocal microscopy was used to scan the scaffold from the top to the bottom. ECs were grown on the surface of the scaffold (Fig. 44A), whereas SMCs migrated and grew inside of the scaffold (Fig. 44B).



Picture 44. Different Growth Behavior of ECs and SMCs on the Scaffold.
ECs and SMCs were seeded on the surface of collagen scaffold by drop-on method separately. After 24 hours, cell nuclei were stained with DAPI, the cell migration was evaluated with confocal fluorescent microscopy. ECs grew on the surface (A) while SMCs (B) migrated towards the center of the scaffold. The schemes underneath the picture A, B present the location of the cells within the picture.

3.7.3 Decellularization of a Bovine Artery

Bovine arteries were decellularized by enzyme digestion to remove cellular components. The histological staining were performed to identify the success of

decellularization. H&E staining showed the bovine cells were removed since cell nuclei could not be found after decellularization (Fig. 45). Nevertheless, the extracellular matrix of the decellularized bovine artery remained highly preserved without signs of disintegration: collagen (pink) was shown by van Gieson staining; elastin fibers (black) were shown by Verhoeff's staining (Fig. 45).

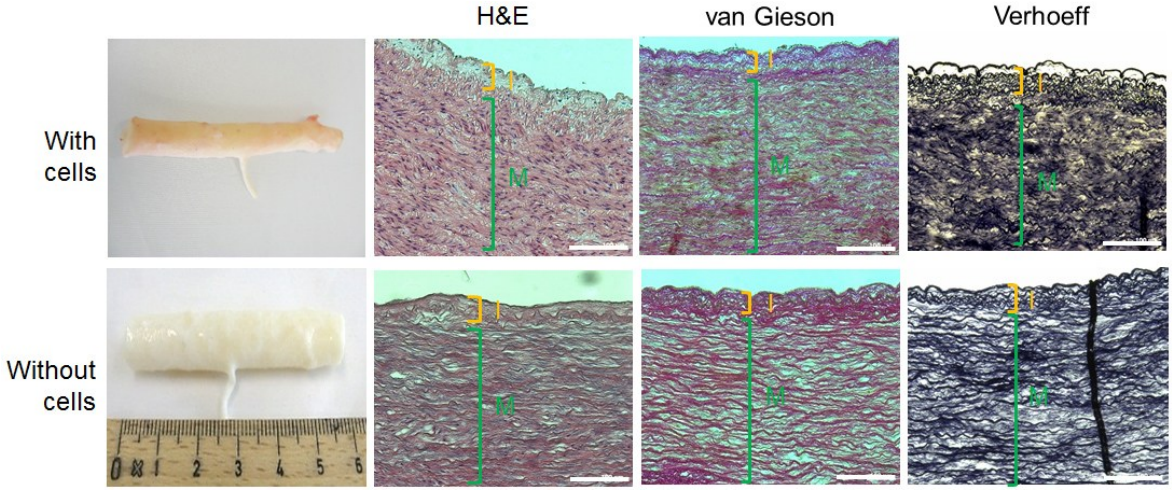


Figure 45. Histological Staining of a Decellularized Bovine Artery
Fresh bovine artery was treated with trypsin/EDTA, DNase, RNase. Sections were cut and prepared for histological staining. H&E staining was performed to confirm the removal of the bovine cells. Collagen (pink) was marked by van Gieson staining. Elastin (black) was labeled by Verhoeff's staining. The luminal surface is at the upper side for both fresh and decellularized group. The yellow line represents the intima as indicated with "I", the green line represents the media labeled with "M".

3.7.4 Decellularization of Human Varicose Vein

To investigate if a scaffold can be isolated from human material, a similar decellularization procedure of enzyme digestion as for a bovine artery (see 2.3.8) was performed with a human varicose vein. The success of decellularization was identified by histological staining. H&E staining showed that the human cells were removed since cell nuclei (Fig. 46, blue dots) were not found after decellularization. At the same time the ECM of decellularized human varicose veins remained less

preserved with signs of disintegration: collagen (pink) was stained by van Gieson staining, elastin (black) was shown by Verhoeff's staining (Fig. 46). Compared with artery, human varicose vein showed less elastin fibers because veins do not need strong vasoconstriction ability.

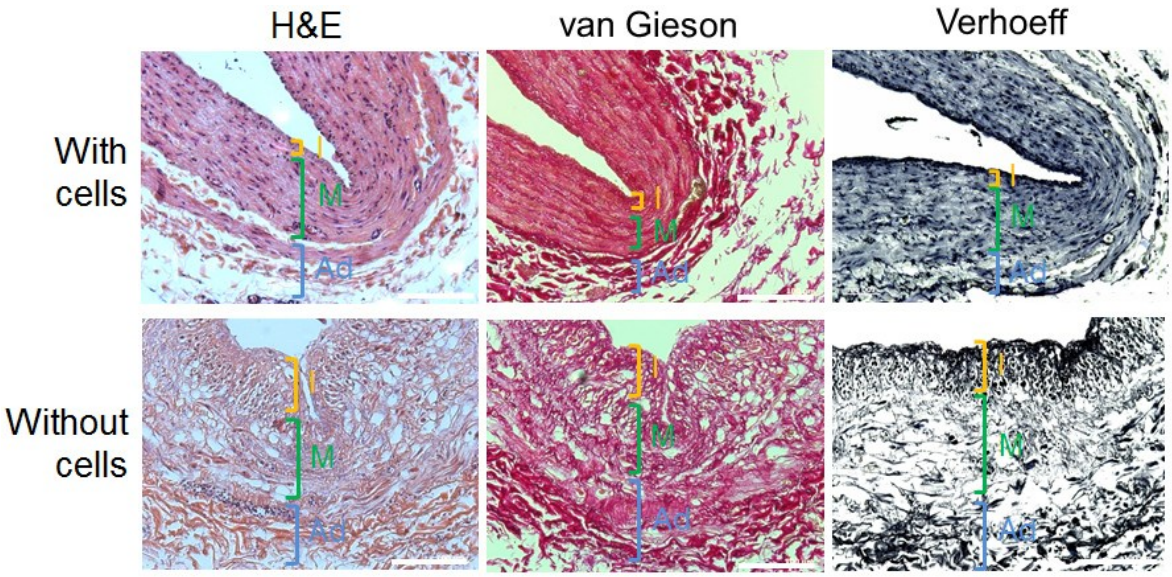


Figure 46. Histological Staining of Decellularized Human Varicose Vein. Human varicose vein was decellularized and subsequently examined with histological staining. H&E staining confirmed the removal of host cells since cell nuclei were not found after decellularization. Collagen was stained by van Gieson staining showing in pink. Elastin was stained by Verhoeff's staining showing the fibers in black. The luminal surface was at the left side of picture with cells and at the upside of the picture of the decellularized. The yellow line represents the intima as indicated with "I", the green line represents the media labeled with "M", the blue line represents the adventitia labeled with "Ad".

3.7.5 Comparison of Different ECs and SMCs Cultured on a Scaffold

To compare the co-culture model with human cells differentiated from MSCs to original ECs and SMCs, ECs isolated from bovine aorta and differentiated from hMSCs were seeded on the scaffold by drop-on, and two types of SMCs were seeded inside of scaffold by drop-in respectively as well. After culturing the cells for 24h, the scaffolds with the cells were fixed in paraffin. Slides with thickness of 15

µM were cut and stained with H&E. ECs from bovine aorta and from differentiated hMSCs grew on the surface of the collagen matrix (Fig. 47A). These two types of ECs showed differences in the distribution with more cluster growth of differentiated ECs. SMCs from bovine aorta and differentiated hMSCs both grew inside of the matrix (Fig. 47B). No growing differences were observed between two types of SMCs.

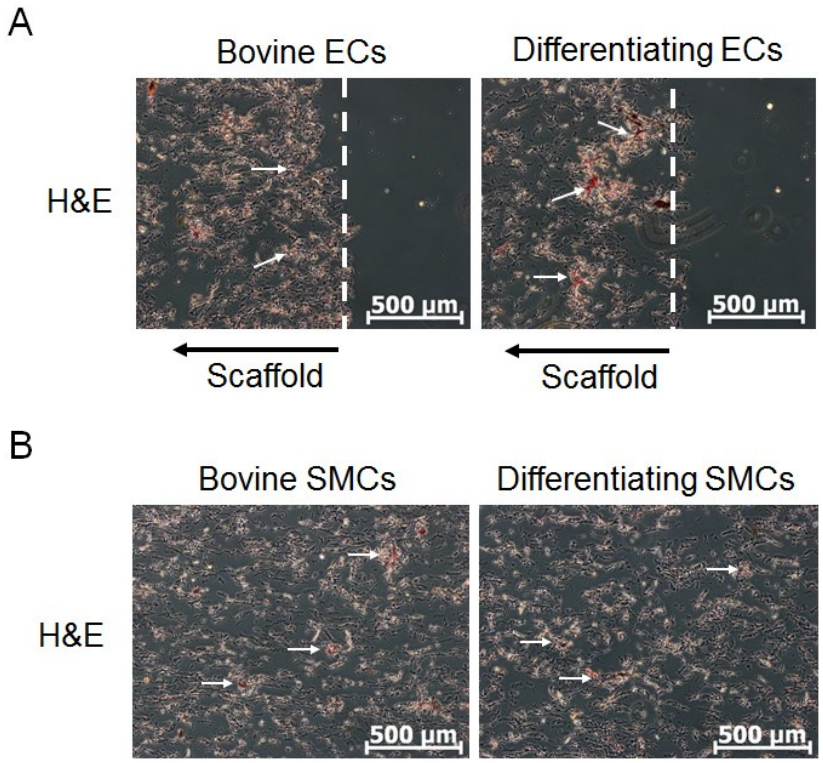


Figure 47. Primary and Differentiated ECs and SMCs Cultured on/in the Scaffold.

The same number of ECs and SMCs derived from bovine aorta and differentiated from hMSCs were seeded on/in the scaffold respectively. After 24h of growth, scaffolds were fixed and embedded with paraffin for H&E staining. The primary and differentiated endothelial cells grew on the surface of scaffold. Whereas two type of smooth muscle cells grew in the scaffold.

4. DISCUSSION

4.1 AT-MSCs as a Promising Source for Obtaining Human ECs and SMCs

Due to the lack of access to human vessel materials, stem cells have been considered as an alternative source to generate human endothelial and smooth muscle cells (Leeper *et al.*, 2010). However, it is critical to identify an appropriate stem cell source. Mesenchymal stem cells are regarded as one of the ideal sources because first, a variety of studies have confirmed that MSCs derived from different sources have the potential to differentiate into smooth muscle cells (Rodriguez *et al.*, 2006; Lee *et al.*, 2006; Heydarkhan-Hagvall *et al.*, 2008; Chong *et al.*, 2011) and "trans-differentiate" into endothelial cells (Wosniza *et al.*, 2007; Heydarkhan-Hagvall S *et al*., 2008; Chong *et al.*, 2011; Zhang P *et al*., 2011; Portalska *et al.*, 2013). Second, MSCs caused less ethical issues compared to ES cells. Third, MSCs trigger a weaker immune response than other stem cells such as ES cells and iPS cells *in vivo* (Mitchell *et al.*, 2006; Nauta & Fibbe, 2007). And fourth, MSCs are less likely to form teratomas. In addition, they migrate faster and secrete beneficial cytokines after implantation (Salem *et al.*, 2010).

Adipose tissue derived MSCs (AT-MSCs) might be more attractive than MSCs derived from other body regions. One reason for this is that MSCs from liposuction materials are easily accessible in abundant numbers and the isolation procedure is less invasive compared to an isolation from bone marrow (Aust *et al.*, 2004; Lin *et al.*, 2010). Another reason is that we do not observe any reduction of AT-MSC differentiation potential between young and old donors. In addition, our previous data showed AT-MSCs derived from older donors appeared to differentiate faster towards adipocytes and there was no decrease for the osteogenic differentiation potential compared to the cells from younger donors (Zippel *et al.*, 2012). In this

study, no significant difference was found in their differentiation potential towards endothelial and smooth muscle cells in donors of different ages. These data was confirmed with the findings from Zhang and colleagues. Their data showed that AT-MSCs were obtained as the same amount numbers and same ability to differentiate towards endothelial cells from elderly patients compared to the younger patients (Zhang *et al.*, 2011). But it must be considered which parameter is investigated because there are donor dependent variances in hMSC differentiation capacity are described in previous studies. For example, the ALP sensitivity after treating with dexamethasone showed a high donor dependency (Siddappa *et al.*, 2007). In addition, a clear variation and senescence was observed on endothelial cell differentiation from BM-MSCs among different donors (Portalska *et al.*, 2013). An explanation for the difference in BM-MSCs as compared to AT-MSCs might be that AT-MSCs are partially positive for CD34 (see Fig. 15). CD34 is a major hematopoietic marker and is also used to identify endothelial progenitor cells. Thus, AT-MSCs might be more prone to differentiated towards endothelial cells compared to the BM-MSCs. A comparative analysis of MSCs derived from bone marrow, adipose and dermal tissues has shown that the highest amount of secreted cytokines such as VEGF-D and in addition an enhanced HMEC tube formation can be detected in AT-MSCs. This indicates that AT-MSCs might have a stronger ability to repair local damaged tissue after injection *in vivo* (Hsiao *et al.*, 2012). Taken together, evidences indicate that human AT-MSCs are a reliable and promising cell source for generating human vascular cells for future vascular tissue engineering or drug testing models.

4.2 The Regulation of P2 Receptor Signaling in EC and SMC Differentiation

Previous data from the laboratory already showed that P2 receptors play a functional role in hMSC adipogenic and osteogenic differentiation (Zippel *et al.*, 2012). Here we further show that P2 receptor agonists and antagonists can directly

influence hMSC differentiation towards endothelial and smooth muscle cells. These findings are in agreement with the findings of respective receptor subtype expressions on both, gene and protein level. P2Y4 and P2Y14 were up-regulated in endothelial and smooth muscle cell differentiation whereas they were down-regulated in adipogenic and osteogenic differentiation (Zippel *et al.*, 2012), which indicates that these two receptors might be important in the early lineage commitment of hMSCs. P2Y1 expression was decreased in smooth muscle cell differentiation and it was increased in endothelial cell differentiation, suggesting it might be a key factor in controlling the differentiation between these two cell lineages. Next to this, further P2X receptors were altered in smooth muscle cell differentiation such as P2X1, P2X3, and P2X7, whereas only P2X5 was changed in endothelial cell differentiation.

In this study UDP-glucose a specific agonist of P2Y14 significantly enhanced calponin expression in smooth muscle cell differentiation and eNOS expression in endothelial cell differentiation. The functional role of P2Y14 was already confirmed in adipogenic differentiation. UDP-glucose decreased the lipid droplet formation after adipogenic differentiation (Zippel *et al.*, 2012). However, its function in osteogenic differentiation required further analysis, since no significant difference was found on Ca^{2+} deposition showed by Alizarin Red S staining (Zippel *et al.*, 2012). Taken together, these data suggest that P2Y14 was functional in the early lineage commitment of hMSCs.

For the P2Y4 subtype there were no selective agonist and antagonist available within the timeframe of this work (Jacobson *et al.*, 2012). However, since UTP is a ligand for P2Y2 and P2Y4 and ATP can activate P2Y2 but inhibit P2Y4 in human (Abbracchio *et al.*, 2006) (see table 1), the administration with UTP and ATP separately could identify the functional role of P2Y4 indirectly. In smooth muscle cell differentiation UTP significantly enhanced calponin expression whereas ATP reduced the expression, which indicates that P2Y4 might promote smooth muscle cell differentiation. In endothelial cell differentiation both, UTP and ATP increased

eNOS expression. Since the ATP sensitive receptors P2Y2 and P2Y11 were also up-regulated in the differentiation, it is difficult to conclude a clear functional role of P2Y4. This needs further investigation. In our previous publication, it was shown that P2Y4 inhibited both adipogenic and osteogenic differentiation (Zippel *et al.*, 2012), which indicates that P2Y4 is likely to be a functional player in early lineage commitment of hMSCs.

4.2.1 P2 Receptor Regulation in EC and SMC Differentiation

Although several specific P2 receptor subtype expressions were altered in the later stage of endothelial and smooth muscle cell differentiation, it required further analysis to identify their function using their agonists and antagonists. Each P2 receptor subtype has its predominant ligand such as P2X receptors are exclusively activated by ATP, while P2Y receptors mainly respond to purine (ATP, ADP) and pyrimidine (UTP, UDP) nucleotides. In details, ATP is the preferential ligand for P2Y2, P2Y11, and P2Y13; ADP is the ligand of P2Y1 and P2Y12; UTP is the ligand of P2Y2 and P2Y4; UDP is the ligand of P2Y6; and UDP-glucose is the ligand of P2Y14 (Burnstock, 2004). Many groups already tested P2 receptor function with the above mentioned natural ligands. However these data might only suggest indirectly the role of a specific receptor function due to their wide interaction potential among each other, and therefore their specific function requires further confirmation (Burnstock, 2010). Thus, potent and selective ligands were synthesized to investigate the specific P2 receptor subtype function (Jacobson *et al.*, 2012).

P2Y1 might be a key factor in determining hMSC differentiation towards endothelial and smooth muscle cells, because it was up-regulated in endothelial cell differentiation and down-regulated in smooth muscle cell differentiation. After adding its predominant natural ligand ADP, calponin expression in smooth muscle cell differentiation was inhibited whereas eNOS expression in endothelial cell differentiation was enhanced. Since ADP is the predominant ligand for P2Y12 and

P2Y13 as well, the highly potent and selective agonist MRS 2365 and the antagonist MRS2500 of P2Y1 were used to confirm its specific function. MRS 2365 inhibited calponin expression while MRS2500 enhanced the expression, which was observed oppositely in endothelial cell differentiation. MRS2365 increased eNOS expression whereas MRS2500 reduced the expression. This result indicated that P2Y1 is very much likely to be a key factor in determining MSC differentiation towards endothelial and smooth muscle cells. The underlying mechanism of P2Y1 differentiation determination is yet unclear. P2Y1 belongs to the Gq family and can activate several signaling transduction pathways such as p38, ERK and Akt. We found that these pathways were activated differently in endothelial and smooth muscle cell differentiation. For example, Akt was only activated in smooth muscle cell differentiation whereas p38 was only activated in endothelial cell differentiation. P2Y1 are found to be highly expressed in hiPS cells, P2Y1 mRNA and protein down-regulation by hypoxanthine guanine phosphoribosyltransferase knockdown is refractory to activation by the P2Y1 receptor agonist ATP and shows aberrant purinergic signaling, as reflected by constitutive activation of the MAP kinases phospho-ERK1/2 and marked deficiency of the transcription factor pCREB (Mastrangelo *et al.*, 2012). P2Y1 was also found to elevate intracellular Ca^{2+} during mouse embryonic stem cell differentiation towards GABAergic neurons (Khaira *et al*., 2009). Thus, the activation or inhibition of P2Y1 might leads to changes in the underlying signaling pathways which subsequent might cause the different lineage commitment. In addition, P2Y1 was shown to regulate keratinocyte proliferation (Greig *et al*., 2003). It has been demonstrated that proliferation and differentiation is balanced in stem cells therefore P2Y1 might regulate the differentiation *via* controlling the proliferation.

Further in smooth muscle cell differentiation P2X1 was up-regulated, while P2X3 and P2X7 were down-regulated. P2X1 is known to be predominantly expressed in smooth muscle cells playing a major role in vasoconstriction and vasodilatation (Burnstock & Knight, 2004). This it is reasonable to assume that the P2X1 up-

regulation might be important in the differentiation process. However since no selective agonist or antagonist of P2X1 is available, its functional role is unproven and requires further investigations. SMCs are also known to express P2X3 and P2X7 (Burnstock & Knight, 2004). However, RO-3, a highly potent and selective artificial P2X3 antagonist, had no significant effect on calponin expression whereas A-740003, a highly potent and selective artificial P2X7 antagonist, significantly enhanced its expression in SMC differentiation. This suggests that P2X7 might be the major functional P2X receptor in smooth muscle cell differentiation. P2X7 is well-known to regulate apoptosis (Zhang *et al.*, 2011) and it was shown to control some critical physiological processes such as cell death and survival in adipose tissue derived MSC differentiation towards Schwann cells (Faroni *et al.*, 2013). Therefore, P2X7 might control the differentiation via apoptosis signaling but this needs further investigations. Another reason could be the unique long intracellular C-terminal tail of P2X7 compared to other P2X receptor subtypes (Erb *et al.*, 2006). This tail contains several binding domains which can initiate a variety of signal transductions such as ERK1/2, Rho and JNK, leading to gene expression alterations (Laurie *et al.*, 2006). Activation or inhibition of P2X7 changed the gene expression and controlled the subsequent differentiation of endothelial and smooth muscle cell differentiation process *via* these signaling pathways.

In endothelial cell differentiation ATP significantly increased eNOS expression and tube formation, which suggests P2X5, P2Y2, or P2Y11 might be responsible for this. P2Y6 was the only down-regulated P2 receptor subtype in this differentiation. UDP, a predominant agonist of P2Y6 significantly reduced eNOS expression. Xiao and colleagues demonstrated that endothelial progenitor cells (EPCs) were negative for P2Y6, which indicates P2Y6 might be an early regulator in differentiation from stem cells to EPCs. Since there is still a lack of identified specific markers of EPCs to distinguish between endothelial cells (Xiao *et al.*, 2012). P2Y6 might be also used as a marker to characterize them.

Administration of the nucleotide hydrolyzing enzyme apyrase decreased eNOS

expression in endothelial cell differentiation, while calponin expression in smooth muscle cell differentiation was enhanced. This indicated that endogenous nucleotide release has an impact on both differentiation processes. MSCs were found to release ATP and induced calcium oscillations (Kawano *et al.*, 2006). Next to this, mature osteoblasts showed a 7-fold amount of releasing ATP compared to un-differentiated cells, which suggested stem cells might release ATP in a differentiation-dependent manner (Orriss *et al.*, 2009). Recent data from the laboratory support that release of nucleotides enhance hMSC osteogenic differentiation but also inhibited adipogenic differentiation (Zippel *et al.*, 2012). All data mentioned above suggest that these released endogenous nucleotides had a direct influence on the differentiation and thus function as feed-back loop.

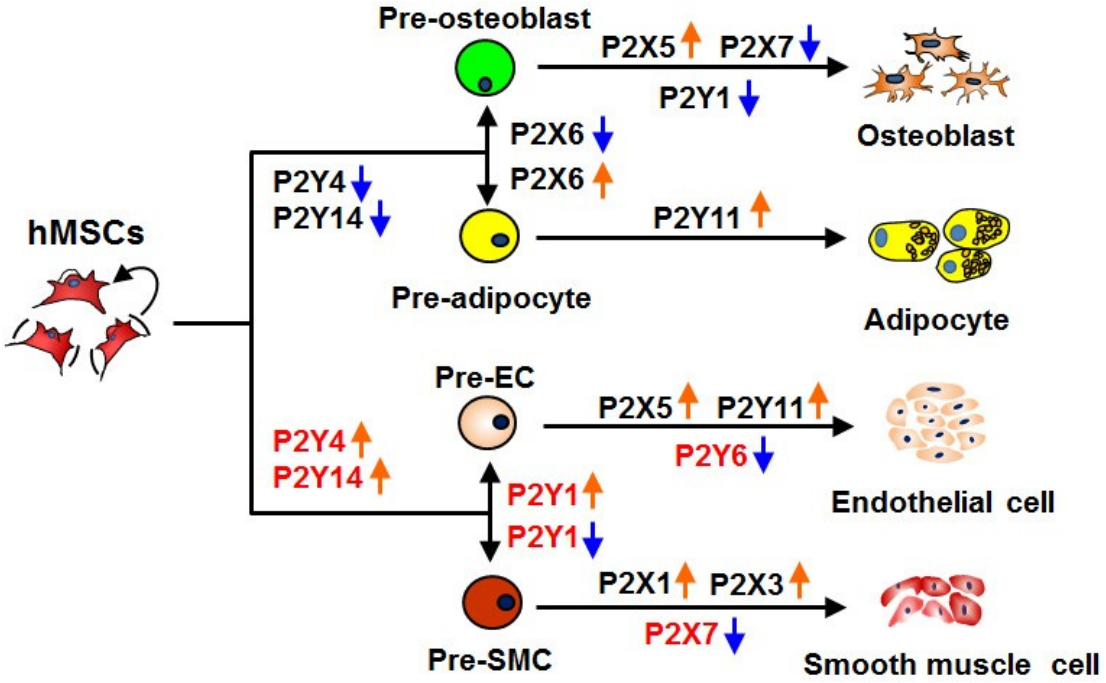


Figure 48. Model of P2 Receptor Function During MSC Commitment.
Taken the previous data from the laboratory together (Zippel *et al.*, 2012), P2Y4 and P2Y14 seem to be involved in the early stage differentiation. P2Y1 might be a key factor in controlling differentiation towards either smooth muscle or endothelial cells. P2X7 mainly regulated smooth muscle cell differentiation while P2X5, P2Y4 and P2Y6 are suggested to be important in endothelial differentiation. The receptor subtypes marked red have been shown to have a functional role.

4.2.2 P2 Receptor Signaling Pathways Involved in SMC and EC Differentiation

P2Y receptors are able to trigger different signaling pathways due to their different subtypes. For example, P2Y1, 2, 4, 6 and 11 belong to the Gq subtype and can activate PI3K/akt/NF-κB or the MKK/p38/CREB pathways. P2Y11 is the only Gs subtype and can initiate the cAMP/ERK/c-jun pathway. P2Y12, 13, 14 are Gi coupled and activate the STAT3 pathway. P2Y11 has the unique feature to couple Gq and Gs, whereas P2Y4 couples to Gq and Gi.

P2X receptors trigger the influx of calcium and can activate subsequent signaling *via* altering the intracellular calcium level. It was shown that blocking the ERK/MAPK pathway enhances BM-MSC smooth muscle cell differentiation (Tamama *et al.*, 2008). However, it the opposite effect and thus inhibiting endothelial cell differentiation in bone marrow derived progenitor cells was published as well (Xu *et al.*, 2008). The phosphorylation levels of several kinases were altered differently in endothelial and smooth muscle cell differentiation in our study. For instance ERK1/2 was down-regulated, but p38 was up-regulated in endothelial cell differentiation while Akt was up-regulated in smooth muscle cell differentiation. These evidences underscored the hypothesis that a balance between different signals triggering and maintaining in specific stem cell differentiation lineages. However, it is difficult to directly control the signaling pathways *in vitro* due to crosstalk. But extracellular nucleotides can drive stem cell differentiation into desired cell lineages *via* P2 receptors by blocking and/or activating respective signals.

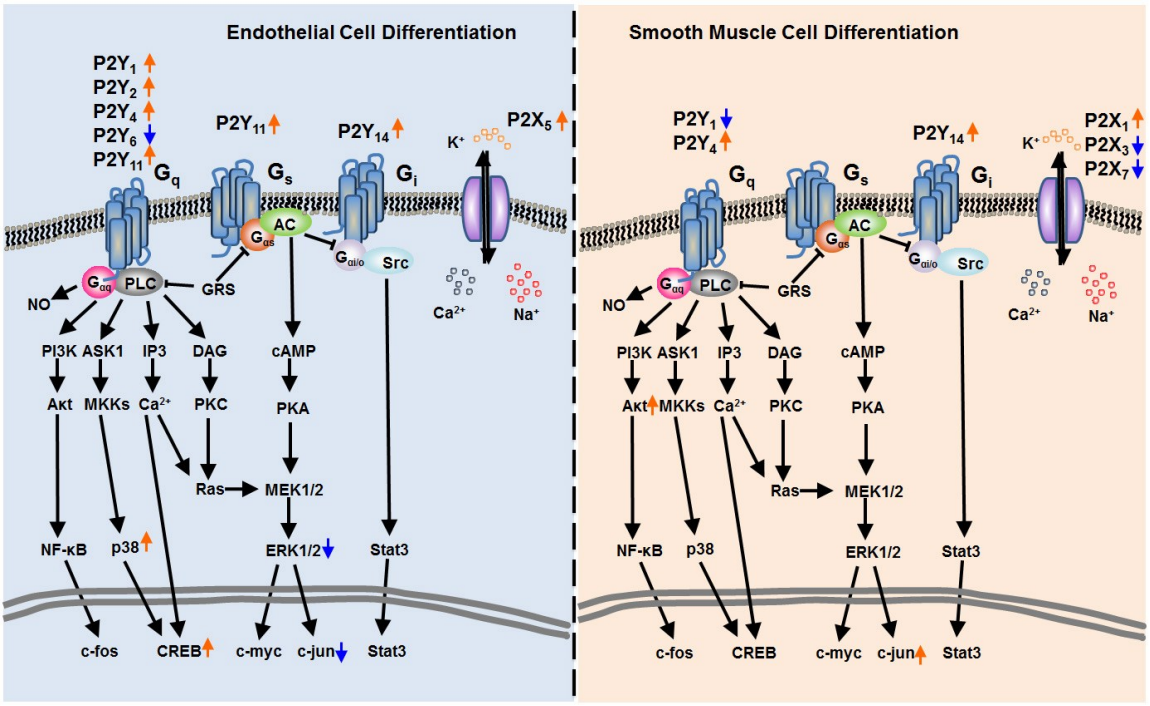


Figure 49. P2 Receptor Signaling Transduction in EC and SMC Differentiation. The differentiation processes are controlled by different signaling pathways. During endothelial cell differentiation of hMSCs the p38/CREB phosphorylation was activated whereas ERK/c-jun was down-regulated. In smooth muscle cell differentiation, the phosphorylation of Akt/c-fos and ERK/c-jun was up-regulated.

4.3 Artificial P2 Receptor Ligand Application on Vascular Tissue Engineering

4.3.1 Clinical Trials Using Human MSCs in Regenerative Medicine

Human MSCs show promising potential for various therapeutic applications and have been used already for various clinical investigations (Ringe *et al.*, 2002; Porada *et al.*, 2006; Wang *et al.*, 2012). A Further understanding of their biological characteristics is needed to successfully use MSCs in various other clinical applications for therapeutic effects. The MSCs are usually considered due to the following properties:

i) The capacity to differentiate into various cell lineages;

ii) The capacity to secrete multiple bioactive molecules such as cytokines or

growth factors that are able to improve the recovery of injured cells and inhibit inflammation (Chen *et al.*, 2004b, Ding *et al.*, 2011);

iii) The capacity to migrate to the inflammatory sites after tissue injury (Denlinger *et al.*, 2003);

iv) The capacity to perform immunomodulatory functions and the lack of immunogenicity. These cells are e.g. known to secrete immunosuppressive cytokines (Aggarwal & Pittenger, 2005). The low-immune characteristics make them suitable candidate cells for allogenic therapeutical use without stimulating the immune response in immunocompetent patients (Pansky *et al.*, 2007; Jung *et al.*, 2009).

The multipotency of hMSCs is considered to be the major feature to make these cells interesting for clinical application (Dan *et al.*, 2006). Recent data showed that MSCs can migrate towards damaged tissues and replace damaged cells *in vivo*. For example, MSCs differentiated towards functional lung cells, such as epithelial or endothelial cells after they were injected into mice which have been irradiation-caused injured in this tissue (Yan *et al.*, 2007). The differentiated donor cells replaced the dead cells, maintained the structural integrity and led to a subsequent tissue repair (Li *et al.*, 2010). However, accumulating data suggest that hMSCs improve the recovery of damaged tissues by secreting cytokines and growth factors as well (Kinnaird *et al.*, 2009).

After the first clinical trial with MSCs was carried out in 1995, a series of these clinical trials had been conducted to test the feasibility and efficiency of these stem cells. By 01/05/2014, 396 clinical trials using MSCs were set on for a wide range of therapeutic applications according to the public clinical trials database http://clinicaltrials.gov. The majority of these trials are in phase I for safety studies (70), phase II for proof of concept and efficiency in human patients (78), or a mixture of phase I/II studies (151). Only a small number of these trials are in phase III for comparison of a new treatment to the standard or best known treatment (20) or phase II/III (10).

Half of the 396 clinical studies that use bone marrow-derived MSCs since this was the first source to isolate and investigate MSCs and until now they are still the most well-studied MSCs. However, there is an increasing number of clinical trials that use adipose tissue-derived MSCs (81 clinical studies) due to the mentioned advantages (see in 4.1.2). Umbilical cord and cord blood-derived MSCs (70 clinical studies) are also applied in some clinical trials due to their simple isolation procedures.

The clinical studies focus on the treatment of various diseases such as heart diseases, cancer, liver diseases, diabetes, bone and cartilage diseases, and graft versus host disease (Horwitz *et al.*, 2002; Liu *et al.*, 2008; Hare *et al.*, 2009; Mizuno *et al.*, 2010) (see figure 50).

Figure 50. Clinical Trials of MSCs Classified by Disease Types.
The current trials (until 01/05/2014) contain 56 studies on heart diseases, 13 on cancer, 33 on liver's diseases, 21 on diabetes, 33 on graft versus host disease, 69 on bone and cartilage diseases, 20 for brain diseases and 15 on multiple sclerosis.

Interestingly, 56 studies focus on cardiovascular disease such as ischemia, myocardial infarction, and heart failure. For example MSCs significantly improved left ventricular function after intracoronary injection of autologous BM-MSCs if compared to the control standard saline in a pilot study (Chen *et al.*, 2004a). The left ventricular ejection fraction had increased significantly in patients who were pre-implanted with MSCs compared to those were implanted without cells after three months (Chen *et al.*, 2003b). The underlying repair mechanism triggered by MSC after transplantation remains unclear. Both beneficial effects of MSCs the differentiating into cardiovascular cells to replace damaged cells and paracrine signaling caused secretion of cytokines/growth factors stimulating local progenitor cells to repair the damaged tissue might be responsible. However, there can be no

doubt that mesenchymal stem cells will be an important tool in vascular regenerative medicine in the future.

4.3.2 Potential of P2 Receptor Ligands as Drug Molecules in Vascular Tissue Engineering

Several P2 artificial ligands have already been used as clinical drugs, such as Plavix (Clopidogrel), which is a P2Y12 antagonist and is the second top-selling drug worldwide (Topol *et al.*, 2011). Since previous data from our laboratory showed for the first time that the P2 receptor expression is altered on both, gene and protein level in differentiation of hMSC towards endothelial and smooth muscle cells, and more importantly, their direct functional role within was proven using their natural and artificial agonists and antagonists. It is reasonable to assume that P2 receptor ligands can be used to improve MSC differentiation towards these two cell lineages. Next to this, artificial P2 receptor ligands can also be used to improve the quality of vascular grafts such as bypass grafts (Zippel *et al.*, 2010). Dual control of endothelial cell differentiation and osteogenesis were already used to improve engineered vascular grafts for large bone defect in some pre-clinical studies (Polykandriotis *et al.*, 2007; Kanczler & Oreffo, 2008). An example for this is the study where AT-MSCs generated osteogenic constructs with intrinsic neovascularization after differentiating towards endothelial cells and osteoblastic progenitor cells (Scherberich *et al.*, 2007). The difficulties of creating re-cellularized graft are: to control stem cell differentiation towards desired cell lineages and to induce differentiated smooth muscle cell migration inside the graft (Schulze & Tobiasch, 2012). According to the clinical trials database http://clinicaltrials.gov, three clinical trials are in process where treat cardiovascular diseases are treated with P2 receptor ligands and stem cells:

i) Clopidogrel, a P2Y12 selective antagonist inhibiting platelet aggregation, is used together with aspirin to study whether it can improve the mobilization of

endothelial progenitor cells *via* angiogenesis which should result in sustained improvement of blood flow in patients with severe peripheral arterial disease.

ii) Clopidogrel is used to evaluate the safety and efficacy of an endothelial progenitor cell capture stent with one month of dual antiplatelet therapy.

iii) Clopidogrel is used to investigate if it can improve endothelial progenitor cell function and thus help to lower the level of harmful markers and simultaneously repair the damaged blood vessels in patients with coronary artery disease.

Until now, the outcome publications from the above mentioned three clinical trials are not available. However, since our data point out several specific P2 receptor subtype functions in endothelial and smooth muscle cell differentiation such as P2X7, P2Y1, and their artificial ligands e.g. A-740003, MRS2365, MRS2500 can be considered as prominent candidates to initiate new clinical studies.

4.4 Treatment of P2 Receptor Ligands with Cardiovascular Deconditioning

4.4.1 The Regulation of P2 Receptors in EC and SMC Co-Culture Model under Simulated Microgravity

Primary endothelial and smooth muscle cells were successfully isolated from bovine aorta and subsequently to simulate microgravity condition using a clinostat. We showed for the first time that several specific P2 receptor expressions were altered on gene and protein level after 24h under this condition as shown in figure 53. Culturing ECs with SMC-conditioned medium under normal gravity and *vice versa* can compensate the P2 receptor expression change of P2X7.

Similar to the data of Wang and colleagues (Wang *et al.*, 2002), our results showed that ECs and SMCs expressed different P2 receptors on the membrane. P2X4, P2Y1, P2Y2 and P2Y11 were predominantly expressed in ECs, while P2X1 and P2Y2 were strongly expressed in SMCs. Macrovascular and microvascular ECs have been shown the several functional differences such as matrix

metalloproteinase expression (Jackson & Nguyen, 1997) and beta-adrenergic regulation of transendothelial permeability (Zink *et al.*, 1995). The expression of P2X3 and P2Y4 is low in bovine aortic ECs if compared to the control HMEC-1 which suggests that macrovascular ECs might differ in the P2 receptor expression pattern compared to microvascular ECs.

The P2 receptor expression patterns of ECs and SMCs have already been shown to play an important role in various cardiovascular functions. For example in controlling vascular tone, ATP and UTP released from ECs act on P2Y1, P2Y2, and P2Y4 leading to the production of NO and subsequent vasodilatation. Simultaneously, ATP released by the sympathetic nerve acts on P2X1, P2X2, and P2X4, resulting in vasoconstriction (Burnstock *et al.*, 2010). We found that the expression of P2X2 and P2X4 in SMCs was significantly increased after clinorotation indicating to may be more vasoconstriction. Next to this P2Y1 and P2Y2 expressions were decreased, which suggests NO production might decrease and cause less vasodilatation. Kang and colleagues found that 72h exposure to clinorotation led to a decreased proliferation but increased the rate of apoptotic SMCs. Additionally, the SMC phenotype was induced and transferred from the contractive to the synthetic type (Kang *et al.*, 2013). Our data showed that the expression P2X7 and P2Y2 was altered differentially between ECs and SMCs under simulated microgravity, which indicates that they could be the key P2 receptor subtypes responding to the change of gravity. To point out, P2X7 has an important role in cell apoptosis and can activate a series of downstream signals due to several protein kinase binding sites on its long intracellular tail (Burnstock *et al.*, 2006b). A mechanical force such as shear stress can induce endothelial cell apoptosis that might be regulated through P2X7. It is of interest that P2X7, which was down-regulated in ECs, was up regulated in SMCs. This change could be compensated by adding conditioned medium from the other cell type. Such compensation was not found in the other P2 receptor subtypes which might point to P2X7 as a major player in the interaction between ECs and SMCs in simulated microgravity with respect to

apoptosis regulation.

4.4.2 Proliferation and Migration of ECs and SMCs in a Co-Culture Model under Simulated Microgravity

Various proteins of endothelial cells are altered under real or simulated microgravity, such as F-actin (Infanger *et al.*, 2007; Zhang *et al.*, 2010), tubulin (Infanger *et al.*, 2006), cell adhesion molecules (Grimm *et al.*, 2010; Zhang *et al.*, 2010), integrins (Infanger *et al.*, 2007), eNOS (Shi *et al.*, 2012), and iNOS (Siamwala *et al.*, 2010). In line with that ECs also showed a decreased proliferation rate, increased apoptosis (Infanger *et al.*, 2006; Kang *et al.*, 2011) and migration (Shi *et al.*, 2012) in simulated microgravity. However, these data were observed based on cultured ECs as single cell type, either under real or simulated microgravity. An EC and SMC co-culture model was successfully created *via* EC-conditioned medium culturing SMCs and *vice versa*. In addition to the paracrine effect on P2 receptor expression such as P2X7, our results showed that this effect found under simulated microgravity could influence EC or SMC behavior for cell proliferation and migration. SMC proliferation has been demonstrated to be a crucial process in atherosclerosis since migrated and proliferating SMCs form a major cell type in the plaque (Dzau *et al.*, 2002). In healthy vessels ECs secrete cytokines that inhibit SMC proliferation and form a monolayer to block small molecules from blood that might cause SMC proliferation. We found that conditioned medium collected from ECs in normal gravity inhibited SMC proliferation, but conditioned medium collected from ECs after clinorotation was not able to do so. On the other hand, EC damage or dysfunction is one of the first steps during the development of pathological change in atherosclerosis. The conditioned medium of SMC grown in simulated microgravity reduced EC proliferation. Enhanced apoptosis was observed when only ECs were cultured in simulated microgravity by Infanger and co-authors (Infanger *et al.*, 2006). They found several caspases such as caspase-3 and -9 activated after simulated

microgravity treatment. Apoptosis might be induced by activation of NF-κB *via* the PI3K/Akt pathway (Infanger *et al.*, 2006; Kang *et al.*, 2011). These data suggest that astronauts may be more prone to suffer from cardiovascular diseases such as atherosclerosis during space missions and the paracrine effects between ECs and SMCs might be the key factors in this process.

Migration of ECs is the first step in angiogenesis and a major factor in metastasis. In addition, it also plays an important role in restenosis in the vascular system after application of a stent. There are evidences that microgravity can promote angiogenesis in both, macrovascular and microvascular ECs when only ECs were cultured under simulated microgravity (Siamwala *et al.*, 2010, Shi *et al.*, 2012). Our data showed an enhanced number of migrated ECs when cultured with SMC-conditioned medium derived after clinostat application, compared to the DMEM control and SMC-conditioned medium collected after 24h exposure to normal gravity. This indicates the effect of microgravity might enhance the angiogenesis *via* both autocrine and paracrine signals.

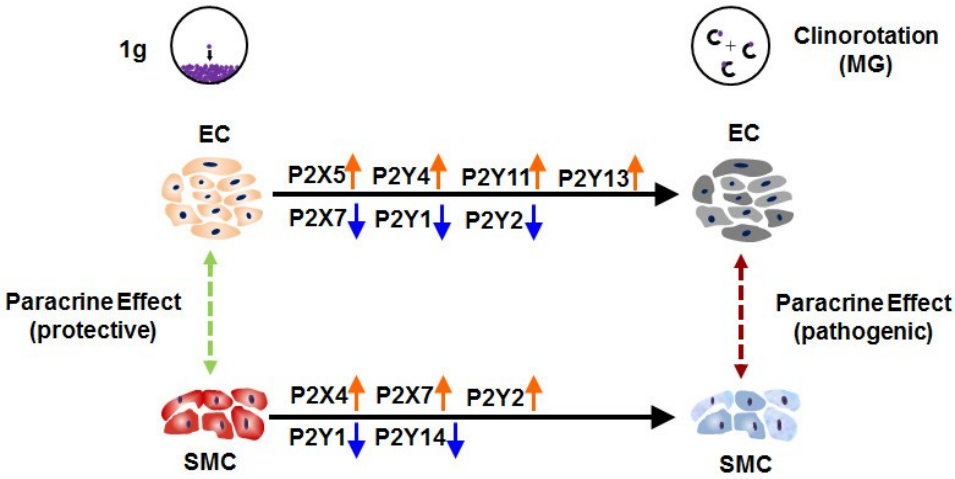


Figure 51. Scheme of P2 Receptor Alteration and the Postulated Paracrine Effect in ECs and SMCs under Simulated Microgravity.

Several P2 receptor expressions were altered in ECs and SMCs under 24h simulated microgravity condition by a clinostat. To point out that the expression P2X7 and P2Y2 was altered differentially between ECs and SMCs under simulated microgravity. Especially, the change of P2X7 in ECs was compensated under SMC-conditioned medium and *vice versa*. The conditioned medium collected under simulated microgravity showed the pathogenic influence of EC and SMC proliferation and migration if compared to condition medium from normal gravity.

Beside the paracrine effect, the cell-cell contact, the reaction to extracellular matrix and 3D environment is also important for vascular function. For example EC migration is inhibited in an *ex vivo* injured artery than in an *in vitro* cell culture and SMC proliferated more slowly in the EC reendothelialization areas than in the EC denuded areas (Lee *et al*., 2010). Here we create the 3D model using artificial and natural scaffolds. The artificial collagen matrix showed the ability for both bovine and human differentiated cell growth. However, the matrix is small (a diameter of 5 mm) and not vessel shape. Thus synthesizing matrix with a vein shape is necessary for further investigation. The natural scaffolds are developed using decellularizing bovine artery and human varicose vein. Both source showed successfully removing of native cells, the structure of artery and vessel such as collagen and elastin fibers. However, the bovine artery is more promising considering the human varicose vein is a disease vessel. The ECs and SMCs will be seeded on and in this decellularized scaffold to build a tissue co-culture model. This model will be subsequently applied into clinostat to investigate the functional changes in ECs and SMCs.

4.4.3 Artificial P2 Receptor Ligands as Potential Drugs for Cardiovascular Deconditioning Therapy

ATP was recognized as a cotransmitter in both, the peripheral and central nervous system and now additional data showed that both purines and pyrimidines were also powerful extracellular messengers to non-neuronal cells, including vascular cells such as endothelial and smooth muscle cells (Burnstock & Knight, 2004).

P2X1, P2X2, P2X3, P2X4, P2Y1, P2Y2, P2Y4, P2Y11 are dominantly expressed in endothelial cells and P2X1, P2X2, P2X4, P2Y1, P2Y2 and P2Y6 are mainly found in smooth muscle cells (Wang *et al*., 2002; Burnstock & Ralevic, 2013). These receptors have also been demonstrated to regulate various vascular functions such as vascular tone, short-term hypertension; vascular remolding, atherosclerosis, and vascular pain as long term effects (Seye *et al*., 2006; Burnstock & Ralevic, 2013).

Compounds involved in purinergic signaling such as receptor agonists and antagonists, ectoenzyme enhancers and inhibitors, and purine transport enhancers and inhibitors have drawn an increasing interest in their therapeutic potential in relation to both, P1 and P2 receptors of the above mentioned cardiovascular disease conditions. A series of these compounds have been patented and various clinical trials have been carried out. According to the clinical trials database http://clinicaltrials.gov by 01/05/2014, 824 clinical trials were set on for a wide range of therapeutic applications. 578 of them focused on P2 receptors. Nearly 70% of these studies based on P2 receptor are carried out to improve the treatment of a wide range of cardiovascular diseases (see figure 52).

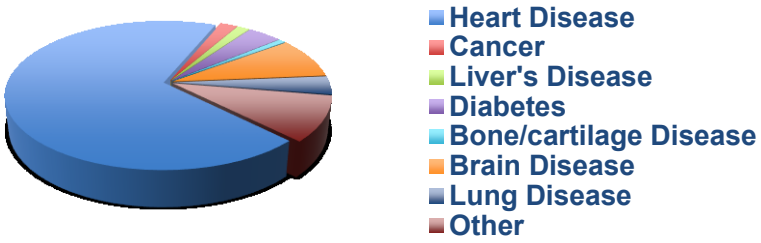


Figure 52. Clinical Trials Based on P2 Receptors Ordered by Disease Types. These clinical trials contain 397 studies for heart diseases, 12 for cancer, 9 for liver's disease, 24 for diabetes, 4 for bone and cartilage disease, 51 for brain disease, and 23 for lung disease.

The role of P2 receptors in these cardiovascular clinical trials can be separated by the disease of which they should improve the outcome such as thrombosis, atherosclerosis, restenosis, heart failure, and hypertension.

i) Thrombosis

Purinergic antithrombotic drugs are the most successful and prominent developments. Several clinical trials have clearly demonstrated that the purinergic antithrombotic drugs clopidogrel and ticlopidine efficiently reduce heart attack and stroke risk, especially when combined with the administration of aspirin (Kam & Nethery, 2003; Kunapuli *et al.*, 2003; Boeynaems *et al.*, 2005). These drugs are

antagonists to the P2Y12 receptor which is mediating platelet aggregation (Gachet, 2001). MRS2500, a highly potent and selective antagonist for the P2Y1 subtype, was also shown to inhibit human platelet aggregation (Cattaneo *et al.*, 2004).

ii) Atherosclerosis and restenosis

Some clinical trials already showed that clopidogrel and ticlopidine treatment of patients with atherosclerosis was significantly better if compared to aspirin. Next to this, in coronary bypass surgery internal mammary and radial arteries as well as saphenous vein have been used as grafts. The P2Y2 subtype expression level of endothelial cells from all three vessels is comparable, but P2X4 subtype expression varies from low in the two arteries to remarkably higher in the saphenous vein. In line with this, saphenous vein grafts showed higher susceptibility to atherosclerosis compared to internal mammary arteries after revascularization surgery (Ray *et al.*, 2002). On the other hand, P2X1 and P2Y6 subtypes regulated more significantly contractions in the saphenous vein compared to the internal mammary artery, which suggests specific antagonists to these two receptors might be used to prevent vasospasm and restenosis in the saphenous vein in and after surgery (Borna *et al.*, 2003). The long-term role of purinergic signaling in vascular endothelial and smooth muscle cell proliferation and death during atherosclerosis and restenosis and will lead to the exploration of new therapeutic strategies (Hou *et al.*, 2002).

iii) Heart failure

Magnesium-ATP has been recommended and used to treat patients with ischemia, shock, sepsis, and radiation injury for many years (Nalos *et al.*, 2003). Injection of ATP inhibits atrioventricular conduction rather than the firing rate of sinoatrial nodes, thus it is only used in those surgeries that require a transient cardiac standstill, for example, in endovascular grafting surgery (Watanabe *et al.*, 2002). On the other hand, 2-deoxy-ATP rather than ATP showed better substantial enhancement of the mechanical performance within the cardiac muscle, which indicates that a ribonucleotide reductase might be a target molecule for the treatment of heart failure (Regnier *et al.*, 2000).

iv) Hypertension

ATP-$MgCl_2$ is widely used for treatment of pulmonary hypertension after cardiac surgery and it is also administered as an effective, safe, and preferential pulmonary vasodilator to children with pulmonary hypertension secondary to congenital heart defects (Brook *et al.*, 1994). Next to this in the hemodialysis treatment of patients with end-stage renal disease, ATP was used as a prognostic indicator in stress myocardial perfusion (Hase *et al.*, 2004).

The outcome of these clinical trials based on using P2 receptor ligands might be also used to treat astronauts suffering from cardiovascular deconditioning and the subsequent cardiovascular diseases. However, before dong so it is important to evaluate their functional efficiency and side effect under microgravity condition such as in space missions. According to the clinical trials database http://clinicaltrials.gov by 01/05/2014, eight clinical trials have been performed to look for effective countermeasures for astronauts to fight against the pathogenic effects of microgravity. Four of them are carried out investigating different countermeasures for cardiovascular deconditioning. Bed rest with “6-degree head-down tilt” models are often used to mimic the blood distribution in space and thus to study the mechanism of cardiovascular deconditioning (Guell, 1995; Watenpaugh, 2001). The outcome of these studies was that exercise such as the flywheel device failed to improve the characteristic manifestations of cardiovascular deconditioning (Belin de Chantemele *et al.*, 2004). However no drug application was used in these trials. As mentioned above, our data show an altered expression pattern of P2 receptors under simulated microgravity, and drugs based on some of these P2 receptor subtypes have been used in a wide range of cardiovascular diseases already. Therefore, it is reasonable to consider drugs based on various potential compounds of P2 receptor signaling as promising candidates for the treatment of cardiovascular deconditioning in astronauts in the future.

Contradictory observations have been demonstrated in several publications, e.g. EC migration increased in simulated microgravity both, in this study and in the study

of Siamvala and colleagues (Siamwala *et al.*, 2010) while Versari and colleagues found a decreased EC migration under simulated microgravity (Versari *et al.*, 2007). One explanation for these findings might be that different endothelial cells were used such as primary endothelial cells from an artery or umbilical vein, or an endothelial cell line (EAhy926), which might give a different response due to its immortalization and thus prolonged time in culture. Another explanation could be that different devices were applied to simulate microgravity conditions such as the clinostat, the RPM, and the RWV. Different equipment might produce different qualities of microgravity as well as a different amount and quality of shear stress during rotation (Herranz *et al.*, 2013). Clinorotation was shown to produce the lowest shear forces and the central area used in our study has an optimized simulated microgravity environment (Eiermann *et al.*, 2013). Furthermore, different ECs from different body parts were used. Macrovascular and microvascular ECs already revealed a difference in promoting angiogenesis under real/simulated microgravity conditions, which is regulated *via* the iNOS-cGMP-PKG pathway in macrovascular ECs but *via* eNOS-PI3K-Akt in microvascular ECs (Siamwala *et al.*, 2010; Shi *et al.*, 2012). Taken together, the simulated micogravity data independently from which ground-based facility we use have to be approved and verified in real microgravity for a final statement on the outcome.

4.5 Future Work and Perspectives

Stem cells have been widely used to generate differentiated cells from various tissues, such human mensenchymal stem cells used to differentiate towards endothelial and smooth muscle cells. Here, we show that natural and artificial ligands of P2 receptors directly influence MSC differentiation towards endothelial and smooth muscle cells. However, not all interesting P2 receptors such as P2X6, P2Y4, have highly potent and selective agonists and antagonists being already synthesized. In the future synthetic artificial P2 receptor ligands should be used to

confirm the specific P2 receptor subtype function during the differentiation process which could not be investigated here. In addition, ECs and SMCs differentiated from hMSCs should be characterized further to define their types, such as are they similar to macro- or micro-vascular endothelial cells? Arterial or vein or type H endothelial cells? Do the differentiated SMCs act as proliferative or contractive phenotype?

Due to the lack of access to human vessel material, bovine ECs and SMCs were used to find interesting P2 receptor candidates. However, these data should be confirmed with human original ECs and SMCs. The human varicose veins were also used to isolate ECs, SMCs and create decellularized scaffolds, but this material is disease vessel and thus not a perfect source. Our data showed for the first time that P2 receptor gene and protein expression in both, ECs and SMCs were altered after the change of gravity influence. However, the functional role of P2 receptors during this procedure requires further investigation with specific artificial ligands. SMC-conditioned medium collected under simulated microgravity influenced some P2 receptor expressions as well as proliferation and migration of ECs and *vice versa*. This suggests that the extracellular environment such as paracrine signals is an important factor and cannot be ignored considering the impact of microgravity on vascular cells. Thus, co-culture tissue models with and without scaffolds should be used to improve the understanding of a 3D environment leading to cardiovascular deconditioning under microgravity.

Although ECs and SMCs differentiated from hMSCs showed a similar growth behavior as the bovine, however, further experiments are required to test for other parameters such as cell apoptosis, proliferation rate, toxicity and biocompatibility to judge the bovine model for usability. Data suggest have shown that cells especially stem cells express morphology and function differently in 2D as compared to 3D culture (Lund *et al.*, 2009). Therefore it is necessary to study the P2 receptor ligand function during the development of cardiovascular deconditioning as well as during the MSC differentiation process on and in artificial and natural scaffolds also to investigate autocrine signaling. Finally, animal experiments with P2 receptor ligands

should be performed to prove their function in a whole organism where next to paracrine signals also autocrine signals play a role.

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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.

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Original Paper

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Zhang Y, Patrick L, Pansky A, Kassack M, Hemmersbach R, Tobiasch E. The Influence of Simulated Microgravity on Purinergic Signaling in Endothelial and Smooth Muscle Cells. ***BioMedical International Research***, 2014 (submitted)

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Book Chapter

Zhang Y and Tobiasch E. The Role of Purinergic Receptors in Stem Cells and in Their Derived Consecutive Tissues. P. Di Nardo (Ed.), ***Adult Stem Cell Standardization, River Publishers***, 2011, 73-98. ISBN: 978-87-92329-74-5

Reviews

Zhang Y, Khan D, Delling J, Tobiasch E. Mechanisms Underlying the Osteo- and Adipo-Differentiation of Human Mesenchymal Stem Cells. ***TSWJ***. 2012, doi:10.1100/2012/793823.

Zhang Y, Chen Sang, Fengyuan Zhuang. Advanced Researches on Cell Adhesion Molecules of Immune System under Microgravity. ***Space Medicine & Medical Engineering***, 2009, 22(3): 231-234.

Zhang Y, Sang C, Zhuang FY. Advanced Researches of Cell Adhesion Molecules in Endothelial Cells. ***Chin. J. Cell Mol. Immunol.***, 2009, 25(1): 89-91.

Invited Talks

Zhang Y. p53 Acts as a Major Regulator in Human Mesenchymal Stem Cell Differentiation towards Various Cell Lineages. University of Palermo, Palermo, Italy, October 15, 2013.

Oral Presentations

Kaebisch C, Zippel N, **Zhang Y**, Limbach C, Winter M, Schulze M, Tobiasch E. Are Dental Follicle Cells Better Than Mesenchymal Stem Cells for Future Bone Regeneration? Deutscher Zahnärztetag, Symposium Transdisziplinärer Arbeitskreis Regenerative Medizin, Frankfurt, Germany, November 9th, 2013. (presented by Kaebisch)

Zhang Y, Zippel N, Schulze M, Tobiasch E. P2 receptors Influence the Commitment of Human Mesenchymal Stem Cells Towards Several Distinct Lineages Interesting for Approaches in Regenerative Medicine. The World Conference on Biological and Biomedical Science, Dar es Salaam, Tanzania, September 23rd-27th, 2013. (presented by Tobiasch)

Zhang Y, Gu C, Güneri D, Khan D, Pansky A, Tobiasch E. Is p53 signaling playing an Important Role During Mesenchymal Stem Cell Differentiation towards Multiple Cell Lineages? 3rd International Satellite Symposium AICC-GISM Mesenchymal stem cells: growth factors and cytokines. Palermo, Italy, November 21st -23rd, 2012.

Zhang Y, Lange M, Khan D, Pansky A, Tobiasch E. Establishment of a Blood Vessel Cell Culture Model with MSCs for Microgravity. Internal Retreat of the Stem Cell Network North Rhine-Westphalia. Herne, Germany, May 04th - 05th, 2012.

Zhang Y, Lange M, Khan D, Giorgi E, Pansky A, Tobiasch E. Investigation of Vascular Functional Changes in Microgravity. Summer Academic Salon, Symposium of German and Chinese exchange of Life Science Technology, Essen, Germany, June 25th, 2011.

Zhang Y, Sang C, Zhuang FY. PKC and Cell Cytoskeleton in Human Umbilical Vein Endothelial Cells under Simulated Microgravity**.** 61st International Astronautical Congress (IAC), Prague International Center, Prague, Czech Republic, September 27th - October 1st, 2010.

Zhang Y, Sang C, Han GJ, Ma X, Zhuang FY. Expression of ICAM-1 and VCAM-1 in Human Umbilical Vein Endothelial Cells under Simulated Microgravity. 60st International Astronautical Congress (IAC), Deajeon, Republic of Korea, October 12th - 16th, 2009.

Poster

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.

Kaebisch C, **Zhang Y**, Schulze M, Tobiasch E. Biomaterial Scaffolds Functionalized

with P2 receptor Ligands Used for Future Bone Tissue Engineering. Zing Conference: Nanomaterials. Playa del Carmen, Mexico, November 13th-17th, 2013.

Zhang Y, John T, Liu K, Pansky A, Tobiasch E. p53 Acts As A Major Regulator In Human Mesenchymal Stem Cell Differentiation Towards Various Cell Lineages. The World Conference on Regenerative Medicine. Leipzig, Germany, October 23rd-25th, 2013.

Güneri D, **Zhang Y**, Kaebisch C, Pansky A, Tobiasch E. Is the Ecto-5'-Nucleotidase CD73 a Suitable Mesenchymal Stem Cell Marker? Molecular life Sciences 2013: International Symposium of the German Society for Biochemistry and Molecular Biology. Frankfurt, Germany, October 3rd-6th, 2013.

Klose J, **Zhang Y**, Babczyk P, Conzendorf C, Tobiasch E, Harre K. Modified Cryoprocessed Collagen Scaffolds for Future Use of Mesenchymal Stem Cells Differentiated Towards Endothelial Cells. IIR Workshop on Cryoprocessing of Biopharmaceutical and Biomaterials. Dresden, September 4th-5th, 2013.

Zhang Y, Pansky A, Kassack M, Tobiasch E. IAA Humans in Space Symposium 2013. Cologne, July 7rd-12th, 2013.

Zhang Y, Pansky A, Schulze M, Tobiasch E. P2 receptor Signaling Controls Human Mesenchymal Stem Cell Differentiation towards Vascular Cell Lineages. 7th International Meeting of the Stem Cell Network NRW. Cologne, Germany, April 23rd - 24th, 2013. (Poster Prize)

Zhang Y, Gu C, Limbach C, Khan D, Pansky A, Tobiasch E. The MAPK Signal Pathway is Critical when Mesenchymal Stem Cells Differentiate into Distinct Cell Lineages. 3rd International Satellite Symposium AICC-GISM Mesenchymal stem cells: growth factors and cytokines. Palermo, Italy, November 21st - 23rd, 2012.

Zhang Y, Dilaware Khan, Martin Lange, Andreas Pansky, Edda Tobiasch. P2 receptors Influence Human Adipose Tissue Derived Mesenchymal Stem Cells Differentiation towards Endothelial and Smooth Muscle Cells. Frontiers in Molecular Biology: Epigenetics in Development and Disease (SIBBM 2012). Palermo, Italy, May 24th - 26th, 2012.

Khan D, **Zhang Y**, Barman S, Pansky A, Brand-Salieri B, Tobiasch E. Mesenchymal Stem Cells Isolated from Different Genders and Body Regions Show Differences in Plasticity Linked to the Regulation of Hox Genes Frontiers in Molecular Biology: . Epigenetics in Development and Disease (SIBBM 2012). Palermo, Italy, May 24th - 26th, 2012.

Zhang Y, Grossmann C, Müller T, Lange M, Khan D, Pansky A, Schulze M, Harre K, Tobiasch E. Models and Methods for Atherosclerosis Treatment. Neue Methoden der Polymercharakterisierung, Rheinbach, Germany, November 17th, 2011.

Delling J, Khan D, Lange M, Giorgi E, **Zhang Y**, Pansky A, Tobiasch E. A2b

Receptor as a Marker for a Selectable Mesenchymal Stem Cell Character Specific for Different Body Regions. 21st Annual Conference of German Society for Cytometry, Bonn, Germany, October 13th - 15th, 2011.

Zhang Y, Sang C, Zhuang FY. Cell Cytoskeleton and Adhesion Molecules in Human Endothelial Cells are Sensitive to Gravity. Annul German and Chinese exchange of Life Science Technology Meeting, Bonn, Germany, September 24th - 25th, 2011

University Thesis

Bachelor thesis: “Cytoskeleton Recombination in Microgravity”. 2007, Beihang University, Beijing, China

Master thesis: “The Impact of Simulated Microgravity in Endothelial Cell Adhesion Molecule and Signal Pathways”. 2010, Beihang University, Beijing, China

Other talks:

Zhang Y. Build Models to Investigate Cardiovascular Deconditioning in Microgravity. Sciences Slam in Tag der Foschung, Hochschule Bonn-Rhein-Sieg. St Augustin, Germany, December 5th, 2013.

Zhang Y. Establishment of a Blood Vessel Cell Culture Model, Testing its Applicability in Microgravity and Examination of the Underlying Signaling Pathways. Helmholtz Space Life Autumn School. October 22nd - 26th, 2012.

Zhang Y, Pansky A, Tobiasch E. Establishment of a Blood Vessel Cell Culture Model, Testing its Applicability in Microgravity and Examination of the Underlying Signaling Pathways. Hauskolloquium des Fachbereichs Angewandte Naturwissenschaften. Bonn-Rhein-Sieg University of Applied Sciences, Rheinbach, Germany, December 29th, 2011.

Zhang Y. How to Be an Astronaut and Live in Space. 93rd Lecture of Rheinforum. Bonn, Germany, October 6th, 2011.

Zhang Y. Establishment of a Blood Vessel Cell Culture Model, Testing its Applicability in Microgravity and Examination of the Underlying Signaling Pathways. 2nd Helmholtz Space Life Workshop. Kiel, Germany, March 14th - 18th, 2011