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Hypoxia and Trypsin enhance the Proangiogenic Properties of human Adipose-derived Stem Cells

Master's Thesis

Simone E. Riis

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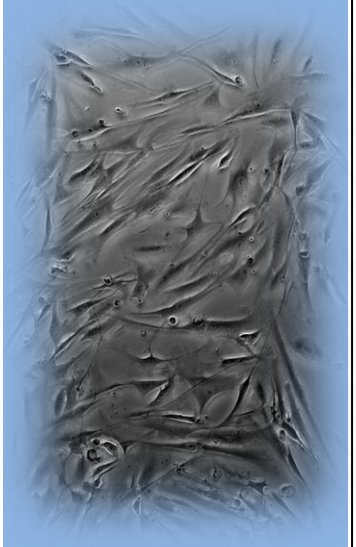

MEDICINE WITH INDUSTRIAL SPECIALIZATION

Title Page

Hypoxia and trypsin enhance the proangiogenic properties of human adipose-derived stem cells

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Preface

This master's thesis was conducted in collaboration with the Laboratory of Stem Cell Research at Aalborg University from September 2011 to June 2012.

I have earlier worked with Adipose-derived Stem Cells in the project "Effect of Hypoxia and Trypsin on the Wound Healing Properties of Adipose-derived Stem Cells". The knowledge and inspiration gained from that project have been utilized for the construction of this master's thesis.

The purpose of this thesis was to investigate research questions which were left open by a former PhD student, Jeppe Rasmussen, in the Laboratory of Stem Cell Research.

I would like to thank Helle Skjødt Møller, Laboratory technician, for laboratory assistance during the compilation of this thesis.

Abstract

Background aims. Adipose-derived stem cells (ASCs) are seen as a promising candidate for the treatment of diabetic foot ulcers, and it was hypothesized that treating ASCs with hypoxia and trypsin would increase the pro-angiogenic effect of these cells.

Methods. The possible increased pro-angiogenic effect was investigated using ASCs treated with hypoxia, trypsin, and a combination of these. The pro-angiogenic effect of ASCs was measured in two ways. First, the expression and secretion of VEGF by the ASCs was assessed. The expression of vascular endothelial growth factor (VEGF) was determined by real-time RT-PCR normalized to the reference genes PPIA and YWHAZ. Moreover, the secretion of VEGF was assessed by ELISA. Second, the influence of ASCs on endothelial cell migration was investigated.

Results. The hypoxic treatment of the ASCs resulted in a significant increased expression of VEGF ($P = 0.000$) compared to untreated ASCs, as did the trypsin treatment ($P = 0.001$). The combinational treatment of hypoxia and trypsin resulted in a significant higher up regulation of the relative expression of VEGF ($P = 0.000$), indicating an additive effect. The secretion of VEGF was both after hypoxic treatment and after trypsin treatment significantly increased ($P = 0.000$, $P = 0.005$). The combination of hypoxic and trypsin treatment increased the secretion even further, which was significantly higher than both control, hypoxic and trypsin treated cells ($P = 0.000$, $P = 0.002$, and $P = 0.000$, respectively). No results were obtained from the endothelial migration assay.

Conclusion. In conclusion, the combination of hypoxic and trypsin treatment of ASCs increased the expression and secretion of VEGF in these in an additive manner. These findings indicate the combinational treatment of hypoxia and trypsin as a potentiating preconditioning of ASCs to be used for clinical application. In the area of endothelial migration the influence of ASCs has yet to be determined.

Abbreviations

ASC	Adipose-derived stem cells
CM	Conditioned media
CO2	Carbon dioxide
D	Days
EC	Endothelial cell
ELISA	Enzyme-linked immunosorbent assay
EPC	Endothelial progenitor cell
ERK 1/2	Extracellular-signal-regulated kinases 1/2
H	Hours
HIF-1	Hypoxia-inducible factor 1
HDMEC	Human dermal microvascular endothelial cells
MAPK	Mitogen-activated protein kinase
O2	Oxygen
PAR	Protease-activated receptor
PBS	Phosphate-buffered saline
PI3K	Phosphatidylinositol 3-kinases
PPIA	Cyclophilin A
Q-PCR	Real-time reverse transcriptase polymerase chain reaction
SEM	Standard error of the mean
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor
YWHAZ	Tyrosine 3/tryptophan 5-monooxygenase activation protein

1 Background

1.1 Diabetes Mellitus and Chronic Foot Ulcers

Diabetes mellitus is an increasing international health burden. The prevalence of diabetes mellitus is during the next 20 years expected to increase with more than 50 % to a total of 439 million incidences worldwide. This is likely due to demographic changes in ageing, obesity, and a sedentary lifestyle becoming more common and also due to better health care, improving the lifespan of people with diabetes mellitus (Shaw et al., 2010). One of the serious consequences of diabetes mellitus is diabetic foot ulcers which in worst case can lead to leg amputations, increasing the 5-year mortality rate from 39 % to 69 %. Effective treatment of diabetic foot ulcers can decrease the number of major amputations and thereby decrease the mortality rate (Ulicna et al., 2010), making such treatment very desirable.

1.1.1 Diabetic Foot Ulcers

Diabetic foot ulcers affect 15 % of people with diabetes mellitus. These wounds are a major morbidity associated with diabetes mellitus often leading to pain, suffering, and poor quality of life for the patients (Brem and Tomic-Canic, 2007). The developmental pathway towards ulceration is multifactorial, but a critical triad of neuropathy, minor foot trauma, and foot deformity is thought to be responsible for more than 50 % of diabetic foot ulcers making these together with fungal infection, abnormal pressure points, arthritis, peripheral vascular disease, and insulin usage serious risk factors for diabetic foot ulcers (Leung, 2007). The treatment of diabetic foot ulcers depends on the diagnostic assessment, as they are very heterogeneous depending on the underlying predominant abnormality. The treatment is therefore often a combination of several treatments. Of possible treatments to be mentioned are pressure relief, surgical corrections, local wound management inclusive debridement, revascularization, and reconstructive methods, and control of infections. However, there is only very weak to moderate evidence of the effectiveness of these treatments (Sundhedsstyrelsen, 2011).
To understand the complexity of diabetic foot ulcers and the treatment it is important to have insight into normal wound healing.

1.1.2 Wound Healing

When the skin is wounded the barrier function and mechanical integrity of the skin are compromised. To restore this, a complex but well-orchestrated process of events will normally occur (Schultz et al., 2011). This process will now be elucidated on a cellular level followed by the diversities seen in diabetic foot ulcers. An overview of the central elements in normal wound healing is shown in Figure 1B.

Normal Wound Healing

Wound healing can theoretically be divided into three phases; the inflammatory, the proliferative and the maturation phase. However, one must be aware of the simplification of this and that the phases overlap and their time of duration can vary.

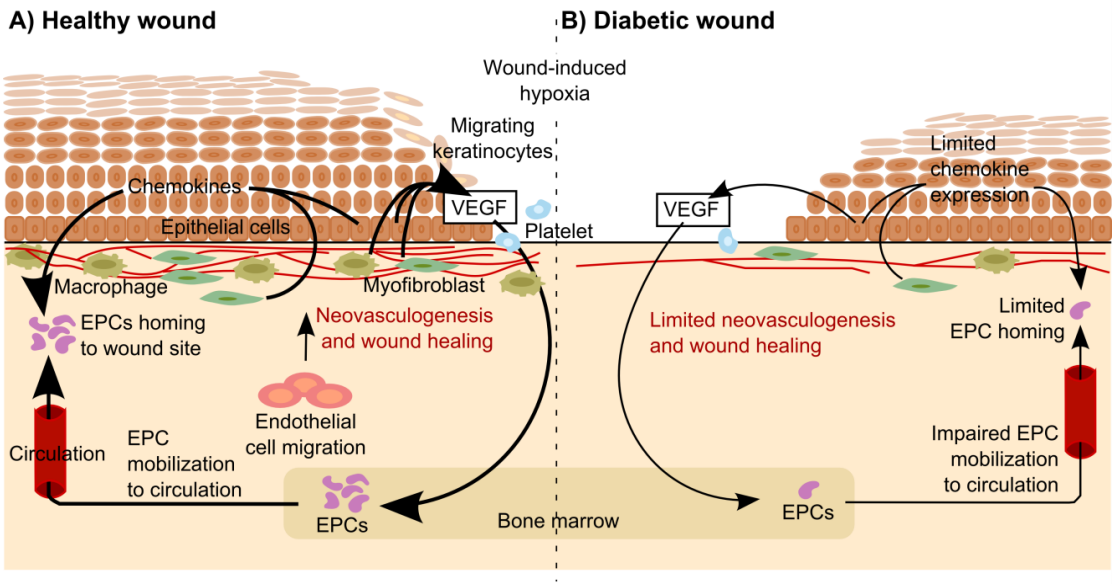

Figure 1. Overview of the mechanisms in normal wound healing versus diabetic wounds. In healthy persons (left) the process of wound healing is controlled through release of chemochines from the kerationocytes, fibroblasts, epithelial cells, macrophages, and platelets. In response to hypoxia in the wound, VEGF is secreted by macrophages, fibroblasts, and epithelial cells which results in mobilization of endothelial progenitor cells (EPCs) from the bone marrow to the circulation. Chemokines promote the homing of the EPCs to the site of injury, where they participate in neovasculogenesis. In patients with diabetes mellitus (right) EPC mobilization and homing is impaired due to decreased chemochine and VEGF secretion. This result in limited wound healing EPC, endothelial precursor cell; VEGF, vascular endothelial growth factor. Inspired by (Brem and Tomic-Canic, 2007).

The Inflammatory Phase

The inflammatory phase initiates the wound healing and includes hemostasis and inflammation. Wound healing starts with hemostasis where vasoconstriction occur and a blood clot of fibrin is formed, to minimize blood loss. Vascular endothelial growth factor (VEGF) binds among other growth factors to fibrin in the cloth and stimulates angiogenesis. Neutrophils migrate to the wounded area and recruit circulating monocytes. Once in the tissue the monocytes mature into macrophages. The neutrophils and macrophages together remove cellular debris, foreign particles, and bacteria. Additionally macrophages release factors as VEGF, which attracts more monocytes to the wound and stimulate angiogenesis (Baum and Arpey, 2005).

The Proliferative Phase

During the proliferative phase the granulation tissue is formed. The granulation tissue is rich in invading capillaries formed by angiogenesis, the process by which damaged blood vessels are replaced by sprouts from intact capillaries in the local area of the wound (For further details see section 1.2.1). One of the most critical growth factors involved in angiogenesis is VEGF. Around two days after wounding, endothelial cells (EC) and endothelial progenitor cells (EPC) begin to migrate into the wound stimulated by growth factors as VEGF. For this migration to occur, the presence of an extra cellular matrix (ECM) and no neighboring ECs are needed. The ECM is secreted by fibroblasts which have migrated to the wound and proliferated (Baum and Arpey, 2005). Additionally reepithelialization of the wound occurs as basal epithelial cells divide and keratinocytes begin to migrate as sheets from the wound edge (Schultz et al., 2011).

The Maturation Phase

In the maturation phase the capillaries are obliterated and the wound becomes firm and relatively avascular, turning into highly collagenized scar tissue. The wound is closed partly due to the differentiation of some of the residing fibroblasts into myofibroblasts, which are able to provide traction forces. The strength of the scar tissue is enhanced over time by the cross binding of collagen fibers (Fenger et al., 2005).

Diabetic Foot Ulcers

The complete and efficient process of wound healing is critical for the general well being of any patient, as it otherwise renders these patients vulnerable to a number of pathologic conditions such as infection and fluid loss (Baum and Arpey, 2005). In the light of this, diabetic foot ulcers are critical, as they often are neither complete nor efficient in healing.

Clinical and experimental evidence suggest that diabetic ulcers do not follow an orderly and reliable progression of wound healing as parts of the chronic wound may be stuck in different phases, having lost the ideal synchrony of events normally leading to rapid healing (Loot et al., 2002). As shown by Sharma *et al.* diabetic wounds exhibit delayed wound closure, prolonged inflammation, poor angiogenesis, and less matrix deposition in the wound bed when compared to normal wound healing (Sharma et al., 2006). It is believed that more than 100 physiologic factors interacting in multiple complex pathophysiological mechanisms contribute to these wound healing deficiencies in individuals with diabetes mellitus as for example decreased amount of chemokines and growth factors (Brem and Tomic-Canic, 2007). This may cause or be caused by a dysregulation of many cellular functions as impaired endothelial and epithelial function (Falanga, 2005, Guo and Dipietro, 2010). See Figure 1B for a schematic overview of wound healing in diabetic persons.

In diabetic foot ulcers, angiogenesis is impaired possible due to impaired EPC mobilization and homing, and decreased levels of VEGF (Felmeden and Lip, 2005). The VEGF gene was by Sharma *et al.* found to be significantly less expressed in diabetic wounds in comparison to normal wounds (Sharma et al., 2006) (Figure 1). This results in diabetic foot ulcers always being hypoxic, which prolong the healing process (Guo and Dipietro, 2010). Sharma and colleagues also found lack of neo-vascularization at day four of diabetic wounds, where in comparison a significant vessel formation took place in a normal wound. At day seven, they found a few newly formed vessels in the diabetic wounds but these were disorganized. In the normal wound there was an extensive and organized vascularization in dermis with the presence of mature vessels. At the 11^{th} day of wound healing extensive vascularization were still present in the diabetic wounds, where it in the acute wound were reduced as expected in a normal wound healing process (Sharma et al., 2006).

1.2 Angiogenesis and VEGF

As angiogenesis is a fundamental element in the process of wound healing and of special interest when considering treatment of diabetic foot ulcers, this will now be further elucidated.

1.2.1 Angiogenesis

Angiogenesis is the formation of new blood vessels from a preexisting vascular network (Goodwin, 2007). Wound-healing angiogenesis involves multiple steps, including vasodilatation, basement membrane degradation, EC migration, and EC proliferation. The angiogenic sprouting

is controlled by the balance between pro-angiogenic and anti-angiogenic factors. When conditions are favorable for angiogenesis, as in normal wound healing, some ECs sprout, while their neighboring ECs are suppressed and thereby stay in place. Sprouting requires flipping of apical-basal polarity of the sprouting ECs, modulation of cell-cell contacts without complete disruption of cell-cell junctions and vessel-wall integrity, and local matrix and subendothelial basement membrane degradation (Adams and Alitalo, 2007). Matrix degradation can further facilitate angiogenesis by releasing matrix bound growth factors (Goodwin, 2007)(Figure 2A).

The induction of motile and invasive activity of the sprouting ECs by activating secreted or cell-surface-anchored proteases is the next step in a successful process of angiogenesis. The growing EC sprout is guided especially by a VEGF165 gradient in the ECM consistent with the prominent expression of vascular endothelial growth factor receptor-2 (VEGFR2) in these cells. EC proliferation is supported by the soluble VEGF121 and extension of the sprout involves migration and proliferation of ECs behind the tip (Adams and Alitalo, 2007) (Figure 2B).

To form new vascular connections tip cells need to form strong adhesive interactions and EC-EC junctional contacts at the joining point with their targets, tips of other sprouts or existing capillaries. The establishment of blood flow requires the formation of a lumen in the sprout. The lumen is formed by intracellular and subsequently intercellular fusion of large vacuoles. Fusion processes at the EC-EC interfaces and establishes a continuous lumen (Adams and Alitalo, 2007)(Figure 2C).

EC-EC junctions need to be maintained after lumen formation to prevent excessive leakage. Blood flow in the newly formed lumen

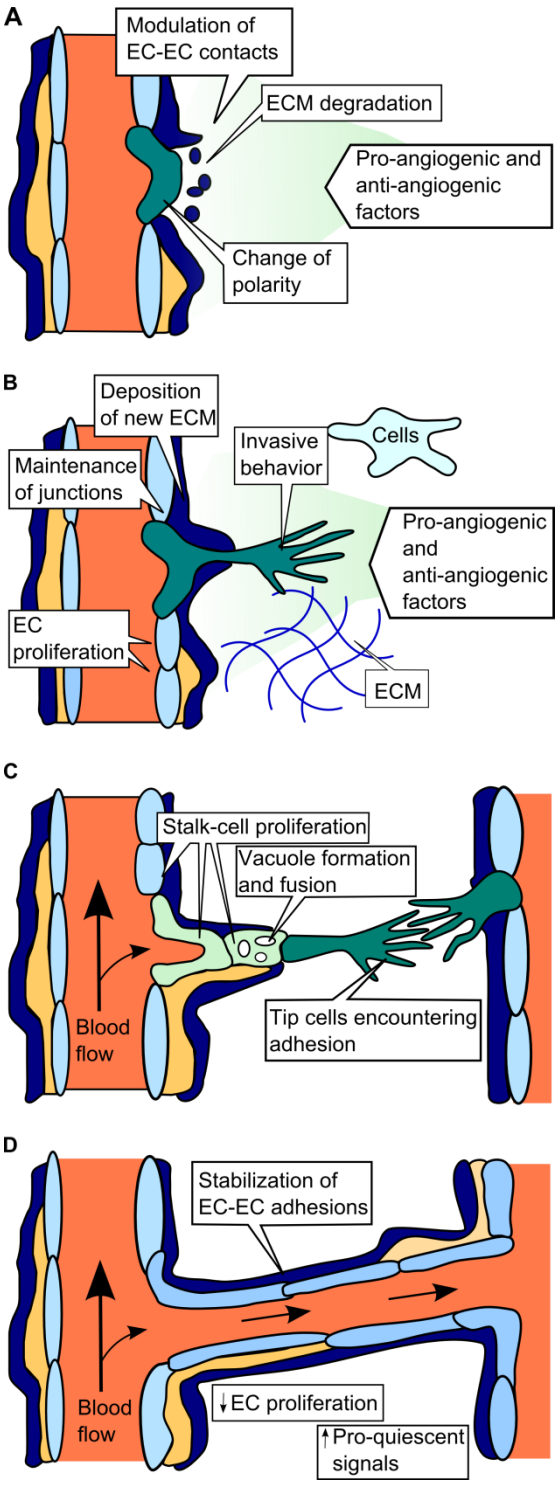

Figure 2. Angiogenesis. A) Selection of sprouting ECs and degradation of ECM. B) Sprout outgrowth and guidance. C) Sprout fusion and lumen formation. D) Perfusion and maturation of newly formed vessel. EC, endothelial cell; ECM, extra cellular matrix. Inspired by (Adams and Alitalo, 2007)

improves the oxygen delivery and thereby reduces the pro-angiogenic signals, as VEGF, which are induced by hypoxia. The blood flow also promotes the maturation processes such as stabilization of cell junctions, matrix depositions, and tight EC attachment (Adams and Alitalo, 2007)(Figure 2D).

1.2.2 Vascular Endothelial Growth Factor

As earlier mentioned the growth factor VEGF is crucial for proper angiogenesis and thereby also for complete wound healing.

As shown in Figure 3, VEGF is a family of six members, VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E, and placenta growth factor (PIGF) and they exert their biological functions by binding to three different transmembrane tyrosine kinase receptors, VEGFR-1, VEGFR-2, and VEGFR-3 (Werner and Grose, 2003). The gene for VEGF-A, often just referred to as VEGF, has been shown to be strongly induced after cutaneous injury (Werner and Grose, 2003), which is in agreement with recent studies, which have shown that VEGF-A possess a particularly important role in wound healing as addition of neutralizing VEGF-A antibodies resulted in a prominent reduction in wound angiogenesis (Howdieshell et al., 2001). At a cellular level, VEGF-A function as an EC mitogen, chemotactic agent and inducer of vascular permeability (Bao et al., 2009).

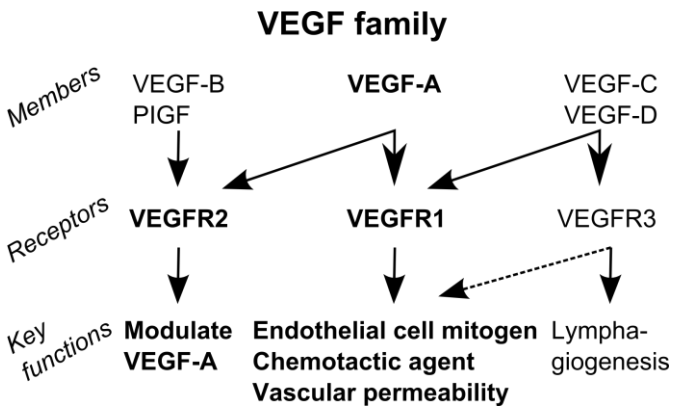

Figure 3. The vascular endothelial growth factor (VEGF) family. The family consists of five members; VEGF-A, -B, -C, -D, and PIGF. The members bind to three receptors; vascular endothelial growth factor receptor-1, -2, and -3 (VEGFR1, 2, and 3). The binding to the receptors result in endothelial cell growth, migration and vascular permeability, modulation of VEGF-A, and lymphagiogenesis, respectively.

VEGF-A have five isoforms with chain lengths of 121, 145, 165, 189, and 206 amino acids. As the chain length increases, the VEGF isoforms change from a weakly acidic to a basic form. This enhances the ability to bind to heparin in the ECM, whereby the bioavailability is decreased. $VEGF_{165}$ is the most studied and available isoform of VEGF (Bao et al., 2009).

Additionally the receptors for VEGF-A, VEGFR-1 and VEGFR-2, have been detected on the endothelial surface of developing and mature blood vessels and specifically on blood vessels of granulation tissue (Werner and Grose, 2003, Bao et al., 2009). VEGFR-1 is required for organization of blood vessels, maybe responsible for the mediation of vascular permeability and induction of anti-apoptotic proteins. VEGFR-2 has been shown to be important for EC differentiation, membrane ruffling, chemotaxis, proliferation, and is thereby thought to mediate the mitogenic and chemotactic activities of VEGF. The expression pattern indicates that VEGF-A stimulates wound healing in a paracrine manner (Werner and Grose, 2003), and further induces VEGF expression in a positive feedback loop (Bao et al., 2009).

VEGF-A plays a role in several of the angiogenic processes of wound healing. It has the ability to induce vasodilatation and vascular permeability through VEGFR-2, increasing the infiltration of cells as neutophils and monocytes from the circulation to the wounded area. VEGF-A induces a

balance of enzymatic promoters and inhibitors setting the stage of basement membrane degradation, destruction of extracellular matrix and thereby facilitates endothelial migration into the extravascular space. Additionally, VEGF-A stimulates EC migration through chemotaxis, as it enhances the expression of $\alpha_v\beta_5$ integrin, and induces osteopontin which is chemotactic for ECs. It also stimulates migration through increment of the vascular permeability, as the leakage of fibrinogen turning to fibrin gel in the extracellular space stimulates EC migration. Summarized, VEGF is a mitogen selectively for ECs, it delays senescence, and prevents apoptosis of these cells, all favorable for angiogenesis and wound healing (Bao et al., 2009). For an overview of the cellular functions of VEGF see Figure 4.

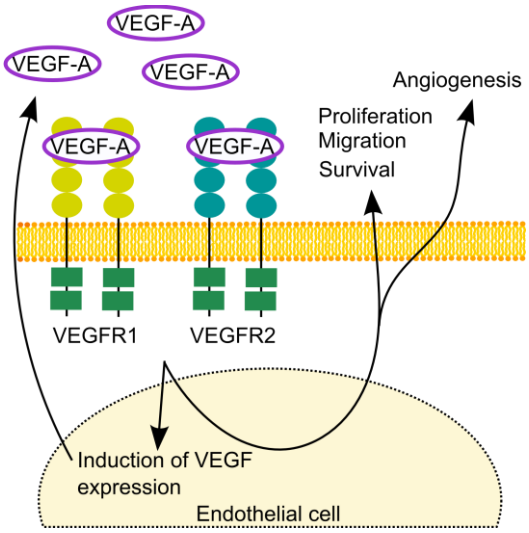

Figure 4. Endothelial cell response to vascular endothelial growth factor-A (VEGF-A). VEGF-A binds to one of its receptors, vascular endothelial growth factor receptor 1 or 2 (VEGFR1 or VEGFR2), which both by intracellular pathways leads to proliferation, migration and survival of the endothelial cell. Additionally, the binding of VEGF-A to its receptors promote angiogenesis. Furthermore, VEGF-A induces its own expression through a positive feedback

As diabetic foot ulcers have severe problems with correct and complete healing, and the evidence of the effectiveness of the current treatments is very weak, new approaches for treatment of these wounds are desirable. Purified growth factors and cultured cells have been approved by the Food and Drug Administration to accelerate the closure of non-healing wounds (Bao et al., 2009). However, the administration of single angiogenic growth factors to increase neovascularization in patients has only shown modest success, indicating the complex process of wound healing to require multiple growth factors (Henry et al., 2003). This enable the possibility of using adipose-derived stem cells (ASCs) as a potential treatment for non-healing wounds as diabetic foot ulcers, as these have been shown favorable for this purpose.

1.3 Adipose-derived Stem Cells and Wound Healing

Adipose-derived stem cells (ASCs) are mesenchymal stem cells isolated from adipose tissue. Stem cells in general are unspecialized cells characterized by their ability to selfrenew by mitosis, their long-term viability, and the ability to differentiate into one or several mature cell types, which renders them suitable for regenerative applications (Gimble et al., 2007). The majority of studies investigating the use of stem cells in skin repair and wound healing have focused on the effect of bone marrow-derived mesenchymal stem cells (Kim et al., 2009, Wu et al., 2007). However, alternative sources have been investigated due to the clinical limitations of bone marrow biopsies. This led to the discovery of related mesenchymal stem cells in adipose tissue (Zuk et al., 2002). The ASCs are a promising candidate for therapeutic applications due to several advantages (Cherubino et al., 2011). The ASCs can relatively easily be obtained by minimally invasive liposuction from adults and have high capacity for *in vitro* expansion (Kim et al., 2009). Furthermore, they possess multilineage developmental potential as they *in vitro* have been ob-

served to be able to differentiate into chondrocytes, osteocytes, myocytes, ECs, and neurons (Zuk et al., 2002, Planat-Benard et al., 2004).

ASCs are located in the perivascular space in subcutaneous adipose tissue, where they seem to function in the repair of injured tissue and in interaction with other cells according to the stimuli they receive (Lee et al., 2009). However, the mechanism of action by which ASCs accelerate skin regeneration is not well characterized. In general, stem cells have been assumed to home to injured tissues and organs, where they repair by differentiating into tissue specific cells (Chung et al., 2009). However, the theory of repopulation appears unlikely because the survival of engrafted stem cells is too low to be therapeutically relevant and no studies report ASCs to differentiate into neither keratinocytes nor dermal fibroblasts, required for proper and complete wound closing (Song et al., 2010). Therefore, it has been suggested, that ASCs, instead of replacing the damaged tissue by differentiation, modulates the surrounding cells and the ECM to facilitate the regenerative effect through secretion of soluble factors (Song et al., 2010). Supporting this, ASCs have been confirmed to secrete several growth factors including VEGF, platelet-derived growth factor, fibroblast growth factor, insulin-like growth factor, hepatocyte growth factor, and transforming growth factor β (Rehman et al., 2004, Kim et al., 2007). Hence, the observed wound healing effect can partially be explained by a complex paracrine mechanism. Supporting this theory, cell-free conditioned medium (CM) from ASCs have been seen to stimulate both growth and survival of human ECs (Rehman et al., 2004, Li et al., 2006).

The angiogenic properties of ASCs have been elucidated in several studies. It has been demonstrated that ASCs can differentiate directly into cells with endothelial phenotype (Planat-Benard et al., 2004), and in an *in vitro* study in our lab a rat aortic ring was cultured with ASCs CM resulting in enhanced endothelial sprouting, supporting the notion of ASCs having a pro-angiogenic effect (Rasmussen et al., 2011).

1.3.1 ASCs and Hypoxia

Damage of dermis involves inflammation and oxidative stress resulting in local tissue ischemia (Lee et al., 2009). This is a key feature of chronic wounds as diabetic foot ulcers. Therefore, it is interesting to investigate how ASCs are affected by hypoxia and in which way this influences the wound healing process. In general, the cellular response of oxygen deficit depends widely on the cell type and environmental conditions (Lee et al., 2009). *In vivo* cells are exposed to various oxygen tensions in different tissues (Zachar et al., 2011). In highly perfused tissues, such as lung, liver, kidney, and heart tissue, the oxygen levels range between 4 and 14 %. In the adipose tissues, ASCs are located in the perivascular space and here the oxygen tensions are less than 3 %. Although ASCs are exposed to these relatively low oxygen concentrations *in vivo*, they are commonly cultured *in vitro* at approximately 20 % O_2, corresponding to atmospheric oxygen tension. When cultured under hypoxic conditions ASCs response with enhanced survival, proliferation, adhesion, and migration and inhibited adipogenesis (Chung et al., 2009). The optimal *in vitro* oxygen tension for ASCs regarding growth and multipotency has been found to be 1-5 % (Lee et al., 2009, Zachar et al., 2011, Rasmussen et al., 2011).

The major response to hypoxia is mediated through activation of hypoxia-inducible factor (HIF) transcription complex as shown in Figure 5. HIF is a heterodimeric compound composed of an oxygen degradable α-subunit and a continuously synthesized oxygen-stable β-subunit. There are three isoforms of HIF-α (HIF-1α, HIF-2α, and HIF-3α) and three of HIF-β (HIF-1β, HIF-2β, and HIF-3β) (Zagorska and Dulak, 2004). HIF-1α is the most investigated in regard to angiogenesis. HIF-

1α, is constantly expressed, but during normoxic conditions enzyme-mediated hydroxylation leads to degradation by a proteasome complex. During hypoxic conditions no such hydroxylation takes place and HIF-1α are not degraded. This leads to an increased level of stabilized HIF-1α in the cytoplasm and translocation to the nucleus, where the HIF-1α dimerize with HIF-β subunits, forming a complex functioning as a transcription factor for more than 60 target genes (Brahimi-Horn and Pouyssegur, 2009). These target genes includes VEGF-A, as shown by Rasmussen et al., as long-term culturing of ASCs under hypoxic conditions increased the expression and secretion of VEGF (Rasmussen et al., 2011). Through transcription promoted by HIF, cellular functions of metabolism, angiogenesis, and sensing of oxygen are regulated (Zachar et al., 2011).

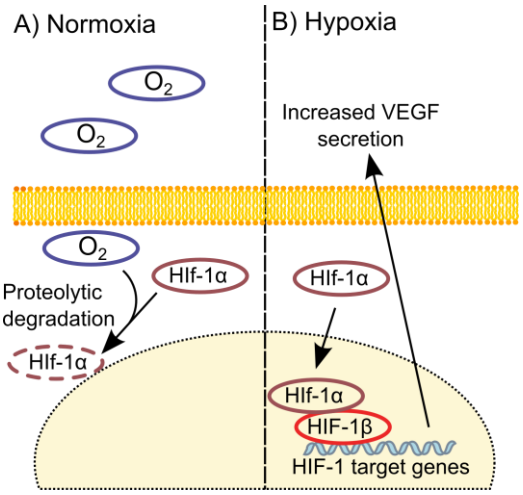

Figure 5. Transcriptional regulation by HIF-1. A) Under normoxic conditions, HIF-1α is, in the presence of oxygen (O_2), degraded by protosomal degradation. B) Under hypoxic conditions, the absence of O_2 prevents the HIF-1α from degradation, resulting in accumulation and translocation to the nucleus where it dimerizes with HIF-1β. The formed complex then binds to the hypoxia response element of hypoxia responsive genes as the vascular endothelial growth factor (VEGF) gene, resulting in gene transcription.

Cellular responses of ASCs during hypoxia involve enhancement of proliferation and inhibition of adipogenesis through the HIF-1α stabilization (Chung et al., 2009). A study conducted by Lee and colleagues investigated the wound healing functions of ASCs during hypoxia (Lee et al., 2009). The study showed that culturing ASCs under hypoxic conditions led to elevated proliferation and survival of the stem cells. Furthermore, the ability of ASCs to migrate toward ischemic tissues was enhanced. A study by Rehman *et al.* also showed that ASCs cultured under hypoxic conditions both expressed and secreted significantly more VEGF and had a significantly enhancing effect on EC survival and proliferation. All these observations suggest that hypoxic culture conditions are advantageous for ASCs regarding paracrine wound healing effects (Rehman et al., 2004).

1.3.2 VEGF and Trypsin

Like hypoxic conditions can stimulate the expression of VEGF and the wound healing effects of ASCs, other conditions may have similar effects. It has been demonstrated that trypsin increases the expression of growth factors as VEGF through activation of the G-protein coupled protease-activated receptor (PAR), see Figure 6 (Zhu et al., 2006, Liu and Mueller, 2006). The proteolytic enzyme trypsin is usually used in the laboratory for detachment of cells, due to the ability to cleave integrins (Brown et al., 2007). PAR2 is a membrane-bound receptor activated via cleavage of an extracellular domain, resulting in a tethered ligand, which then activates the receptor. The activation of the receptor propagates through mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinases (PI3K) pathways leading to increased expression of VEGF (Zhu et al., 2006, Dutra-Oliveira et al., 2012). PAR2 is expressed on several cell types in the skin including keratinocytes, fibroblasts during wound healing, ECs, and cells of the immune system, and the receptor appears to play a role in epidermal barrier construction, endothelial proliferation, in-

flammation, and skin pigmentation (Rattenholl and Steinhoff, 2008, Liu and Mueller, 2006). Activation of PAR2 is believed to be involved in angiogenesis, as it in several studies has been found to increase neo-angiogenesis and the blockage of it, by genetic deletion or inhibitors, abolished the neoangionetic effect. (Zhu et al., 2006, Milia et al., 2002, Uusitalo-Jarvinen et al., 2007) Furthermore, the presence of the receptor (data not published) and the up-regulation of VEGF expression in response to trypsin has now been confirmed for ASCs (Rasmussen et al., 2011).

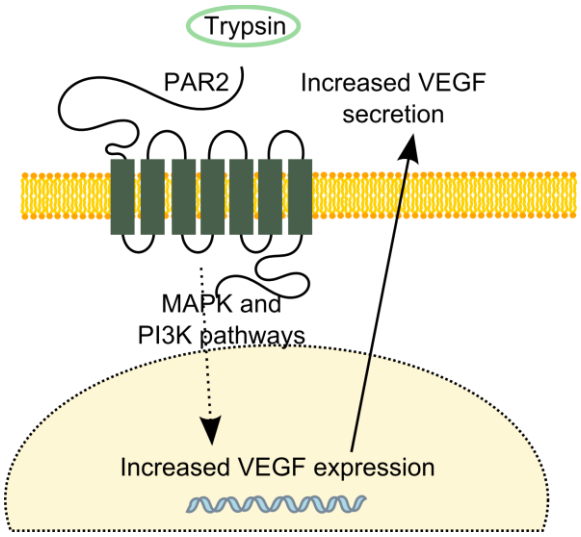

Figure 6. VEGF expression activated by trypsin. Trypsin activates the protease-activated receptor 2 (PAR2) by cleavage of an extracellular domain, leaving a tethered ligand, which then activates the receptor. This activation then results in the increased expression and secretion of vascular endothelial growth factor (VEGF). This effect is possibly through mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinases (PI3K) pathways

2 Aims and Hypotheses

As ASCs have been shown to have wound healing properties combined with their practical advantages they are of special interest for this thesis. This combined with the fact that hypoxia enhances the wound healing properties of ASCs in regard to the expression and secretion of VEGF and that trypsin have been shown to have the same effect, could maybe even further increase the wound healing effect of ASCs. Based on this, the thesis had two major aims:

1. To examine the proangiogenic properties of human adipose-derived stem cells in relation to short-term hypoxic culture and trypsin.

2. To examine the influence of human adipose-derived stem cells on endothelial cells in relation to wound healing.

The hypotheses were:

1. Short-term hypoxia and trypsin will separately have an increasing effect on VEGF expression and secretion, and in combination they will have an additive effect. This will be mediated through HIF-1α stabilization.

2. Human adipose-derived stem cells will have an activating effect on endothelial cells and hypoxia and trypsin will further increase this.

To address the study aims and test the hypotheses two studies were conducted:

1. Human adipose-derived stem cells from three healthy donors were cultured at normoxia and hypoxia, and treated with trypsin, and the expression and secretion of VEGF were measured. Additionally, the HIF-1 activation was investigated.

2. Human microvascular endothelial cells were co-cultured with pre-treated human adipose-derived stem cells in a migration assay and the tendency to migrate was measured and compared.

3 Materials & Methods

3.1 Cell Cultures

3.1.1 Human Adipose-derived Stem Cells

Human adipose derived stem cells (ASCs), line ASC-12, ASC-21, and ASC-23, were previously isolated in our laboratory from lipoaspirate obtained from Grymer Priveta Hospital, Skejby, Denmark (Pilgaard et al., 2008). In brief, the lipoaspirate was harvested from subcutaneous adipose tissue by elective liposuctions approved by the regional committee on biomedical research ethics of Northern Jutland, Denmark. Patients (age 42, 58, and 52 years) had given written informed consent and were healthy and not taking any medication. ASCs were cultured in α-Minimum Essential Medium w. GlutaMAX™ (Invitrogen™ Cat. no. 32561) with 10 % fetal calf serum (Invitrogen™ cat. no. 10106-169), 100 U/mL penicillin, 0.1 mg/mL streptomycin (Invitrogen™, Cat. no. 15140), and 0.05 mg/mL gentamicin (Invitrogen™, Cat. no. 15710-072). This medium is for future use referred to as ASC medium. For all experiments, if not otherwise stated, ASCs were seeded at a density of 8.000 cells/cm^2 in 6-well plates (Corning® Costar ®, Cat. no. 3516) and experiments were conducted on biological replicates of the cell lines ASC-12, -21, and -23.

3.1.2 Human Dermal Microvascular Endothelial Cells

Adult Human Dermal Microvascular Endothelial Cells (HDMECs) were chosen for endothelial experiments as dermal microvascular endothelium is known to have a crucial role in wound healing and therefore these cells are commonly used as an *in vitro* model of wound healing (Talavera-Adame et al., 2011). HDMECs were purchased from PromoCell (Germany), and cultured in Endothalial Cell Growth Medium MV2 (PromoCell, Cat. no. c-22121).

3.1.3 General Conditions

When passaging cells, a mixture of 0.125 % trypsin (Invitrogen™, Cat. no. 15090-046) and 0.01 % ethylene diamine tetra acetic acid (EDTA, VWR & Bie & Berntsen, Cat. no. 1.08418.0250) was used.

3.1.4 Standard Incubation

All cells were propagated in a classical laboratory setup involving incubation in a Steri-Cycle CO_2 standard incubator at 37 °C in an atmosphere containing 5 % carbon dioxide (CO_2) and 20 % oxygen (O_2). These incubation conditions were also applicable when using the standard incubator for experiments. When processing the cells, they were directly transferred to a laminar air flow (LAF) bench with atmospheric air and room temperature. (Figure 7)

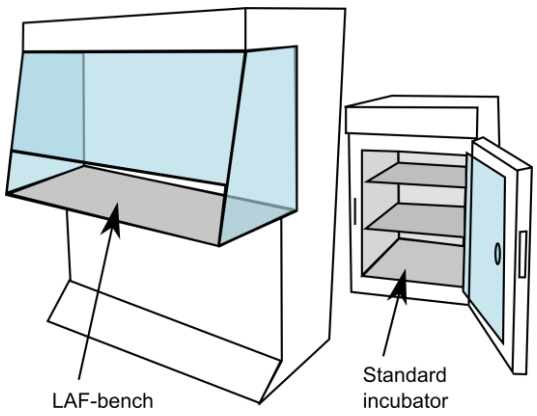

Figure 7. Laminar air flow (LAF) bench and Steri-Cycle CO_2 standard incubator. In the LAF bench the air was atmospheric and the temperature corresponding to room temperature. In the Steri-Cycle CO_2 standard incubator the air contained 5 % carbon dioxide and 21 % oxygen, and the temperature was 37 °C.

3.1.5 The BioSpherix Glove Box

To expose the ASCs to short-term hypoxia a BioSpherix glove box (Xvivo, BioSpherix, Redfield, NY, USA) was used. (Figure 8)

The BioSpherix glove box is created with a work area, a buffer chamber, and several separate incubators. In the work area of the BioSpherix glove box the oxygen tension is kept at 5 % regulated with nitrogen and 5 % CO_2 and the temperature is constantly 37 °C due to a heating element in the tabletop. The buffer chamber functions to ensure a stable oxygen tension in the work area when transferring equipment from the outside atmospheric air into the glove box. The time it takes the buffer chamber to establish an oxygen tension of 5 %, corresponding to the oxygen tension in the work area, is around ten minutes. Therefore the transit time when bringing equipment in to the station is ten minutes. The BioSpherix glove box has as mentioned integrated incubator boxes, in which the oxygen tension can be regulated individually of each other and of the work area. The oxygen tension is regulated with nitrogen and there is no pure oxygen inlet, only an atmospheric air inlet. The main function of this glove box is that it has integrated incubators where the oxygen tension individually can be very well controlled and that there is a direct access from the incubators to a work area where cultures may be manipulated.

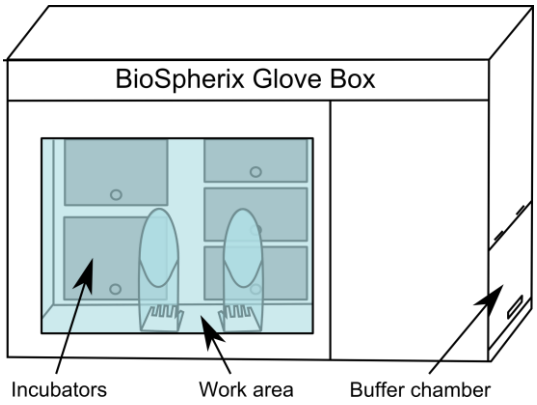

Figure 8. BioSpherix glove box with integrated incubators and a heated O_2 stable work area. The buffer chamber ensures equalized oxygen tensions before transfer of equipment to the work area.

3.1.6 Verification of Incubator Systems

To verify the comparability of the two incubator systems in regard to VEGF expression in ASCs, ASCs were seeded and incubated in the standard incubator for three days before half of the cells were transferred to an incubator in the BioSpherix glove box set to 20 % O_2 and 37 °C in an atmosphere containing 5 % CO_2. The other half was kept in the standard incubator. For both systems the cells were incubated for another 24 h before analysis of the VEGF expression. This was done as described in section 3.4.

3.2 Hypoxic- and Trypsin Treatment

3.2.1 Hypoxic Treatment

ASCs were for all experiments with hypoxic treatment incubated at 37 °C in an atmosphere containing 5 % CO_2 balanced with nitrogen to reach an oxygen concentration of 1 % in an incubator in the BioSpherix glove box. They were incubated at these hypoxic conditions for 24 h. Medium and PBS added to these cells were always allowed to equalize for a minimum of 3 h to reach 1 % O_2 before addition.

3.2.2 Trypsin Treatment

Trypsin treatment was first investigated according to the time dependent effect, then optimized, and finally, the actual trypsin treatment procedure was established.

Time Dependent Effect

To investigate the time dependent effect of trypsin on the expression of VEGF in ASCs, the cells were incubated for 3 days in the standard incubator. Then, one group was lysed and the rest of the cells washed with s-PBS and exposed to a mixture of 0.125 % trypsin in s-PBS at room temperature until the cells drew together and became round of appearance. Then, ASC medium was added to inactive the trypsin and the cells were incubated for additional 1 h. After this, medium was changed to fresh ASC medium and the incubation continued. Cells were lysed after both 6, 12, 18, 24, 36, and 48 h of incubation after trypsin treatment. Expression of VEGF was analyzed by real time RT-PCR as described in section 3.4. One replicate of each cell line was analyzed (n = 3).

Optimization

Three similar optimization experiments were conducted; the only difference was within the trypsin treatment procedure. Applicable for all three experiments was, that ASC-21 was seeded and incubated for 3 days in the standard incubator before half the cells were transferred to an incubator in the BioSpherix glove box with an atmosphere of 20 % O_2, 5 % CO_2, and 37 °C. The ASCs grown in the BioSpherix glove box and in the standard incubator were both treated with a mixture of 0.125 % trypsin in s-PBS either 6 or 12 h prior to analysis. The cells were allowed to reattach for 1 h before medium was change to fresh ASC medium. Afterwards, the cells were incubated in the respective incubator systems for a total of 24 h after which the VEGF expression was analyzed by real time RT-PCR as described in section 3.4.

The cells incubated in the BioSpherix glove box were all processed in the work area of the glove box (Figure 8). To visualize the cells, a microscope (SteREO Lumar.V12, Carl Zeiss International) inside the BioSpherix glove box was used. The cells in the standard incubator were processed in a LAF-bench (Holten LaminAir, Holm & Halby A/S) and a microscope (Olympus CK40 Culture Microscope) next to the bench was used to visualization of the cells (Figure 7). The experiment was conducted on biological replicates (n =2).

Objectively Assessment

First part of this experiment was the same as described above. The trypsin treatment took place outside the incubators under the respective microscopes and was completed when the cells drew together and became round of appearance. Hereafter, trypsin was inactivated using ASC medium.

Fixed Time

First part of this experiment was the same as described above. The trypsin treatment lasted for 7 min under the respective microscopes and was subsequently inactivated with ASC medium.

Total trypsinization

First part of this experiment was the same as described above. The trypsin treatment took place under the respective microscopes and lasted until the cells detached from the culture surface and was then inactivated with ASC medium.

The Trypsin Treatment Procedure

Based on the optimization experiments, the final trypsin treatment was designed as follows: For trypsin treatment all ASCs, regardless of incubation system, were transferred to the work area of

the BioSpherix glove box and washed with equalized s-PBS. Then a mixture of 0.125 % ice cold trypsin in s-PBS was added for 4 min before the trypsin was inactivated with equalized ASC medium. The cells were allowed to reattach for 1 h in their respective incubator systems before the medium was changed to equalized fresh ASC medium in the work area of the BioSpherix glove box (Figure 9).

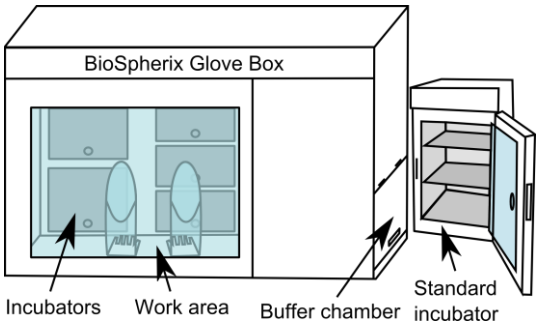

Figure 9. Final trypsin treatment procedure. All cells treated with trypsin were transferred to the work area of the BioSpherix glove box for manipulation, relocated to their respective incubators for incubation and reattachment of cells and then again transferred to the work area of the BioSpherix glove box for media change. Hereafter, they were incubated in their respective incubators again.

3.3 Combinational Treatment

To test the best combination of the hypoxic treatment and the trypsin treatment, a pilot study was conducted investigating the effect of trypsin treatment at different time points when combined with hypoxic treatment.

3.3.1 Response Time of Trypsin Treatment

Pilot Study

To determine the time point of trypsin treatment giving the maximum VEGF expression, ASCs were seeded and incubated in the standard incubator. After three days of incubation, medium was change and the cells were transferred to an incubator in the BioSpherix glove box to receive hypoxic treatment. At the time points corresponding to 6, 12, and 18 h before the 24 h of hypoxic treatment was ended, a group of ASCs was trypsin treated. One group was left untreated representing pure hypoxic treatment. After 24 h of hypoxic treatment the expression of VEGF was analyzed for all cells as described in section 3.4.

Main Experiment

To find the optimal time point for trypsin treatment, during the period of hypoxic treatment, giving the maximum VEGF expression, ASCs were seeded in duplicates and incubated in the standard incubator. After three days of incubation medium was change and one group was kept in the standard incubator representing the untreated control. The other cells were transferred to an incubator in the BioSpherix glove box to receive hypoxic treatment. At the time points corresponding to 2, 4, 6, 12, and 18 h before ending of the 24 h of hypoxic treatment a group of ASCs was trypsin treated. One group was left untreated representing pure hypoxic treatment. After 24 h of hypoxic treatment the expression of VEGF was analyzed by real time RT-PCR for all cells as described in section 3.4.

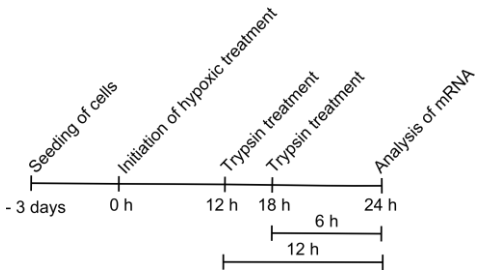

Figure 10. Timeline for the investigation of an additive effect of the combinational treatment of hypoxia and trypsin on the expression of VEGF. The timeline is not drawn to scale.

3.3.2 Investigation of an Additive Effect

To investigate the presence of an additive effect when using both hypoxic and trypsin treatment, ASCs were seeded and incubated for three days in the standard incubator. Then, half the cells were transferred to an incubator in the BioSpherix glove box to receive hypoxic treatment and the other half were incubated in the standard incubator. At time points corresponding to 6 or 12 h before termination of the 24 h of hypoxic treatment, some cells from each incubator system were trypsin treated and the others kept as controls for hypoxic treatment and standard culturing conditions respectively. After termination of the hypoxic treatment, all cells were lysed and expression of VEGF analyzed by real time RT-PCR as described in section 3.4. For a timeline over the experiment see Figure 10.

3.4 Expression of VEGF

The expression of VEGF was used to assess the effect of the different treatments on the pro-angiogenic properties of ASCs.

3.4.1 RNA Extraction

ASCs from a confluent 6-well plate were lysed and total RNA extracted using an Aurum™ Total RNA Mini Kit (Bio-Rad, cat. no. 732-6820) according to manufacturer's instructions using the spin protocol. The concentration and purity of the RNA in each sample were determined by spectrophotometry (Nanodrop; Thermo Science, Wilmington, DE, USA). RNA was stored at – 80 °C.

3.4.2 cDNA Synthesis

cDNA were produced with an iScript cDNA Synthesis Kit (Bio Rad, Cat no. 170-8891) using 210-900 ng RNA. The cDNA synthesis was performed in a thermocycler (PERKIN ELMER GeneAmp PCR system 2400) at 25 °C for 5 min then 42 °C for 30 min, and finally 85 °C for 5 min to terminate the reaction. cDNA was stored at – 80 °C.

3.4.3 Quantitative real-time RT-PCR

The amplification reactions were performed on a MyiQ™ Single-color Real-Time PCR Detective System using iQ™5 Optical System Software (Bio-Rad) in a final volume of 25 µL containing 5 pmol of each of the two primers, 0.25 µL cDNA and iQ SYBR Green Supermix (Bio-Rad, Cat. no. 170-8880). Thermal cycling were initiated with denaturation at 95 °C for 5 min. Amplification were performed by 50 cycles of denaturation at 95 °C for 10 sec and primer annealing and elongation at 61 °C for 30 sec. After amplification, a melt curve was created to test the specificity of the product. A standard curve from pooled aliquots of cDNA from each sample, diluted in a four-fold serial dilution, was used to calculate the relative expression of each gene. All real-time RT-PCR reactions were performed in technical replicates.

Primers

To analyze the effect of trypsin and hypoxia on the expression of VEGF, primers for hVEGF (DNA Technology A/S) was used (Table 1). The expression of VEGF was, if not otherwise stated, normalized to the geometric mean of the expression of the two reference genes cyclophilin A (PPIA) and 3-/tryptophan 5-monooxygenase activation protein (YWHAZ) (DNA Technology A/S). Researchers in the Laboratory of Stem Cell Research have previously shown that these reference genes are expressed relatively stable in human ASCs during hypoxic culture (Fink et al., 2008).

Table 1 Primer sequences for primers used in quantitative real-time RT-PCR analysis.

	Forward Primer Sequence	Reverse Primer Sequence
hVEGF	5'- CGA TTC AAG TGG GGA ATG G -3'	5'- CAT TGA TCC GGG TTT TAT CC-3'
PPIA	5'- TCC TGG CAT CTT GTC CAT G -3'	5'- CCA TCC AAC CAC TCA GTC TTG -3'
YWHAZ	5'- ACT TTT GGT ACA TTG TGG CTT CAA -3'	5'- CCG CCA GGA CAA ACC AGT AT -3'

3.5 Secreted VEGF

3.5.1 Best Time to Harvest

To indentify the most optimal time point to harvest medium for analysis of the amount of VEGF secreted by the ASCs into the medium, ASCs were seeded in duplicates and incubated in the standard incubator for three days. After this, the cells were transferred to an incubator in the BioSpherix glove box to receive hypoxic treatment. 6 h before the hypoxic treatment was completed the cells were treated with trypsin in the work area of the BioSpherix glove box as described in section 3.2.2. After the 24 h of hypoxic incubation medium was changed. Medium was harvested after additional 4, 8, 12, 24, 48, and 72 h of incubation, centrifuged at 1200 rpm and supernatant collected and kept at -20 °C.

3.5.2 Additive Effect of Trypsin and Hypoxia

To investigate a possible difference in the amount of VEGF secreted by the ASCs depending on the treatment they had received, ASCs were seeded in duplicates and incubated in a standard incubator for three days. After this, half of the cells were transferred to an incubator in the BioSpherix glove box to receive hypoxic treatment. 6 h before the hypoxic treatment was completed a group of cells from each incubator system was treated with trypsin as described in section 3.2.2. After the hypoxic treatment was completed, medium was changed for all cells and they were all incubated for further 12 h in their respective incubator systems. After this, medium was harvested, centrifuged at 1200 rpm and supernatant collected and kept at -20 °C. Cells were lysed in 0.02 % sodium dodecyl sulfate and kept at - 80 °C. For a timeline over the experiment see Figure 11.

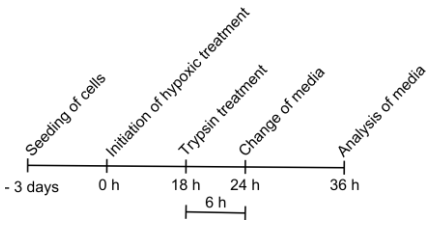

Figure 11. Timeline for the investigation of an additive effect of the combinational treatment of hypoxia and trypsin on the secretion of VEGF. The timeline is not drawn to scale.

3.5.3 Enzymed-Linked Immunosorbent Assay (ELISA)

The concentrations of VEGF in the medium were assessed using a Quantikine® ELISA kit for human VEGF (R&D Systems, Cat. no. DVE00) according to manufacturer's instructions. Fresh ASC medium was used as negative control. All assessments were run in technical replicates. The absorbance was measured using a Wallac 1420 Victor Multilabel Counter (PerkinElmer,

Hvidovre, Denmark) at a wavelength of 450 nm and corrected to 540 nm.

3.5.4 DNA

The amount of DNA matching the number of cells secreting the VEGF was determined using a PicoGreen dsDNA Quantitation Kit (Invitrogen™, Cat. no. P7589). The fluorescence was measured using a Wallac 1420 Victor Multilabel Counter (PerkinElmer, Hvidovre, Denmark) with excitation and emission at 485 nm and 535 nm, respectively. All assessments were run in technical replicates. The secretion of VEGF was normalized to the amount of DNA.

3.6 Activation of Hypoxia Inducible Factor-1

The activation of Hypoxia Inducible Factor-1 (HIF-1) was investigated in order to assess if trypsin increased the expression and secretion of VEGF through activation of this. The timeline for this experiment is shown in Figure 12.

3.6.1 Harvest of Nuclear extract

To investigate the influence of trypsin treatment on the activation of HIF-1, the ASCs were seeded in duplicates in T75 Cellstar® Tissue Culture Flasks (Greiner Bio-One, Cat. no. 658175) and incubated in the standard incubator for three days. Half of the cells were transferred to an incubator in the BioSpherix glove box to receive hypoxic treatment. 6 h before the hypoxic treatment was completed a group of cells from each incubator system were treated with trypsin; see section 3.2.2 for trypsin treatment procedure. After completion of the hypoxic treatment, all cells were lysed using a Nuclear Extract Kit (Active Motif, Cat. no. 40010) and the concentration of nuclear extract determined using a Pierce BCA Protein Assay Kit (Thermo Scientific, Cat. no. 23227) according to manufacturer's instructions. Nuclear extract was kept at – 80 °C.

3.6.2 HIF-1 Activation

The HIF-1 activation was measured in technical replicates using a TransAM HIF-1 kit (Active Motif, Cat. no. 47096). For each sample 20 µg was used per well. The absorbance was measured using a Wallac 1420 Victor Multilabel Counter (PerkinElmer, Hvidovre, Denmark) at a wavelength of 450 nm.

3.7 Migration of Endothelial Cells

To examine the influence of ASCs on ECs in relation to wound healing, HDMECs were co-cultured with pre-treaded ASCs in a ring assay and the tendency to migrate was measured and compared between treatments.

To clearly distinguish the HDMECs from the ASCs, the cells were transfected.

3.7.1 Transfection of Cells

The HDMECs were transfected with p^{CMV} LifeAct-TagRFP (Ibidi, Cat. no. 60102) and the ASCs with pLenti6.2-GW/EmGFP Expression control vector (Invitrogen, Cat. no. V369-20). The transfected ASCs were

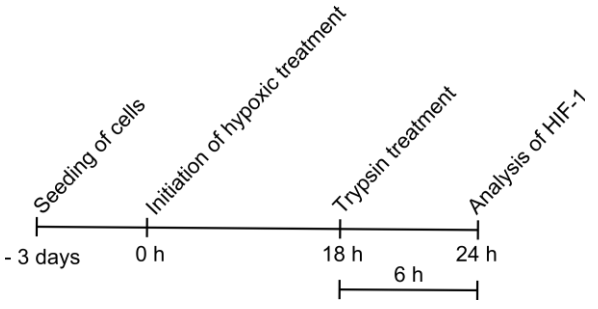

Figure 12. Timeline for the investigation of activation of HIF-1 through hypoxic, trypsin, and combinational treatment. The timeline is not drawn to scale.

kindly provided by fellow student Morten Juhl.

Human Dermal Microvascular Endothelial Cells

The HDMECs were grown confluent and transfected with the plasmid using Lipofectamine™ 2000 (LifeTechnologies, Cat. no. 11668). 8 µg plasmid and 20 µL Lipofectamine™ 2000 were diluted in Opti-MEM I medium (LifeTechnologies, Cat. no. 31985) in separate containers and incubated for 20 min at room temperature. Afterwards, they were mixed and additionally incubated for 20 min at room temperature to allow complexes to form. 1,000,000 HDMECs were suspended in MV2 medium and mixed with the plasmid-Lipofectamine™ 2000 complexes and seeded in a T25 Cellstar® Tissue Culture Flasks (Greiner Bio-One, Cat. no. 690175). After 48 h, medium was changed to fresh MV2 medium. When the HDMECs had recovered from the transfection, selection and establishment of a stable transfection was initiated using 250 µg/mL geneticin (G418, Invitrogen™, Cat. no. 11811).

The cells were regularly controlled under a fluorescence microscope (ZEISS Axiovert 200M) and medium changed regularly with fresh medium containing G418.

3.7.2 Ring Assay

To investigate the effect of pre-treated ASCs on HDMECs, a co-culture system was established. ASC-21 were seeded in T25 Cellstar® Tissue Culture Flasks and incubated in the standard incubator for three days. Then, half of the cells were transferred to an incubator in the BioSpherix glow box to receive hypoxic treatment. 6 h before the hypoxic treatment was ended a group of cells in each incubator system was treated with trypsin as described in section 3.2.2. At this time point, HDMECs were detached from their culture flask using Cell Dissociation Solution (Sigma-Aldrich®, Cat. no. C5914) for 60 min at 37 °C and stained with 5 µg/mL Hoechst 33342 for 20 min (Molecular Probes®, Cat. no. H-3570). Stainless steel rings were placed in the periphery of the wells of a Costar® 24-well plate (Corning Life Sciences, Cat. no. #3524). The rings were used to control and separate the areas of cell seeding (Figure 13). HDMECs were seeded outside the ring with a seeding density of 80,000 cells/cm^2 in Endothelial Cell Growth Medium MV2. When the hypoxic treatment ended ASCs were detached in the same way as the HDMECs, but seeded in the middle of the rings in ASC medium with a seeding density of 25,000 cells/cm^2. The co-culture system was transferred to the standard incubator and after attachment of the cells to the culture surface the rings were gently removed from each well leaving a 4 mm cell free area called the "wound". The medium was changed to Endothelial Cell Growth Medium MV2 to support the HDMEC viability.

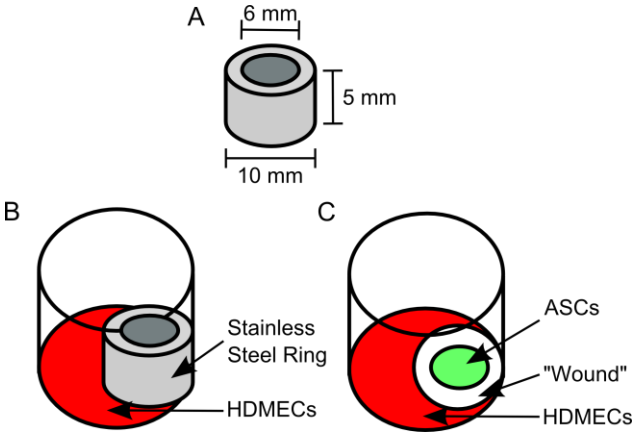

Figure 13. Design of the ring assay. A) Dimensions of the stainless steel ring used for the ring assay. B) A stainless steel ring was placed in the periphery of the well. Human dermal microvascular endothelial cells (HDMECs) were seeded outside the stainless steel ring. C) Adipose-derived stem cells (ASCs) were seeded inside the stainless steel ring. When all cells had attached the ring was removed creating a cell free area called the "wound".

Time-lapse Photography

The culture plate with the “wounded” cells was to assess “wound” closure by time-lapse photography transferred to a PeCon Incubator System. This incubator system consists of an incubator box connected to a CTI-controller 3700 digital, which was further connected to a Tempcontrol 37-2 digital and an O_2-Controller. The incubator was placed on top of a microscope. Phase contrast and fluorescence images were obtained during the time-lapse photography with an AxioCam MRm attached to a Zeiss Axio Observer.Z1 microscope with HAL 100 light source and ApoTome slider (Carl Zeiss International). Pictures were captured with AxioVision (ver. 4.7). For each well, a mosaic picture consisting of two overlapping images were taken at 100x magnification at the cell-free area, the “wound”, once every hour until wound closure was detected. This sequence of pictures was used to determine migration speed of the HDMECs.

3.8 Statistical Analysis

Data were analyzed using IBM® SPSS® Statistics version 19 (IBM Company, Inc.). Paired Sample t-test was used for analysis if data were normally distributed. This was tested by a Shapiro-Wilk normality test. In case the data were not normally distributed a Paired-Sample Wilcoxon Signed Rank test was used. Data are presented as mean ± standard error of the mean (SEM). Statistical significance was assigned to p values < 0.05.

4 Results

4.1 Investigation of ASC's Response to Hypoxia and Trypsin through VEGF

To examine the proangiogenic properties of human adipose-derived stem cells in relation to short-term hypoxic culture and trypsin treatment a series of experiments was performed and in the following sections the considerations and results from these experiments are presented.
The results from the verification of incubator systems are shown in section 9 (Appendix I: Verification of Incubator Systems). No difference in baseline VEGF expression was detected and both incubators therefore used in the following experiments when needed.

4.1.1 The Time-Dependent Effect of Trypsin Treatment

Previously a similar trypsin treatment had been performed in our laboratory indicating an effect of trypsin treatment on the VEGF expression when performed 12 h before analysis (data not published). To investigate whether this time point of treatment was the most optimal, an experiment investigating the timeline of the effect of trypsin on the VEGF expression was conducted. The results are shown in Figure 14.

Before trypsin treatment (0 h after treatment) the relative expression of VEGF was 0.59 ± 0.13, this increased almost two fold to 1.13 ± 0.17 6 h after treatment and dropped again 12 h after treatment to 0.34 ± 0.05. 48 h after trypsin treatment the relative expression of VEGF had increased to 0.47 ± 0.03.
Because of these observations of the effect of trypsin on the VEGF expression, the effect of trypsin treatment was suspected to peak earlier than 12 h after treatment. Therefore, both the 6 h and 12 h time point was used in the following experiments.

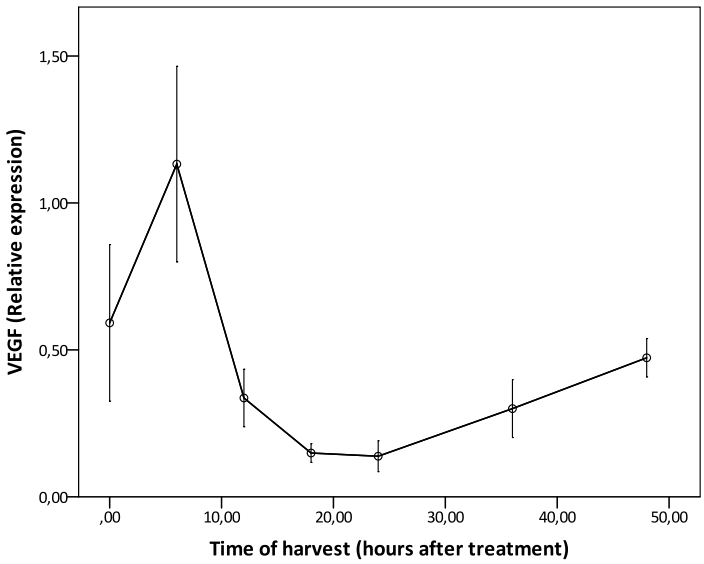

Figure 14. The timeline of the effect of trypsin treatment on the expression of vascular endothelial growth factor (VEGF). The expression was determined by real-time RT-PCR and normalized to the expression of the two reference genes PPIA and YWHAZ (n = 3). Data are represented as mean ± SEM.

4.1.2 Optimization of ASCs Treatment

The main goal with the trypsin treatment was to make it reproducible. To test the reproducibility of different trypsin treatment approaches a row of experiments were performed. They were all evaluated by measurement of the expression of VEGF relative to the expression of the reference gene PPIA.

Objective assessment of Rounding of the Cells in Trypsin Treatment

In an attempt to treat the cells with a uniform degree of trypsinization independently of differences in incubation systems, an objective assessment of the rounding of the cells was made to determine the length of the trypsin treatment. Results are shown in Figure 15.

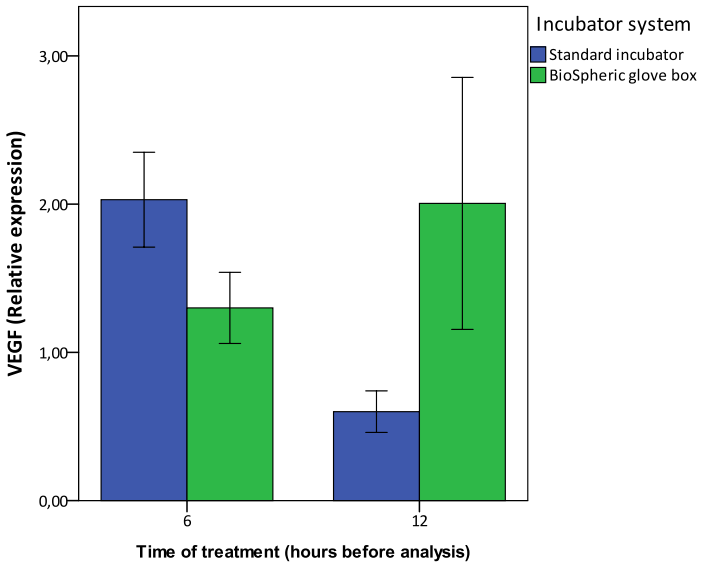

Figure 15. Objective assessment of rounding. The effect of trypsin treatment on vascular endothelial growth factor (VEGF) expression was determined by real-time RT-PCR and normalized to the expression of the reference gene PPIA (n = 2). Data are represented as mean ± SEM.

The trypsin treatment 6 h before analyzing the cells resulted in the following observations. The ASCs incubated in the standard incubator system (Figure 16A) became round, but had not fully reattached after 1 hour of incubation after treatment. These cells had a VEGF expression of 2.03 ± 0.16. Likewise, the ASCs incubated in the BioSpherix glove box (Figure 16B) became round and had not completely attached after 1 h. These cells had a VEGF expression of 1.30 ± 0.12.

For the trypsin treatment conducted 12 h before harvest of the cells, the ASCs incubated in the standard incubator system became round, and had reattached after 1 h of incubation. These cells had a VEGF expression of 0.60 ± 0.07. The ASCs incubated in the BioSpherix glove box became round, but had not fully attached after 1 h. These cells had a VEGF expression of 2.01 ± 0.43.

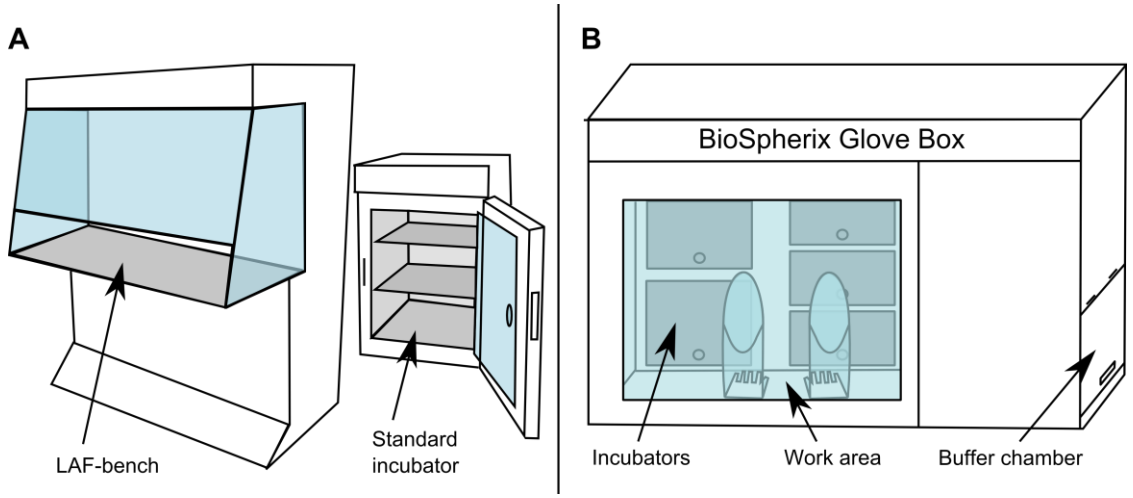

Figure 16. Incubator systems used for the standardization of the trypsin treatment. A) The standard incubator system was used for incubation at 21 % O_2, 5 % CO_2 and 37 °C and the LAF-bench was used when cells were to be processed. This was done at room temperature and atmospheric air. B) The BioSpherix glove box was used for both incubation in the integrated incubators set to 21 % O_2, 5 % CO_2, and processing of the cells in the work area set to 5 % O_2, 5 % CO_2. In both cases the temperature was kept at 37 °C.

Even though no statistical difference between the trypsin treatments in the two incubator systems was found at any of the time points, concerns regarding the procedure were present. It was very difficult to determine when the cells had rounded to the same degree between systems and treatment time points, as the cells did not round up simultaneously and it thereby became an overall assessment of rounding. Additionally the lack of reattachment was a problem.

Total Trypsinization

In an attempt to treat the cells with the same degree of trypsin and overcome the objective assessment, the cells were trypsin treated until they detached from the cell culture plate. (Figure 17)

The treatment 6 h before analysis of the cells showed that the ASCs incubated in the standard incubator system had a VEGF expression of 0.80 ± 0.16. Additionally, the ASCs incubated in the BioSpherix glove box had a VEGF expression of 1.53 ± 0.22.

When treating the cells 12 h before analysis of the cells, the ASCs incubated in the standard incubator system had a VEGF expression of 0.23 ± 0.02, and the ASCs incubated in the BioSpherix glove box had a relative expression of VEGF of 1.35 ± 0.22.

When using this method for trypsin treatment it was clear, that the cells did not detach at once but over a longer period of time, and it was difficult to detach all cells without damaging others due to the long exposure time to trypsin. Additionally, reattachment of the cells was still a problem, as they were very slow to reattach, even when supplemented with higher serum concentrations.

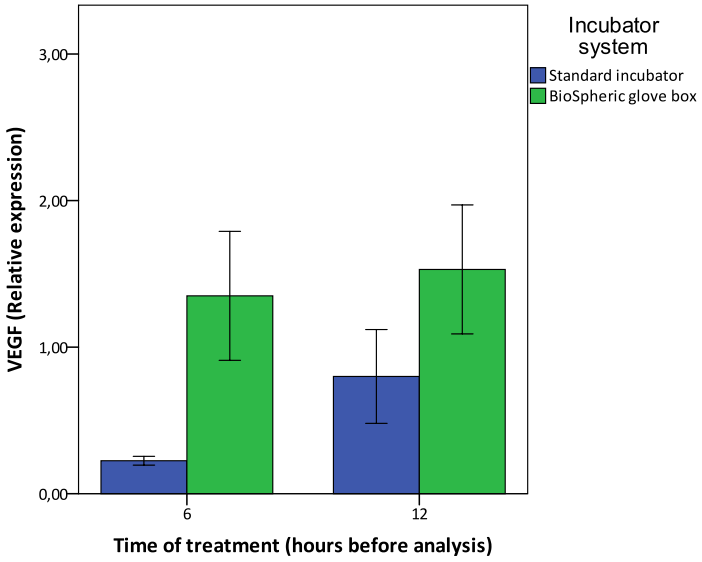

Figure 17. Total trypsinization. The effect of trypsin treatment on vascular endothelial growth factor (VEGF) gene expression was determined by real-time RT-PCR and normalized to the expression of the reference gene PPIA (n = 2). Data are represented as mean ± SEM.

Time fixed trypsin treatment

To ensure reproducibility the idea of a fixed time of trypsin treatment was appealing, excluding the objectiveness and thereby also excluding investigators bias. Therefore, the response to trypsin was investigated for different durations of time (data not shown). This was done to ensure a rounding of the cells, reflecting an effect of the trypsin, but also a firm reattachment of the cells within an hour, to have an overall reasonable treatment time. From this it was concluded that 7 min at room temperature was appropriate. (Figure 18)

The treatment taking place 6 h before analysis of the cells showed, that the ASCs incubated in the standard incubator system had a VEGF expression of 0.83 ± 0.09. In comparison, the ASCs incubated in the BioSpherix glove box had a VEGF expression of 1.23 ± 0.14.

When treating the ASCs 12 h before analysis, the ASCs incubated in the standard incubator system had a VEGF expression of 0.34 ± 0.06, and the ASCs incubated in the BioSpherix glove box had a VEGF expression of 1.15 ± 0.49.
When applying trypsin for the same amount of time, when treating the cells in the two incubator systems, it was clear, even though not statistical significant, that the trypsin treatment were more effective in the BioSpherix glove box. This increased enzymatic activity was probably due to the constant temperature of 37 °C when using the BioSpherix glove box.

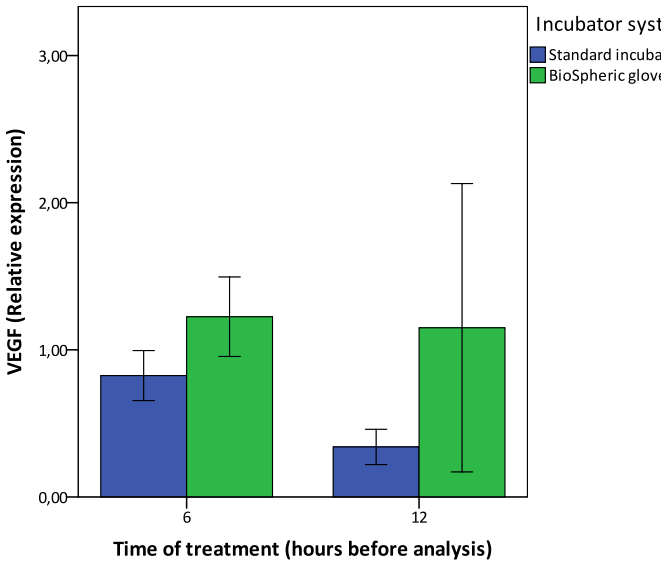

Figure 18. Time fixed trypsin treatment. The effect of trypsin treatment on vascular endothelial growth factor (VEGF) gene expression was determined by real-time RT-PCR and normalized to the expression of the reference gene PPIA (n = 2). Data are represented as mean ± SEM.

From this it was concluded that it was not possible to trypsin treat in a comparable and reproducible manner using the two different incubator systems as it had been done until now. Instead the cells were for future experiments incubated in the two incubator systems, but all trypsin treatments and cell processing took place in the work area of the BioSpherix glove box. (Figure 9)
To ensure comparable enzymatic activity of the trypsin between all experiments, the same lot was used, the trypsin was handled in the same way every time, and it was kept on ice until applied to the cells to avoid warming and thereby changed enzymatic activity due to the climate in the BioSpherix glove box.
Based on the above mentioned experiments and earlier experiences, the final trypsin treatment was designed as described in section 3.2.2.

4.1.3 Response Time of Trypsin Treatment

As earlier mentioned experiments had prior to this thesis been made treating the cells with trypsin 12 h before harvest. However, preliminary results of this thesis indicated that the effect of trypsin treatment on VEGF expression peaked much closer to the time point of treatment (Figure 14). Because of this, a pilot study was performed investigating the effect of trypsin treatment at different time points. This was combined with hypoxic treatment. (Figure 19)
The group H24 was cells only treated with hypoxia. They had a VEGF expression of 0.75 ± 0.05. The group H24T6 received hypoxic treatment and trypsin treatment 6 h before termination of the hypoxic treatment. They had a VEGF expression of 2.28 ± 0.08, three times higher than the only hypoxia treated cells. The group H24T12 was cells receiving hypoxic treatment and trypsin treatment 12 h before the termination of the hypoxic treatment. They had a VEGF expression of 1.86 ± 0.03. H24T18 was cells treated with hypoxia and trypsin treated 18 h before termination of the hypoxic treatment. They had a VEGF expression of 1.40 ± 0.01.

From this pilot study it looked like trypsin treatment 6 h before analysis was more beneficial than the 12 h previously used in our laboratory.

To more thoroughly investigate the time point of trypsin treatment giving the largest increase in VEGF expression, the experiment was expanded to include trypsin treatment closer to the ending of the hypoxic treatment. (Figure 20)

The control group was cells receiving no treatment. Their VEGF expression was 0.23 ± 0.04. The group H24 received hypoxic treatment for 24 h and had a VEGF expression of 0.45 ± 0.07. The group H24T2 received hypoxic treatment for 24 h and trypsin treatment 2 h prior to termination of this. They had a VEGF expression of 1.07 ± 0.08. The group H24T4 received hypoxic treatment for 24 h and trypsin treatment 4 h prior to termination of this. This group of cells had a VEGF expression of 1.33 ± 0.12. The group H24T6 received hypoxic treatment for 24 h and trypsin treatment 6 h prior to termination of the hypoxic treatment. They had a VEGF expression of 1.33 ± 0.16. The group H24T12 received hypoxic treatment for 24 h and trypsin treatment 12 h prior to termination of the hypoxic treatment. This group of cells had a VEGF expression of 0.67 ± 0.09.

A paired samples t-test showed, that all treatments significantly increased the VEGF expression compared to the control (P = 0.001, P = 0.000, P = 0.000, P = 0.000, P = 0.001). Trypsin treatment 2, 4, and 6 h before termination of the hypoxic treatment gave all rise to a significant increase in the VEGF expression when compared to the pure hypoxic treatment (P = 0.000, P = *0.000,* P = 0.002). No statistical difference between pure hypoxic treatment and trypsin treatment 12 h before ending of the hypoxic treatment was found. No significant difference was found when comparing H24T4 and H24T6 and

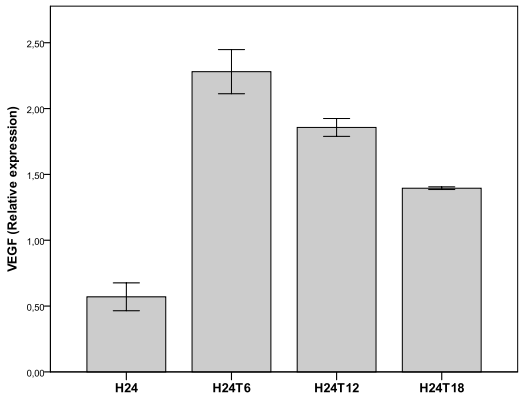

Figure 19. Pilot study investigating the response time of the trypsin treatment on the relative expression of vascular endothelial growth factor (VEGF). The effect of trypsin treatment on the relative VEGF gene expression was determined by real-time RT-PCR and normalized to the expression of the two reference genes PPIA and YWHAZ (n = 3). H, hypoxia; T, trypsin, H24, cells treated with hypoxia for 24 h; H24T6, cells treated with hypoxia for 24 h and trypsin 6 h before termination of the hypoxic treatment.; H24T12, cells treated with hypoxia for 24 h and trypsin 12 h before termination of the hypoxic treatment; H24T18, cells treated with hypoxia for 24 h and trypsin 18 h before termination of the hypoxic treatment. Data are represented as mean ± SEM.

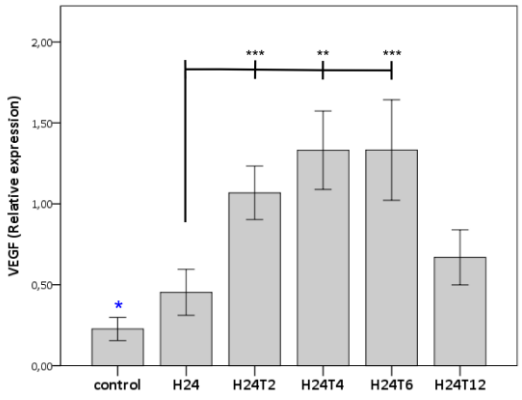

Figure 20. Response time for trypsin treatment. The effect of treatment on the relative expression of vascular endothelial growth factor (VEGF) was determined by real-time RT-PCR and normalized to the expression of the two reference genes PPIA and YWHAZ (n = 12). Control, cells without treatment; H, hypoxia; T, trypsin, H24, cells treated with hypoxia for 24 h; H24T6, cells treated with hypoxia for 24 h and trypsin 6 h before termination of the hypoxic treatment.; H24T12, cells treated with hypoxia for 24 h and trypsin 12 h before termination of the hypoxic treatment; H24T18, cells treated with hypoxia for 24 h and trypsin 18 h before termination of the hypoxic treatment. * : The control group has a significantly lower VEGF expression than all treated groups (P = 0.001). ** P = 0.005, *** P = 0.001. Data are represented as mean ± SEM.

H24T2 was only significantly lower than H24T4, not H24T6 (P = 0.008).
From this it seemed like the time interval between 4 and 6 h before analysis were the most effective period to perform the trypsin treatment in.

4.1.4 Hypoxic and Trypsin Treatment – an Additive Effect?

Since the time response experiment showed that trypsin treatment was most beneficial when performed in the 4 to 6 h interval before termination of the 24 h hypoxic treatment, both time points and the 12 h used in earlier experiments in our laboratory were included in the investigation of a possible additive effect of hypoxia and trypsin when used in combination.
ASCs were seeded and incubated in the standard incubator for 3 days. After this half the cells were transferred to an incubator in the BioSpherix glove box to receive hypoxic treatment. 2, 6, and 12 h before the hypoxic treatment was terminated cells in both incubator systems were trypsin treated. Additionally, a group of cells in both systems was left untreated.
As shown in
Figure 21 in the front row (Red), the cells incubated in the standard incubator (normoxia) without any trypsin treatment, designated the control, had a VEGF expression of 0.22 ± 0.04. The VEGF expression was increased by trypsin treatment 2 h before analysis to 0.72 ± 0.11, and even further increased by trypsin treatment 6 h before analysis (0.98 ± 0.12). However, when applying the trypsin treatment 12 h prior to analysis the expression of VEGF was only 0.56 ± 0.06.
A Related Samples Wilcoxon Signed Rank test showed, that trypsin treatment at any of the tested time points significantly increased the VEGF expression (P = 0.01, P = 0.001, P = 0.028 respectively) when compared to the control. Additionally, trypsin treatment 6 h before analysis was found significantly more effective in increasing the relative expression of VEGF than both trypsin treatment 12 h and 2 h before analysis (P = 0.001 and P = 0.046 respectively). No difference was found between treatment 12 h before and 2 h before analysis.
As shown in
Figure 21 in the back row (blue), the cells incubated in hypoxia without any trypsin treatment (designated the control) had a VEGF expression of 0.76 ± 0.12. The VEGF expression was increased when trypsin treated 2 h before analysis to 1.07 ± 0.08, and even further when treated 6 h before analysis (1.60 ± 0.14), but treatment 12 h before analysis did almost not result in any increase of the VEGF expression

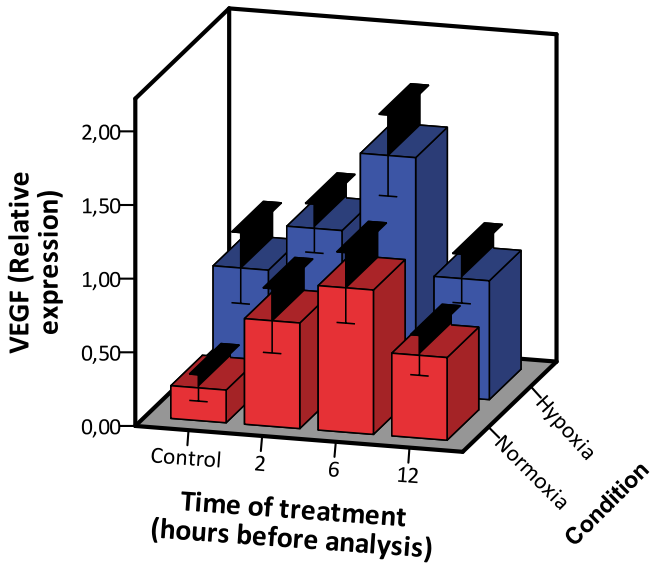

Figure 21. Investigation of the combination of trypsin and hypoxic treatment on the expression of vascular endothelial growth factor (VEGF). Red) Normoxia corresponds to 20 % O_2. These cells have been exposed to pure trypsin treatment. The red control corresponds to cells cultured without any treatment. Blue) Hypoxia corresponds to 1 % O_2. These cells have been exposed to the combinational treatment of hypoxia and trypsin. The blue control corresponds to pure hypoxic treatment. The effect of treatment on the relative VEGF gene expression was determined by real-time RT-PCR and normalized to the expression of the two reference genes PPIA and YWHAZ (n = 6-20). Data are represented as mean ± SEM.

(0.81 ± 0.08) when compared to the control.
A related Samples Wilcoxon Signed Rank test showed, that trypsin treatment 2 and 6 h before analysis significantly increased the expression of VEGF (P = 0.002 and P = 0.000) when compared to both the control and trypsin treatment 12 h before analysis. However the difference between trypsin treatment 2 and 6 h before analysis was not statistically different. When trypsin treating the cells 12 h before analysis no statistical difference was found when compared to the control.
When comparing the expression of VEGF across oxygen tensions, the hypoxic control (
Figure 21, back row) gave rise to a significantly higher expression than the normoxic control (
Figure 21, front row, P = 0.001). Treating the cells both 2, 6, and 12 h before analysis was also more effective when combined with hypoxic treatment than the effect of trypsin treatment alone (P = 0.046, 0.023, and 0.028).

4.1.5 Secretion of VEGF

In order to investigate whether the observations regarding the effect of hypoxic and trypsin treatment on the VEGF expression in ASCs was reflected in the culture medium as increased amount of secreted VEGF, the cells were treated as for the earlier experiments. However, where the cells earlier were harvested for analysis, they were now continued in culture. Medium was later harvested for analysis of the VEGF content using an ELISA for secreted $VEGF_{165}$.

Best Time to Harvest

To ensure harvesting medium with a detectable concentration of VEGF, and registering a possible effect of trypsin, an experiment was designed exposing ASC-21 to hypoxic treatment and additionally expose them to trypsin treatment 6 h before the hypoxic treatment was terminated. At this time point medium was changed. After this, medium was harvested from separate wells at the time points 4, 8, 12, 24, 48, and 72 h after treatment and the concentration of VEGF analyzed by ELISA. (Figure 22)
The VEGF concentration in the ASC medium itself gave not rice to any detectable absorbance. This was serving as 0 h control. The medium harvested 4 h after medium change gave rise to an absorbance of 0.63 ± 0.04, the medium harvested after 8 h to an absorbance of 1.16 ± 0.10, the medium harvested after 12 h to an absorbance of 1.93 ± 0.01, the medium harvested after 24 h to an absorbance of 1.94 ± 0.08, the medium harvested after 48 h to an absorbance of 2.43 ± 0.08, and the medium harvested after 72 h to an absorbance of 3.59 ± 0.01.
From this we wanted to determine the most optimal time of media harvest based on the maximal secretion rate, leaving the cells with enough time to secrete a detectable amount of VEGF, but still maintaining a reasonable amount of nutrients in the medium in case this should be used for other experiments as CM, and short enough time not make hypoxia obscuring the possible effect of trypsin.

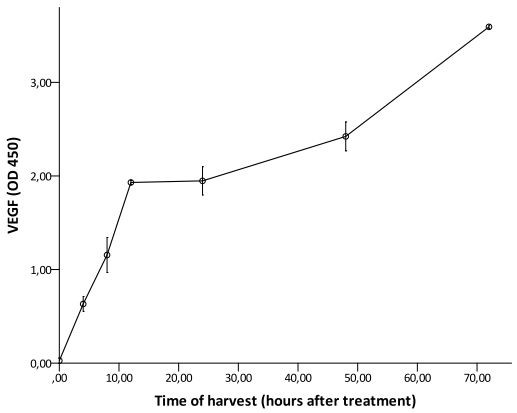

Figure 22. Time dependent secretion of vascular endothelial growth factor (VEGF) after the combination of trypsin and hypoxic treatment. Medium was analyzed 4, 8, 12, 24, 48, and 72 h after treatment. The absorbance corresponding to the concentration of VEGF was determined by ELISA (n = 2). Data are

Base on these criteria, medium was harvested after 12 h.

Is the Same Effect seen Secreted in the Medium

To test if the observations regarding the effect of trypsin treatment on the VEGF expression also showed as increased amount of secreted VEGF in the medium, ASCs were treated as for the expression experiments, but instead of analysis after termination of the hypoxic treatment, the cells were incubated for additionally 12 h. At this time point, 12 h after termination of the hypoxic treatment, the medium was harvested and the concentration of VEGF analyzed by ELISA and the amount of DNA per well, representing the number of cells secreting the VEGF, analyzed by Quant-iT™ PicoGreen® dsDNA analysis. ELISA data was normalized to the amount of DNA.

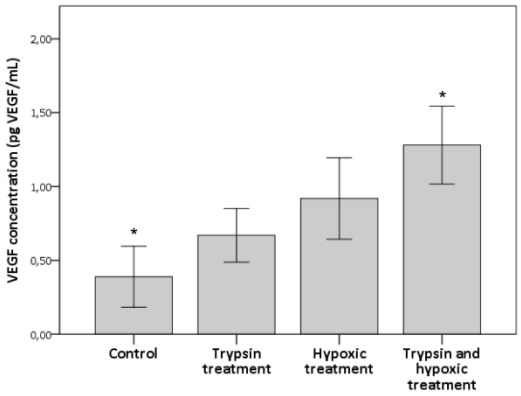

Figure 23. Secretion of vascular endothelial growth factor (VEGF) by ASCs exposed to standard culturing conditions, trypsin treatment, hypoxic treatment, and a combination of trypsin and hypoxic treatment. The VEGF concentrations were determined by ELISA and normalized to the amount of deoxyribonucleic acid (DNA) of each sample (n = 6). *a statistical significant difference was detected between this group and all the others groups. Data are represented as mean ± SEM.

A Paired Sample t-test determined that trypsin treated ASCs secreted significantly more VEGF than untreated ASCs (0.67 ± 0.09 pg VEGF/mL vs. 0.39 ± 0.10 pg VEGF/mL, P = 0.005), and that hypoxic treated ASCs secreted significantly more VEGF than the untreated ASCs, but no difference was detected when compared to the trypsin treated (0.92 ± 0.14 pg VEGF/mL, *P* = 0.000). Finally the ASCs treated with the combination of trypsin and hypoxic treatment secreted significantly more VEGF than both untreated, trypsin treated, and hypoxic treated ASCs (1.28 ± 0.13 pg VEGF/mL, *P* = 0.000, *P* = 0.002, and *P* = 0.000 respectively).

4.2 Endothelial Cells

To examine the influence of ASCs on ECs in relation to wound healing an *in vitro* model of migration was to be established.

4.2.1 Migration Assay

Wound Closure Time

Untreated ASCs not transfected with GFP was used for this experiment. The experiment showed that HDMECs were beginning to close the “wound” after 48 h of incubation after the removal of the ring. (Figure 24)

This was only satisfactorily completed for one sample and was therefore only used as a guide line for the main experiment.

Ring Assay

For the main migration assay using the stainless steel ring approach the cells were to be kept in the time lapse system until complete closure of the wound. HDMECs transfected with p^{CMV} LifeAct-TagRFP was not used for this experiment as the transfection efficiency was below 1 %. Instead Hoechst 33342 stained HDMECs were used.

However, after 96 h of incubation in the time-lapse system no migration of the HDMECs was detected (Appendix II: Results of Migration Experiment). This was seen as a sign of inappropriate conditions and therefore the experiment was ended after 96 h without wound closure.

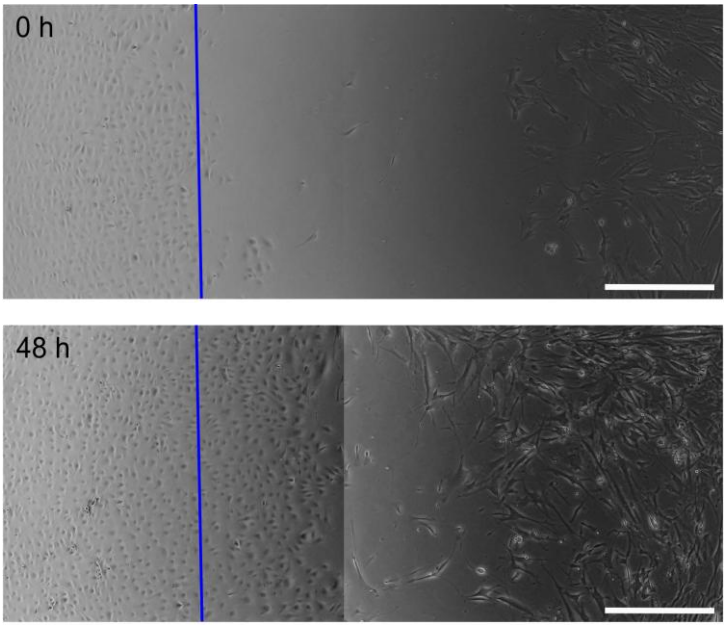

Figure 24. Investigation of wound closure time. The co-culture system was incubated in a time lapse system for 48 h. The white stripe marks the front of the endothelial layer at 0 h. The pictures are mosaics of two phase contrast pictures at 100x magnification. Scale bar denotes 500 µm.

5 Discussion

In summary, both hypoxic and trypsin treatment significantly up regulated both the expression and secretion of VEGF. Additionally, the combination of hypoxic and trypsin treatment increased the expression and secretion of VEGF even further, indicating an additive effect.

The main benefit of using ASCs compared to other stem cells is that they can be easily harvested from patients in a simple, minimally invasive lipoaspiration procedure and then be readily available for autologous cell therapy (Rehman et al., 2004). ASCs are shown to be important stromal cells that repair injured tissue. They migrate and interact with other cells and with the extracellular environment in response to different stimuli, as for example when injury of capillaries generates a hypoxic environment during wound healing (Chung et al., 2009). The experimental findings of this thesis support the paracrine function of ASCs, as being capable of secreting growth factors as VEGF in response to injury like hypoxia, as VEGF was detected both at transcriptional and at translational level. VEGF was used as a measure of the pro-angiogenic response of the ASCs. This growth factor has been shown to play a significant role in the ASC mediated tissue regeneration, as the positive effect of CM from ASCs was reversed by VEGF neutralizing antibodies or by adding VEGFR inhibitors (Kim et al., 2007, Rehman et al., 2004, Lee et al., 2009, Rasmussen et al., 2011).

5.1 Hypoxia increases VEGF Expression and Secretion

Investigators have shown that hypoxia induces the expression and secretion of VEGF in ASCs (Rehman et al., 2004, Lee et al., 2009). It has been found that the expression of VEGF was approximately 7 times higher in ASCs cultured in hypoxia compared to ASCs cultured under standard conditions (Rehman et al., 2004, Lee et al., 2009). The effect of hypoxia on the VEGF expression in ASCs has previously been investigated in our laboratory, and a change in the expression of VEGF was observed after 24 h of hypoxic incubation compared to standard conditions (Rasmussen et al., 2011). This is a longer incubation time than used in other laboratories where a difference was detected after 6 h (Lee et al., 2009). Based on the findings from our laboratory, 24 h of hypoxic culturing was applied as hypoxic treatment. This resulted in ~ 3 fold increase in the expression of VEGF compared to the control cultured under standard conditions. As a result of the increased VEGF expression an increased VEGF secretion would be expected. When investigating the CM from ASCs differences in methodology exist. The secretion of VEGF has after 72 h of hypoxic incubation been found to increase approximately 4 fold compared to when incubated in normoxia (Rehman et al., 2004, Lee et al., 2009). In the present study, a secretion time of 12 h was used and a 2 fold significant increase in the amount of secreted VEGF was detected for ASCs treated with hypoxia. Based on this, hypoxic treatment of ASCs resulted in both increased expression and secretion of VEGF, which is considered to be consistent with the literature.

5.2 Trypsin increases VEGF Expression and Secretion

In the present study, it was found that trypsin treatment significantly induced both VEGF expression and secretion in ASCs. This correlates with previous findings in our laboratory by Rasmussen *et al.*, who have shown that ASCs increase the expression of VEGF in response to trypsin treatment (Rasmussen et al., 2011). The effect of trypsin treatment on ASCs is not fully investigated. Trypsin is known to activate PAR2 (Zhu et al., 2006). It is hypothesized that activation of G-protein coupled receptors as PAR2 results in activation of PI3K and MAPK pathways and through

these, result in increased expression and secretion of VEGF (Zhu et al., 2006, Dutra-Oliveira et al., 2012, Zhong et al., 2000). The direct activation of PAR2 has been shown to result in increased expression and secretion of VEGF both *in vitro* and *in vivo* (Liu and Mueller, 2006, Dutra-Oliveira et al., 2012, Milia et al., 2002, Zhu et al., 2006). Furthermore, the activation of PAR2 by trypsin has been shown to play a major role in angiogenesis (Uusitalo-Jarvinen et al., 2007). PAR2 has been identified on different cells and recently also on ASCs (Data not published). Trypsin treatment as a stimulator of VEGF expression could maybe be used in the clinical application of ASCs to increase their pro-angiogenic effect.

5.3 The combination of Hypoxic- and Trypsin Treatment has an Additive Effect on the Expression and Secretion of VEGF

The additive effect on VEGF of the combination of hypoxic and trypsin treatment found in this thesis has not previously been investigated. It is well known that hypoxia induces increased expression and secretion of VEGF through stabilization of HIF-1α (Zagorska and Dulak, 2004). It is also known that trypsin activates PAR2, and that the effect of this most likely is propagated through MAPK and PI3K pathways, also leading to induced expression and secretion of VEGF (Liu and Mueller, 2006, Zhu et al., 2006, Dutra-Oliveira et al., 2012, Zhong et al., 2000). Furthermore, some researchers have suggested that activation of PI3K and MAPK pathways leads to either stabilization or induced expression of HIF-1α, which would link the mechanisms of action of hypoxia and trypsin (Fukuda et al., 2002, Zhong et al., 2000). Moreover increased expression of HIF-1α has been shown to be additive to the stabilizing effect of hypoxia on HIF-1α (Fukuda et al., 2002). However, such a connection has not previously been found in our laboratory as trypsinization of ASCs increased the expression of VEGF but not affected the stability of HIF-1α, indicating a HIF independent pathway for trypsin mediated VEGF expression (data not published).

In summary, trypsin treatment may act through a number of mechanisms. One might directly increase the expression and secretion of VEGF as seen under normoxic conditions. The other might lead to increased expression of HIF-1α, which under normoxic conditions will be degrad-

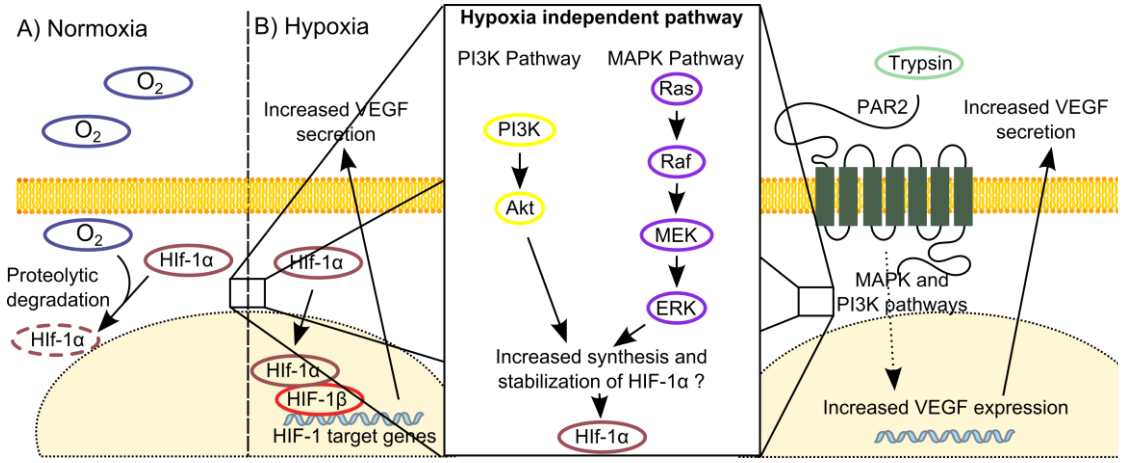

Figure 25. The hypoxia independent pathway of increased synthesis and stabilization of hypoxia inducible factor 1α (HIF-1α). Activation of protease activated receptor 2 (PAR2) leads to activation of phosphatidylinositol 3-kinases (PI3K) and mitogen-activated protein kinase (MAPK) pathways resulting in increased synthesis and stabilization of HIF-1α. This may lead to increased vascular endothelial growth factor (VEGF) expression and secretion if HIF-1α is not degraded.

ed. However, when combined with hypoxic treatment the increased quantity of HIF-1α is stabilized by the absence of O_2 resulting in an even higher activity of HIF leading to further increased expression of VEGF and thereby even higher secretion of VEGF compared to hypoxic treatment alone. This effect would then be registered as an additive effect. For a schematic overview of the possible link between trypsin and hypoxia see Figure 25.
It was the intension of this thesis to investigate the activation of the HIF-1 complex by trypsin and hypoxia and the combination of these. All preparations for this analysis were made. However, due to the supplier not being able to deliver the TransAM HIF-1 kit in time, the results from this experiment was at the time of thesis submission not obtained.

5.4 ASCs and Endothelial Cell Migration

To examine the influence of ASCs on ECs in relation to wound healing, HDMECs were co-cultured with pre-treated ASCs in a ring assay and the tendency to migrate were measured and compared. However, a functioning and reproducible migration assay was not established. There are several parameters to take into account when establishing a migration assay, which can influence the outcome of such an assay. An advantage of the ring approach is the uniformity of the wound increasing the reproducibility. Additionally, using a ring makes it possible to make a co-culture system of two different cell types within the same well. Furthermore, by using the ring assay the cells are allowed to migrate into the "wound" as a sheet of cells, as endothelial cells normally do *in vivo* (Liang et al., 2007). A limitation of the ring assay is the incubation time to ensure a confluent monolayer of the ECs with extensive cell-cell contacts before migration can be assessed, as this is important for endothelial cells (van der Meer et al., 2010, Dejana, 2004). In order to distinguish the two cell cultures in the migration assay the HDMECs were transfected with a red fluorescent protein. However the transfection efficiency was below 1 %, not making this method suitable. The low transfection efficiency was in accordance with the literature about transient transfection of ECs and therefore not further investigated (Tanner et al., 1997). Instead the HDMECs were stained with Hoechst 33342. However, Hoechst 33342 staining has been shown to inhibit cell cycle progression, cause DNA damage and mutations, which all were increased by time-lapse photography (Purschke et al., 2010, Siemann and Keng, 1986). This could maybe explain the lack of HDEMC migration.
Others have shown that CM from ASCs and especially VEGF increase the EC migration rate, cell viability, and tube formation (van der Meer et al., 2010, Nakagami et al., 2005). These findings support the paracrine function of ASCs in wound healing. Based on this, a positive effect on EC migration rate would have been expected for our migration assay, as our combinational treatment resulted in a significantly increased VEGF secretion by treated ASCs.

6 Prospects

ASCs from diabetic patients are suspected to be less active than ASCs from healthy donors (Gu et al., 2012). Therefore, it would be meaningful also to test the hypoxic and trypsin treatments on ASCs from diabetic patients, as only ASCs from healthy persons have been tested in this thesis. This would be done in order to investigate if the combination of hypoxic- and trypsin treatment also could trigger VEGF expression in ASCs from diabetic patients as well as in healthy and thereby also increase the pro-angiogenic effects of these, potentiating them for clinical use. An impaired healing diabetic mouse model has shown that autologous ASCs significantly increased

wound healing. This support the idea of autologous ASCs from diabetic patients for clinical application (Nambu et al., 2011). These findings indicate that ASCs from diabetic patients have a potential in clinical application as cell based cytokine therapy, and based on our findings the proangiogenic effect might be even further enhanced by treating the ASCs with hypoxia and trypsin before application.

7 Conclusion

In conclusion, both hypoxic and trypsin treatment of ASCs significantly up regulated the expression and secretion of VEGF. The effect of the trypsin treatment was largest when applied 6 h before ending of the hypoxic treatment. Additionally, the combination of hypoxic and trypsin treatment increased the expression and secretion of VEGF even further, indicating an additive effect. As no results were obtained from the endothelial migration assay, the effect of pretreated ASCs on endothelial migration has yet to be investigated.

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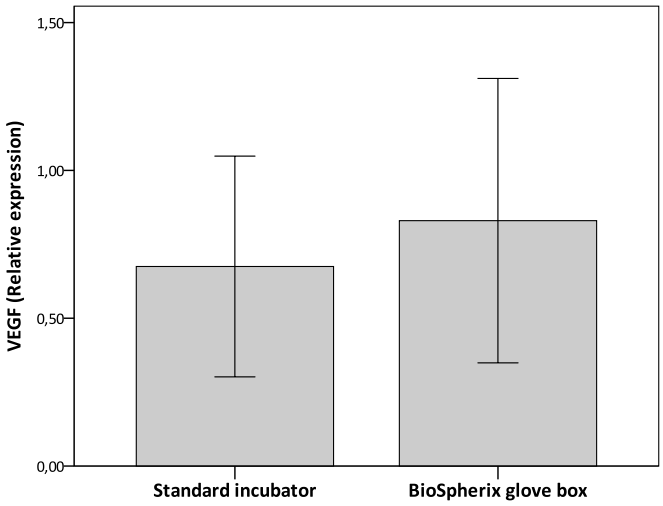
9 Appendix I: Verification of Incubator Systems

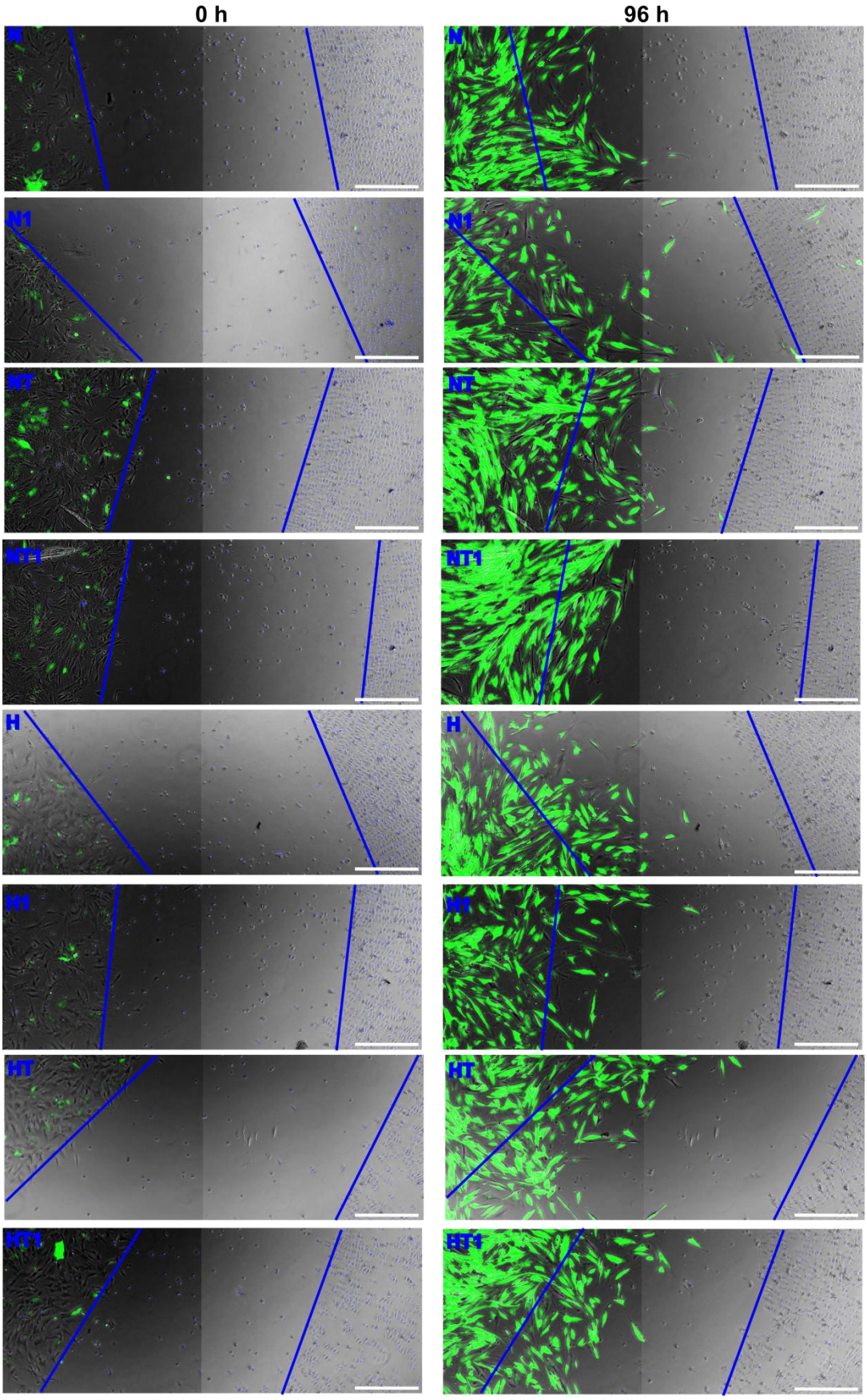
To test the comparability of the standard incubator system and the BioSpherix glove box, in regard to the expression of VEGF in ASCs, both systems were set to 20 % O_2, 5 % CO_2, and 37 °C, and a group of ASC-21 was incubated in each system for 24 h. After this, the expression of VEGF relative to the expression of the reference gene PPIA was analyzed by real-time RT-PCR (see section 3.4).

The experiment was run in biological duplicates and repeated for the total of two experiments. The cells incubated in the standard incubator had a VEGF expression of 0.68 ± 0.19 in comparison to the BioSpherix glove box, where the cells had a VEGF expression of 0.83 ± 0.24. A paired sample t-test showed no statistical difference in the expression of VEGF between the two incubator systems (n = 4; *P* = 0.133), making both systems appropriate to use in future experiments.



Vascular endothelial growth factor (VEGF) expression in ASC-21 cultured and processed in either the standard incubator system or the BioSpherix glove box. The effect of incubation on the expression of VEGF was determined by real-time RT-PCR and normalized to the expression of the reference gene PPIA (n = 4). Data are represented as mean ± SEM.

10 Appendix II: Results of Migration Experiment



Results of migration experiment. ASCs transfected with GFP in co-culture with HDMECs stained with Hoechst 33342. The cultures have been separated by a 4 mm stainless steel ring leaving a “wound”. Left column (0 h); pictures are taken right after the removal of the stainless steel ring. Right column (96 h); pictures taken after 96 h of incubation in the time-lapse incubation system. N, normoxic treated ASCs; NT, trypsin treated ASCs, H, hypoxic treated ASCs; HT, hypoxic and trypsin treated ASCs. All pictures are taken in duplicate wells; the second well is marked 1. Scale bar denotes 500 µm.