Vascularization and Host Response in Bone Tissue Engineering

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Dedication:

This thesis is dedicated to my beloved parents and my husband

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SCIENTIFIC ENVIRONMENT

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ABBREVIATIONS

ANG	Angiopoietin
ASC	Adipose stem cells
AV	Arteriovenous
CD	Cluster of differentiation
CDH	Cadherin
cDNA	Complementary deoxyribonucleic acid
CO_2	Carbon dioxide
CTRL	Control
CXCL	C-X-C motif chemokine ligand
EC	Endothelial cells
EGF	Epidermal growth factor
EGM	Endothelial cell growth medium
ELISA	Enzyme-linked immunosorbent assay
EPC	Endothelial progenitor cells
EPO	Erythropoietin
FBS	Fetal bovine serum
FGF	Fibroblast growth factor
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
HA	Hydroxyapatite
HE	Hematoxylin and eosin
HIF	Hypoxia inducible factor
HRE	Hypoxia response elements
IFN	Interferon
IGF	Insulin growth factor
IGFBP	Insulin-like growth factor-binding protein
IL	Interleukin
KGF	Keratinocyte growth factor
MAPK	Mitogen-activated protein kinase
MCGS	Mesenchymal cell growth supplement

MMP	Matrix metalloproteinase
MSCGM	Mesenchymal stem cell growth medium
MSC	Mesenchymal stem cells
mTOR	Mammalian target of rapamycin
MyD	Myeloid differentiation primary response
N_2	Nitrogen
NF	Nuclear factor
NIMP	Anti-neutrophil antibody
NK	Natural killer
NO	Nitric oxide
NOD/SCID	Non-obese diabetic severe combined immunodeficiency mouse
iNOS/NOS_2	Inducible nitric oxide synthase
NOV	Nephroblastoma overexpressed
O_2	Oxygen
OCT	Optimum cutting temperature compound
RT-PCR	Real-time reverse transcription polymerase chain reaction
PDGF	Platelet derived growth factor
PECAM	Platelet/endothelial cell adhesion molecule
PGA	Polyglycolic acid
PGE	Prostaglandin E
PHD	Prolyl-hydroxylase domain
PI3K	Phosphoinositide 3-kinase
poly(LLA-co-DXO)	poly(L-lactide-co-1,5-dioxepan-2-one)
RNA	Ribonucleic acid
SDS	Sodium dodecyl sulfate
SERPIN	Serine protease inhibitor
SMC	Smooth muscle cells
TCP	Tricalcium phosphate
TEVG	Tissue-engineered vascular graft
TLR	Toll-like receptors
TNF	Tumor necrosis factor

TNFR	Tumor necrosis factor receptor
TPO	Thrombopoietin
VDJ	Variable, diversity, and joining genes
VEGF	Vascular endothelial growth factor
VHL	von Hippel-Lindau

ABSTRACT

The major hurdle in the survival of a tissue-engineered construct after implantation is vascularization. Pre-vascularization is one of the proposed methods to achieve vascularization in tissue-engineered constructs. This thesis is centered on pre-vascularization of the tissue-engineered construct and local host response upon implantation *in vivo*.

In **Study I**, mesenchymal stem cells (MSC) with and without endothelial cells (EC) were cultured in normoxic and hypoxic conditions *in vitro*. The conditioned medium from each group were evaluated with functional assays. The secretomes from hypoxic mono- and co-culture groups demonstrated increased wound healing and tube formation *in vitro* when compared with their normoxic counter-parts.

In **Study II**, pre-vascularized constructs were generated by co-culturing MSC and EC in three dimensional copolymer poly (LLA-co-DXO) scaffolds. After *in vivo* implantation, local tissue response was evaluated with gene and protein analysis. The constructs with both MSC and EC were associated with decreased pro-inflammatory cytokines compared with those having MSC alone.

In **Study III**, three-dimensional constructs with either MSC or EC alone were implanted and their angiogenic and immunogenic property were evaluated. The expressions of anti-inflammatory and vasculogenic cytokines were higher in the constructs with EC than in the constructs with MSC. Furthermore, down-regulation of pro-inflammatory cytokines was observed.

In conclusion, MSC in combination with EC developed microvessels-like structures before implantation *in vivo*. Hypoxia, a common consequence after implantation of constructs, accelerated wound healing and tube formation via paracrine effect of MSC. Combining these two effects might lead to inosculation and survival of a graft. Addition of EC improved the immunomodulatory property of MSC.

List of publications

The doctoral thesis is based on the following studies

I

Bartaula-Brevik S, Bolstad AI, Mustafa K, Pedersen TO. Hypoxia preconditioning of mesenchymal stem cells accelerates wound healing and vessel formation *in vitro*. (S*ubmitted manuscript)*

II

Bartaula-Brevik S, Pedersen TO, Blois AL, Papadakou P, Finne-Wistrand A, Xue Y, Bolstad AI, Mustafa K. Leukocyte transmigration into tissue-engineered constructs is influenced by endothelial cells through Toll-like receptor signaling. *Stem Cell Research and Therapy*. 2014; 5(6):143.

III

Bartaula-Brevik S, Pedersen TO, Finne-Wistrand A, Bolstad AI, Mustafa K. Angiogenic and immunomodulatory properties of endothelial and mesenchymal stem cells. *Tissue Engineering Part A*. 2016; 22(3-4):244-52.

1. Introduction

1.1 Tissue Engineering

In the field of tissue engineering, clinicians, scientists and engineers work together to develop functional substitutes to restore damaged tissues. Cells, signaling molecules, growth factors and scaffolds together hold the potential to generate new functional tissue. Tissue engineering as a field has grown quickly over the past decades, and is no longer confined to building artificial biological tissues in the laboratory. An increasing number of clinical trials have been conducted, bringing tissue engineering closer towards establishing itself as a realistic treatment modality to enhance and restore the function of diseased and damaged tissues in patients (1, 2). In order to reconstruct cranio-maxillofacial hard-tissue defects in humans, tissue-engineered constructs with autologous stem cells and resorbable scaffolds were implanted. Out of 13 patients, 10 were successfully treated and hard-tissue formation was observed (3). Treatment of patients with large bone defects however, still presents a challenge for clinicians.

Bone regeneration is a complex biological process comprised of a series of well-orchestrated events. Bone grafting is a common clinical procedure in orthopedic and maxillofacial surgery and autologous bone grafts are currently considered as the ‘gold standard’ (4). The current approaches for bone replacement or enhancing bone regeneration are autologous bone grafts, but also include allografts and various bone substitute materials, with or without the addition of growth factors.

Synthetic or natural bone substitute materials can be used as alternatives to autologous and allogenic bone grafts, and in order to reconstruct large defects a scaffold may be needed to restore its shape and size. A challenge in dealing with large defects is the replacement of complex vascularized tissue.

The addition of another dimension, the mechanical environment, to the basic 3 point or triangular diagram used to illustrate the concept of tissue engineering (cells, biomaterials and signaling molecules) leads to the formation of a 4 point diamond

shaped structure (Fig. 1). This diamond structure has two V's, which emphasize the importance of vascularization, and thus the need for a functional blood supply in tissue engineering. The diamond concept also indicates the importance of the host response for successful tissue regeneration (5, 6).

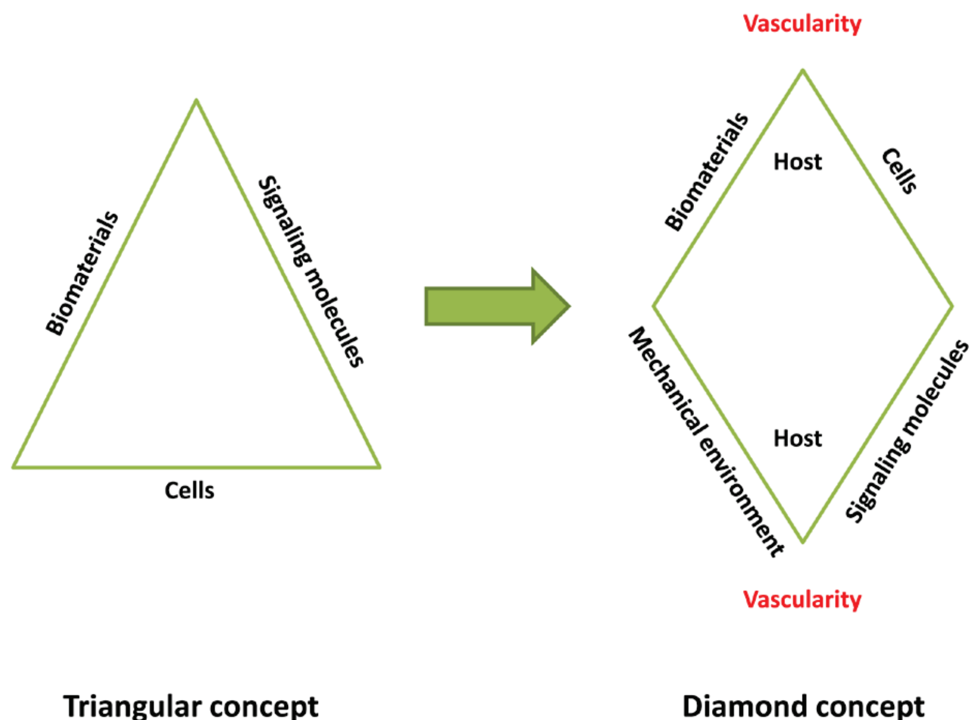


Figure 1. Evolution of the diamond concept from the basic triangular concept. Figure inspired by (5, 6).

1.1.1 Rationale for vascularization

Every year millions of soft- and hard- tissue grafts are performed worldwide. The major challenge is in obtaining vascularization of the implant and its integration with the host vasculature (7, 8). The vascular system provides nutrients and oxygen and removes waste products from cells, tissues and organs via larger blood vessels which ultimately subdivide into small capillaries. The inter-capillary distance is between

150 and 200 µm, and cells remain alive by oxygen diffusion when they are within this distance as shown in Fig. 2 (7, 9).

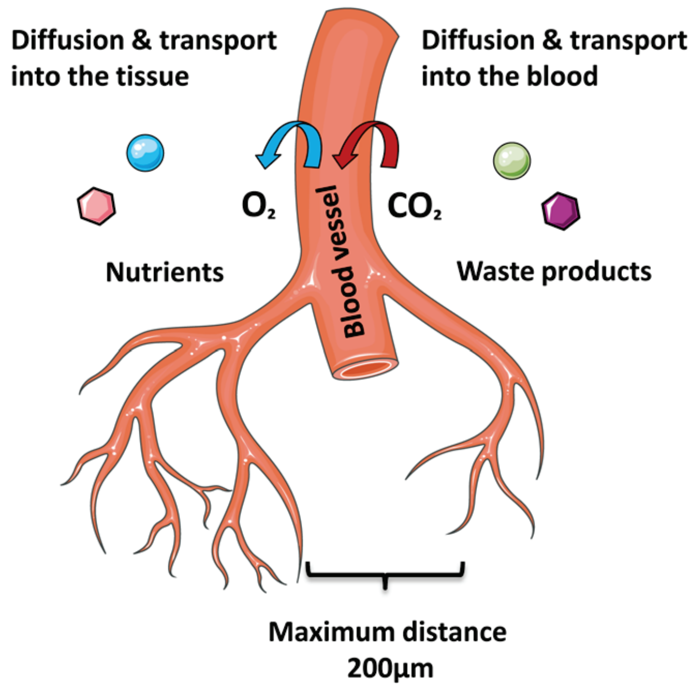


Figure 2. Schematic representation of inter-capillary distance, diffusion, and transport to and from the tissue. Figure modified from (7).

Vascularization is a major determinant for success during healing, tissue regeneration and treatment of ischemic disorders. Blood vessels provide adequate oxygen for the implanted constructs and aid in survival of the tissue-engineered constructs. The vascular system supplies nutrients and oxygen, removes waste products and acts as an immune barrier against foreign bodies (10).

Cells in the epidermis of the skin, cartilage and cornea maintain their viability due to diffusion of oxygen and nutrients from adjacent blood vessels, and regeneration of these tissues has therefore been quite successful. In reconstruction of thicker and more complex tissues, an adequate blood supply is required for cell survival within the grafts. A major concern is to maintain perfusion of tissue-engineered

microvascular networks from *in vitro* culture conditions through the *in vivo* conditions of a transplanted graft.

Vascularization of the implanted construct is needed to prevent graft necrosis and facilitate integration of the host vasculature with the graft. There are two main mechanisms for graft vascularization: inosculation and neovascularization. The term inosculation describes a coordinated process of anastomosis of the host vasculature to the capillaries within the graft (11, 12). Neovascularization is facilitated by a rapid proliferation of endothelial cells (EC) into microvascular networks that undergo a maturation process required for the survival of the graft.

1.1.2 Implantation of endothelial cells for vascularization

In 1999, the first clinical application of a tissue-engineered vascular graft (TEVG) was performed in a 4-year old child. The graft was prepared from autologous cells derived from a peripheral vein and seeded onto a tubular scaffold [50:50 copolymer of ε-polycaprolactone–polylactic acid reinforced with woven polyglycolic acid (PGA) fibers] (13). In 2001, a human trial was done to evaluate TEVG in patients with single ventricle physiology. Mononuclear cells from autologous bone marrow were seeded onto a biodegradable scaffold composed of PGA and ε-caprolactone/L-lactide, and 25 grafts were implanted. Over a follow-up period of 6 years the study showed patent and intact grafts with successful vascular integrity (14). Asahara *et al.* isolated endothelial progenitor cells (EPC) from peripheral blood vessels and demonstrated neovascularization (15), and since then extensive experimental and clinical research has been done. Pre-clinical and clinical studies have been performed using EPC for the treatment of coronary artery diseases, demonstrating promising results (16, 17).

1.2 Vascular Development

During embryonic development the vascular plexus is formed by two physiological mechanisms: vasculogenesis and angiogenesis. During early stages of embryogenesis, the vascular plexus is formed by differentiation of angioblasts or stem cells, termed

vasculogenesis. Angiogenesis is the formation of capillaries from pre-existing blood vessels (18). Vasculogenesis was previously thought to occur only in embryonic development, but it has now been shown that adult vasculogenesis is also possible (15). Angiogenesis is either physiological or pathological. During wound healing and the ovarian cycle physiological angiogenesis takes place. Pathological angiogenesis occurs during tumor development, retinopathies, rheumatoid arthritis, psoriasis and in development of hemangiomas (19, 20).

1.2.1 Vessel formation and maturation

Blood vessel formation is a dynamic process which includes proliferation of EC, migration, lumen formation, branching, remodeling, pruning and recruitment of mural cells (21). The EC are normally in a quiescent state and get activated after receiving pro-angiogenic signals from vascular endothelial growth factor (VEGF), angiopoietin (ANG) and fibroblast growth factors (FGFs) (22). In the quiescent state, EC and pericytes share a common basement membrane. During the process of angiogenesis, the basement membrane is degraded by various proteolytic enzymes such as matrix metalloproteinases (MMP). The matrix degradation facilitates migration and proliferation of EC and release of different growth factors needed for angiogenesis (23). The EC come in contact with the provisional extracellular matrix and this matrix supports proliferation of EC. Then, mural cells are recruited along with the re-establishment of extracellular matrix to stabilize the immature vessel (21). It has been also reported that MSC could act as mural cells supporting EC to form microvascular networks (24).

1.2.2 Role of hypoxia in angiogenesis

Embryonic and developing tissues experience physiological hypoxia which results in angiogenesis. Hypoxia also occurs in wound healing, inflammation and tumor formation (25). Limited oxygen supply leads to a hypoxic environment, which triggers the release of hypoxia inducible factors (HIF). The oxygen concentration determines the fate of HIF transcription and release of angiogenic molecules (26). With normal oxygen concentration, HIF-1α is hydroxylated by the prolyl-

hydroxylase domain (PHD). Hydroxylated HIF is recognized by the von Hippel-Lindau (VHL) tumor suppressor protein and is ubiquitinated. Finally, the ubiquitinated complex results in degradation of HIF-1α. However, under hypoxic conditions, hydroxylation of HIF-1α is inhibited, thus preventing degradation of HIF-1α. As a result, HIF-1α accumulates and dimerizes with HIF-1β or aryl hydrocarbon receptor nuclear translocator (ARNT) and binds to hypoxia-response elements (HRE). This transcription complex results in up-regulation of angiogenic factors (27) (Fig. 3).

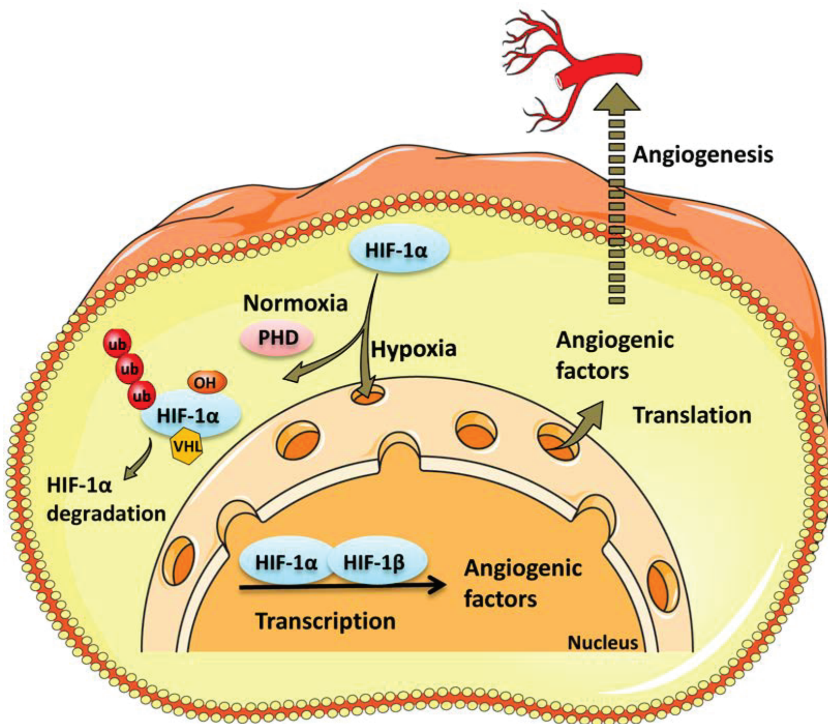


Figure 3. Regulation of hypoxia inducible factor (HIF) activity. Figure inspired by (26).

1.3 Pre-vascularization in Tissue Engineering

1.3.1 Vascular scaffold

Microfabrication techniques such as soft-lithography, photo-lithography and micropatterning have been used to pre-treat the two-dimensional (2D) surface to allow cell adhesion and repulsion on the micrometer scale (28). Further, an elastomer-based capillary network created by micropatterning technique and then seeded with EC has been used to generate tissue-engineered microvasculature (29). In addition, a network pattern created by optical lithography and patterned EC transferred to extracellular matrix has been shown to generate a tubular structure, with functional vessels observed after implantation *in vivo* (30). These approaches, however, were limited by the ability to only perfuse thin tissues. With recent advances, three-dimensional (3D) bio-printing has shown promising potential to engineer thick vascularized tissues. For example, a lumen size of 0.5-1mm was constructed by 3D printing, and EC and mural cells were introduced into those vascular channels. Remodeling and maturation of the channel led to capillary network growth (31). EC printed into hydrogels were able to form a luminar network, with the parameters of biological laser printing adjusted to guide the orientation and size of the networks (32). Production of vascular scaffolds *in vitro* is currently being studied by many in an effort to optimize vascularization *in vivo*.

1.3.2 *In situ* pre-vascularization

A pre-vascularized construct can be achieved using a three-step approach. First, the scaffold is implanted into a well-vascularized and easily accessible part of the body to generate a microvascular network, for example by subcutaneous implantation (33). Then, the scaffold is excised after pre-vascularization and finally, the pre-vascularized construct is transplanted into the defect site. Using this approach, the body acts as a natural bioreactor (34). However, this process is time-consuming and can be associated with unwanted ingrowth of granulation tissue onto the scaffold (33, 34). The reperfusion of the scaffold takes about 3-6 days and the pre-vascularized

construct can survive the first critical days after implantation until they are anastomosed with host vasculature (33).

Another approach is a flap technique where the scaffold is directly implanted to a muscle where vascular ingrowth from the surrounding tissue can take place. The pre-vascularized construct along with the entire free-flap is then transferred to the defect site and the vascular pedicle of the flap is surgically anastomosed to vessels at the site of implantation (35-37). This process gives the opportunity to perfuse the construct from the time of implantation but results in a large defect at the donor site where the pre-vascularized flap is generated (38).

Another approach, the arteriovenous (AV) loop technique, results in an intrinsic vascularization where the center of the construct becomes vascularized first (39). The loop is placed inside a polycarbonate growth chamber with extracellular matrix to generate vascularized tissue, which can later be implanted and anastomosed to the defect site. The greatest advantage of this technique compared to the flap approach is the lack of morbidity at the donor site (40). The AV loop technique has been used to generate pre-vascularized bone to reconstruct a critical-size mandibular defect in goats. In this preclinical model, a β-tricalcium phosphate/hydroxyapatite (βTCP/HA) scaffold with AV-groove was created to allow direct anastomosis of the construct with facial vessels. This approach resulted in vascularized constructs and enhanced bone formation (41, 42).

1.3.3 Co-culture strategies

EC have widely been investigated aiming for pre-vascularized tissue-engineered constructs. However, autologous mature EC have some limitations. Isolation of tissue specific EC is relatively invasive, it can be difficult to harvest large number of cells and the cells may have a low proliferation rate *in vitro* (43). EPC can be isolated from bone marrow or peripheral blood, and can be cultured and expanded *in vitro* (44, 45). They have been suggested as an alternative to mature EC.

During angiogenesis EC interact with other cells, extracellular matrix and growth factors (21). EC and parenchymal cells interact during organogenesis (46). Considering this natural process, researchers have started to make constructs where EC are co-cultured with different cells. This process has resulted in the assembly of EC to form vascular networks (47). EC co-cultured with dermal fibroblasts and keratinocytes have formed pre-vascularized skin grafts that demonstrated inosculation after transplantation *in vivo* in a murine model (48). During this three-way interaction, extracellular matrix is released by fibroblasts and EC can form capillary-like structures within the matrix, while keratinocytes express VEGF and promote angiogenesis (49).

Mural cells are essential to provide the physical support needed for vessel development, remodeling and stabilization (50). Co-culturing EC with mural precursor cells can generate long lasting stable microvascular networks. For instance, microvascular networks created with this approach survived for 1 year *in vivo*, whereas in the experimental group with only EC, the tissue-engineered micro-vessel regressed over time (51). Further, MSC and EC interact to form capillary-like structures in Matrigel. There, MSC differentiate into smooth muscle cells and pericytes which facilitate extracellular matrix formation and vessel stabilization (52). Co-seeding of MSC and EC can generate tissue-engineered constructs with an intrinsic blood supply. Such pre-vascularized constructs can anastomose with the host vasculature after implantation *in vivo* (Fig. 4) (53, 54). MSC act as perivascular cells and promote angiogenesis (24).

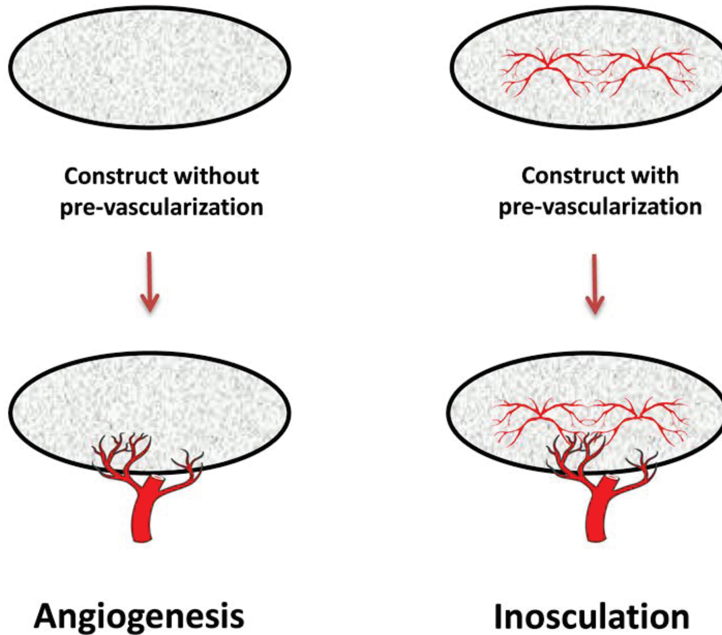


Figure 4. Schematic diagrams of in-growth of host vasculature in tissue-engineered constructs without pre-vascularization by angiogenesis and interconnection of the pre-vascularized construct with host vasculature by inosculation. Figure modified from (12).

1.4 Angiogenesis and Inflammation: Cause or Effect?

Angiogenesis and inflammation are interrelated and share some common molecular pathways. After tissue injury, a cascade of molecular events occurs. The inflammatory markers activate resting EC to initiate angiogenesis. Activation of the Tie-2 receptor by ANG-1 down-regulates nuclear factor-κB (NF-κB), a transcription factor regulating inflammation. On the other hand, inflammatory molecules induce the release of ANG-2 by EC. ANG-2 binds to the Tie-2 receptor and up-regulates NF-κB. ANG-2 stimulates EC to produce VEGF for angiogenesis (55, 56). Similarly, macrophages play an important role during angiogenesis. Macrophages express the Tie-2 receptor and release pro-angiogenic factors to initiate angiogenesis and anastomosis (57) (Fig. 5).

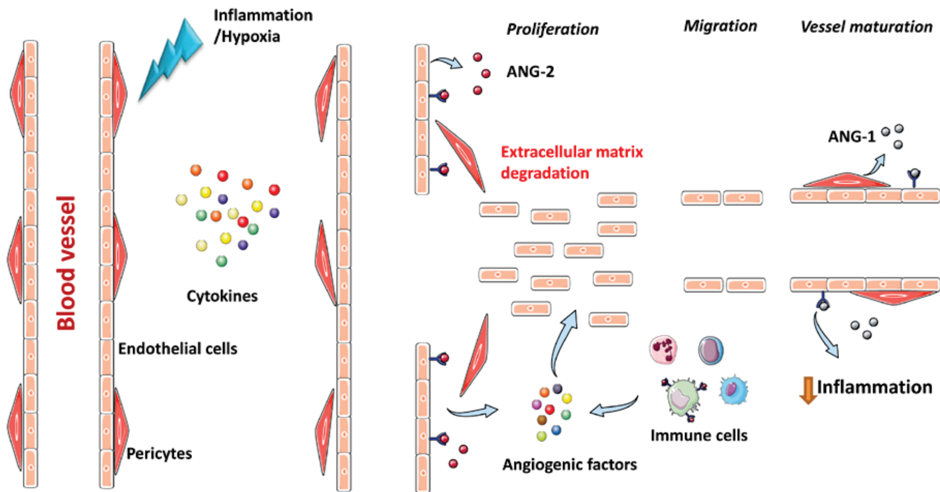


Figure 5. Relationship between angiogenesis and inflammation. Figure based on (23, 57).

1.5 Host Response

1.5.1 Inflammatory response after implantation of biomaterials

The basic function of biomaterials is to provide structural support to the defect area and facilitate tissue regeneration. Biomaterials may restore and augment the bio-physical function of damaged and diseased hard and soft tissues following replacement and subsequent regeneration (58, 59). Synthetic biomaterials are foreign bodies to the host. However, implantation should not induce undesirable local or systemic effects, which is broadly termed as the biocompatibility of a material. The interaction between the scaffold and local tissue greatly influences the outcome of the therapy (60). A recent description of biocompatibility which includes both biomaterial and the host refers to an "intrinsically biocompatible system" described as being a property of the system and its effect on the material (61). The physical, chemical and mechanical properties of the biomaterial can also influence the host response (62). A series of local events take place following implantation of a biomaterial, potentially including tissue injury, blood-material interactions,

provisional matrix formation, acute inflammation, chronic inflammation, granulation tissue formation, foreign body reaction and fibrous capsule development (63, 64).

1.5.2 MSC and inflammatory niches

Adult stem cells are available in almost all parts of the body. MSC have been successfully isolated from bone marrow, adipose tissue, nervous tissue, amniotic fluid, umbilical cord, menstrual blood and dental pulps (65-67). MSC can differentiate into osteoblasts, chondrocytes, adipocytes, myocytes and neural-like cells in specialized culture conditions (68, 69). An *in vitro* study revealed that MSC could also differentiate into endothelial-like cells (70). Intra myocardial injection of MSC in a chronic canine ischemic model resulted in MSC differentiation into EC and increased vascularity (71). Human MSC cultured in endothelial growth supplements, under shear force and extracellular matrix stimuli showed endothelial-like properties. These endothelial-like MSC expressed endothelial markers CD31, VEGF receptor 2 and von Willebrand factor (72). However, MSC differentiation into the endothelial lineage is still controversial (73). Co-culture of MSC and EC can be utilized to create pre-vascularized tissue-engineered constructs which upon *in vivo* transplantation contains an intrinsic blood supply (53, 54, 74, 75). MSC not only differentiate into a variety of tissue lineages but also have immunomodulatory properties (76) (Fig. 6).

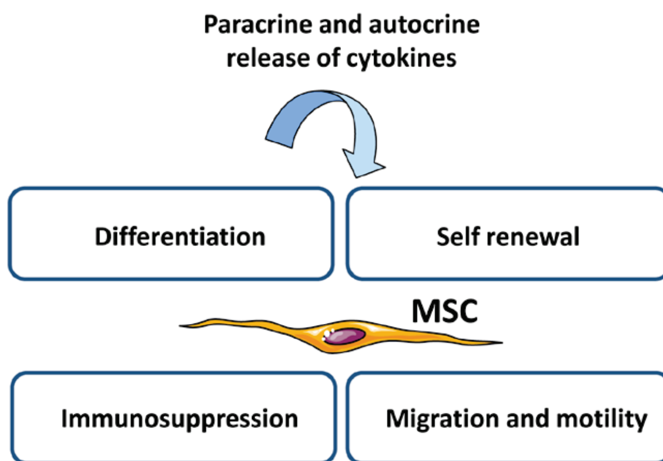


Figure 6. Characteristics of MSC in vitro and in vivo. Figure modified from (76).

Pericytes from capillaries, microvessels, adventitial cells and other blood vessels can produce MSC-like cells *in vitro*, cells that can differentiate into different cell lineages (77). In addition to the differentiation potential of pericytes, like MSC they release different secretomes essential for tissue regeneration (78). Perivascular cells from different parts of the body in fact express the MSC surface markers CD44, CD73, CD90 and CD105 (79).

MSC are found generally in close proximity to blood vessels after implantation *in vivo*. They express the pericyte markers $CD146^{+}$, $CD34^{-}$, $CD45^{-}$ and $CD56^{-}$. These facts support the concept that MSC are pericytes and blood vessels can act as a stem cell niche (80). MSC secrete multiple bioactive molecules, which are activated after tissue injury. Like those from pericytes, these secretomes have tropic and immunomodulatory functions (81) (Fig. 7). However, although there are many similarities between MSC and pericytes, MSC can also be isolated from interstitial tissues and tissues surrounding both arteries and veins, while pericytes are found localized on the basement membrane of capillaries (82, 83).

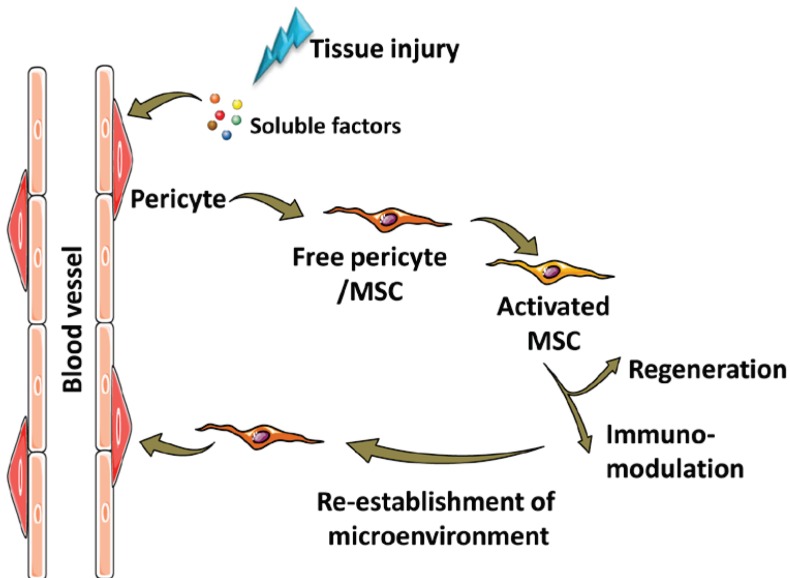


Figure 7. Microenvironment niche: from pericyte to activated MSC for regeneration and immunomodulation. Figure inspired from (81, 84).

It has also been shown that not all MSC exhibit the properties of pericytes (85). Pericytes differentiate into other cell lineages, are involved in inflammatory processes and may be involved in the matrix formation process in wound healing. However, they do not accelerate wound healing in a similar manner to MSC (86).

The exact mechanism of MSC homing to a site of injury is still unclear. It has been proposed that after tissue injury, various cytokines are released and stimulate the newly migrated MSC to release growth factors. This microenvironment orchestrates EC, fibroblasts, immune cells and inflammatory molecules as well as stem cells to promote angiogenesis. These changes locally result in the recruitment and differentiation of intrinsic MSC leading to tissue regeneration (87).

MSC are referred to as guardians of inflammation. After injury or infection a variety of pro-inflammatory cytokines are released from activated macrophages, which activates MSC to secrete anti-inflammatory molecules and results in reduction of pro-inflammatory molecules. MSC can thus suppress inflammation following tissue injury (88, 89).

The immunobiology of MSC is a double-edged sword. It has been reported that MSC have both immunosuppressive and immune-enhancing properties depending on the inflammatory status. That is, the inflammatory cytokine and chemokine levels determine the immunomodulatory properties of MSC (87, 90). This bi-directional immune-regulatory function of MSC is illustrated in Fig. 8.

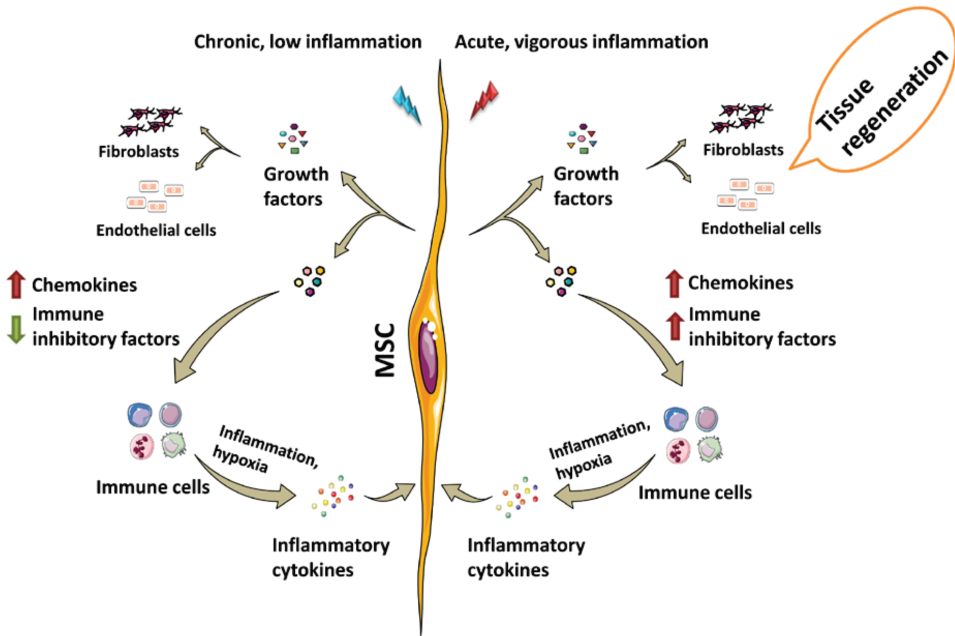


Figure 8. Bidirectional immune-regulatory function of MSC. Figure based on (87).

Bioactive molecules for tissue regeneration are secreted in different amounts in response to the local microenvironment. There is no precise dose of bioactive molecules released and the exact amount of cytokines cannot be measured *in vivo* (84). The inflammatory microenvironment regulates the secretion of bioactive molecules from MSC. The degree of inflammation determines the immunomodulatory properties of MSC, which is also associated with the initial activation of MSC. A wide range of pro-inflammatory cytokines secreted by immune cells activates MSC (91) (Fig.9).

Pro-inflammatory cytokines such as tumor necrosis factor- α (TNF-α), interleukin-1α or interleukin-1β (IL-1α or IL-1β) in the presence of interferon-γ (IFN-γ) induce MSC to secrete increased levels of chemokines and inducible nitric oxide synthase (iNOS/NOS_2). The cytokine induced iNOS produces nitric oxide (NO), which is an essential factor for T-cell suppression by MSC. However, although cytokine primed MSC can attract T-cells, in the absence of NO there is an increase in the inflammatory response (92). In response to tissue injury and released soluble factors

macrophages polarize into the classical M1 phenotype and the alternative M2. The M1 phenotype is pro-inflammatory and is stimulated by the toll-like receptors (TLR) ligand and IFN-γ, whereas the M2 phenotype is anti-inflammatory and activated by IL-4/IL-13, corresponding to polarization of T-cells into Th1 and Th2 (93) (Fig.10). MSC may influence the polarization of macrophages by downregulating the production of pro-inflammatory cytokines and up-regulating the release of anti-inflammatory cytokines. In a sepsis model, it has been shown that tumor necrosis factor receptor (TNFR) and TLR4-mediated NF-κB signaling upregulate synthesis of prostaglandin E_2 (PGE_2) by MSC. PGE_2 binds to PGE_2 receptors 2 and 4 (EP2 and EP4) in macrophages to produce IL-10. MSC ameliorate sepsis by inducing the production of IL-10 by macrophages and alternatively activating M2 macrophages (94).

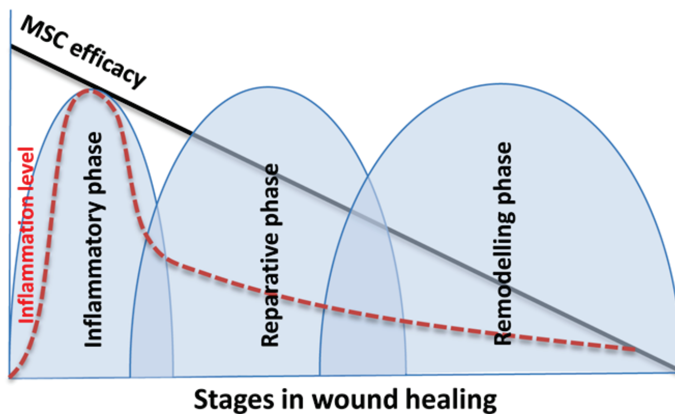


Figure 9. Schematic representation of the stages in wound healing: inflammatory phase, reparative phase and remodeling phase. The degree of inflammation determines the fate of MSC activity. Figure modified from (91).

1.5.3 Modulation of MSC via TLR

TLR are expressed by MSC at the mRNA and protein levels. TLRs 1, 2, 3, 4, 5 and 6 are expressed at the mRNA level and TLRs 2, 3, 4, 7 and 9 are expressed at the protein level (95). TLR stimulation of MSC not only takes part in immune-modulation but also activates survival, proliferation, migration and differentiation of

MSC. TLR activation in MSC may be initiated either by MyD88 dependent or independent signaling. This signal cascade results in downstream activation of NF-κB, MAPKs and PI3K, resulting in secretion of different TLR, cytokines and chemokines (96). MSC isolated from MyD88 knock out mice failed to differentiate into osteocytes and chondrocytes, suggesting the importance of TLR and their ligands in directing the fate of MSC (97). MSC treated with TLR ligands showed increased migration in a transwell system compared to non-treated MSC, while pretreatment with anti-TLR3 antibodies prior to ligand activation inhibited MSC migration. This demonstrated that MSC migration is dependent on the presence of TLR3 (98).

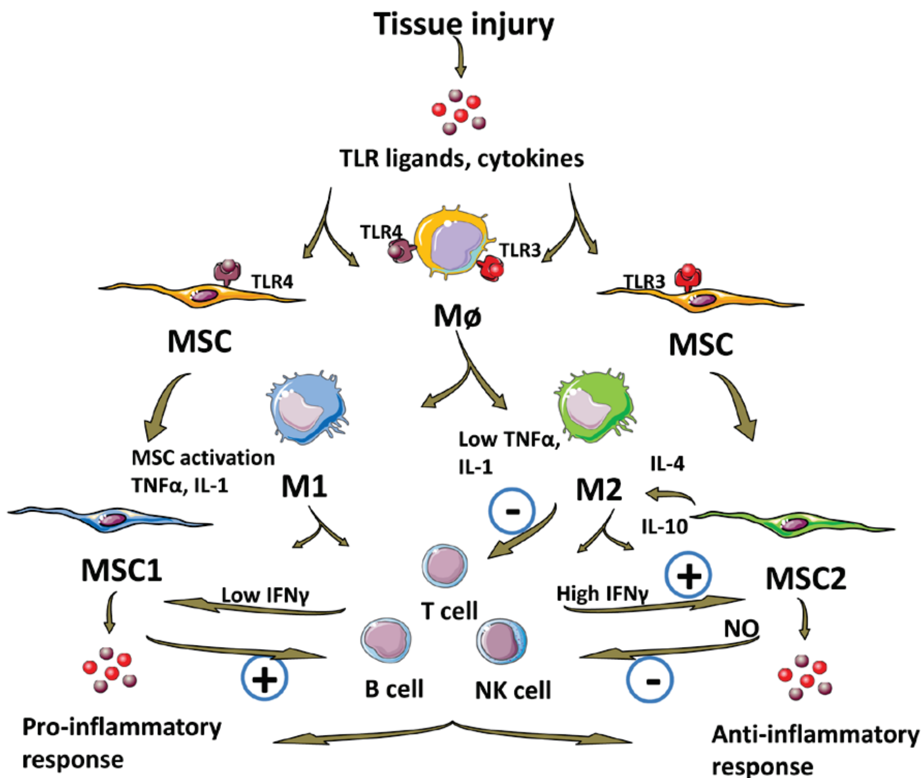


Figure 10. TLR3- and TLR4-dependent polarization of monocytes and MSC. Figure modified from (95).

1.6 Rationale

Survival of MSC after implantation is critical, although the long term effect is dependent on the residing MSC. Therefore, it is important to recruit the resident MSC while simultaneously ensuring survival of the graft. In large grafts the main obstacle is angiogenesis, with the host vasculature unable to perfuse the tissue-engineered constructs immediately after implantation. A pre-vascularized graft is therefore needed to meet this requirement and to keep the implanted MSC-construct viable and facilitate its role in tissue regeneration.

Different co-culture strategies have been employed to increase the osteogenic and vasculogenic properties of MSC. Co-culture of MSC and EC has been used for pre-vascularization of tissue-engineered constructs (47). Inflammatory cytokines and hypoxia can stimulate MSC to produce different growth factors, and these secretomes have direct effects on endothelial cells and fibroblasts promoting both angiogenesis and wound healing.

2. AIMS

The fundamental concept in vascular tissue engineering is to provide sufficient vascularity to the implanted construct. Basically, adequacy of blood supply determines the fate of the tissue-engineered construct.

The main focus of this thesis was to determine the local tissue response to the tissue engineered constructs.

The thesis is based on the following specific aims:

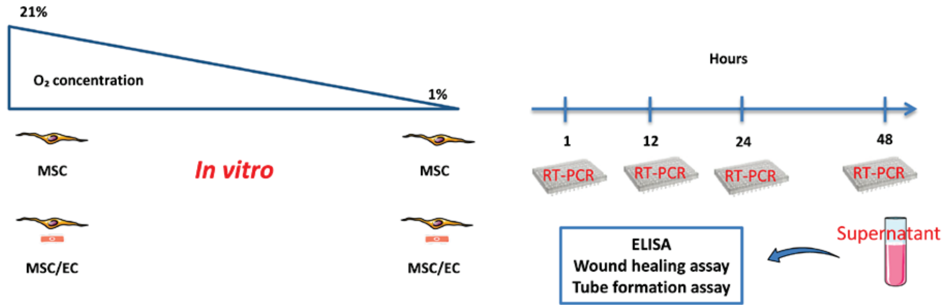
1. To study the effect of MSC and MSC/EC on wound healing and vessel formation *in vitro* under hypoxic conditions (Study I)

2. To assess the influence of pre-vascularized constructs on leukocyte transmigration *in vivo* (Study II)

3. To evaluate the *in vivo* host response and vessel formation after implantation of MSC and EC (Study III)

3. MATERIALS AND METHODS

Study I



Study II

Phase 1

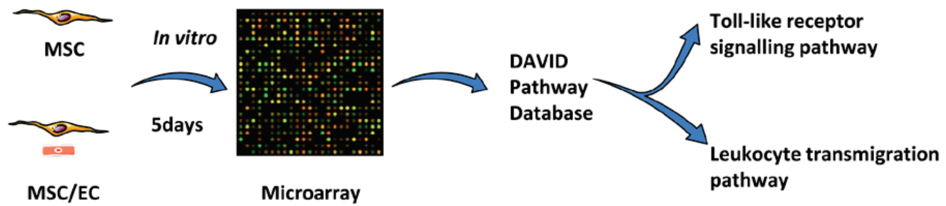


Figure 11a. Schematic summary of the study design.

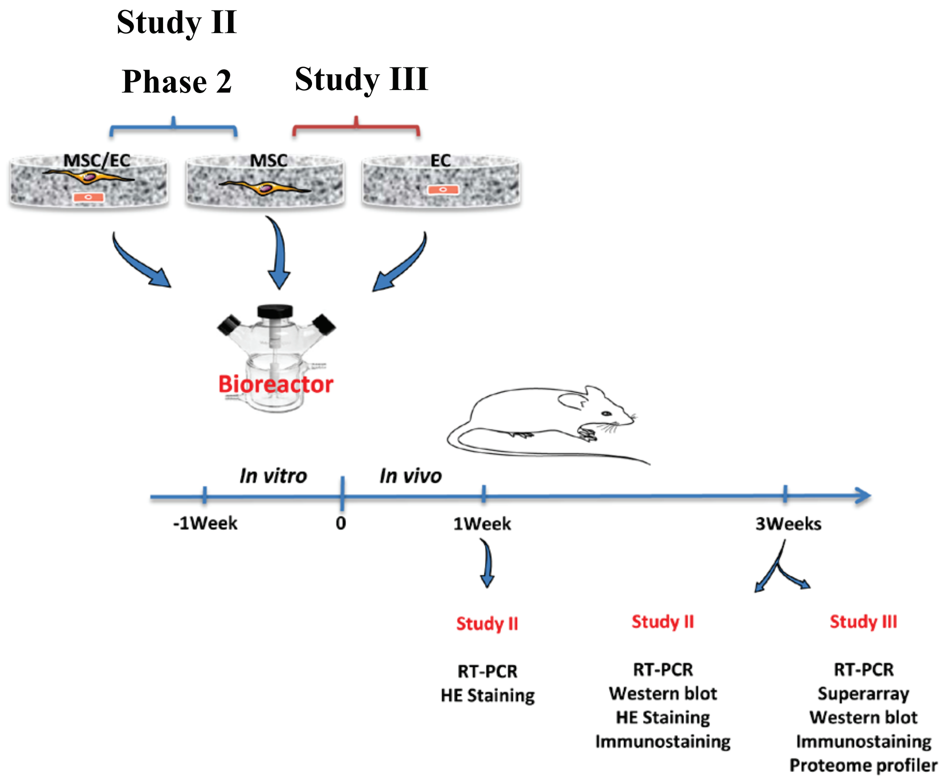


Figure 11b. Schematic summary of the study design.

The studies I, II and III are schematically illustrated in Fig. 11a and 11b. Study I, II and III corresponds to paper 1, 2 and 3 respectively.

3.1 Cell Culture (Studies I-III)

Primary human bone marrow derived MSC were purchased from Lonza (Walkersville, Maryland, United States) and expanded in Mesenchymal Stem Cell Growth Medium (MSCGM™) SingleQuots™ (Lonza) containing mesenchymal stem cell basal medium supplemented with mesenchymal cell growth supplement (MCGS), L-glutamine and GA-1000 (Gentamicin, Amphotericin-B) (**Study I**).

Primary human bone marrow derived MSC were purchased from StemCell Technologies (Vancouver, British Columbia, Canada) and cultured in MesenCult® complete medium (StemCell Technologies) following the manufacturer's instructions (**Studies II** and **III**).

Human umbilical vein endothelial cells (EC) were purchased from Lonza (Walkersville, Maryland, United States) and were cultured in Endothelial Cell Growth Medium (EGM) (Lonza) following the manufacturer's instructions (**Studies I - III**).

3.1.1 Co-culture of MSC and EC (Studies I and II)

MSC and EC were co-cultured in a 5:1 ratio to conduct **Studies I** and **II**. In **Study I** MSC and EC were separated using CD31 Endothelial Cell Dynabeads® (Invitrogen, Carlsbad, California, United States) according to the manufacturer's instructions.

3.1.2 Hypoxic Chamber (Study I)

A Modular Incubator Chamber (MIC-101) (Billups-Rothenberg Inc. Del Mar, California, United States) was used as a hypoxia chamber. First, the chamber was flushed and filled with a gas mixture of 1% O_2, 5% CO_2 and 94% N_2 and then placed inside the incubator.

3.2 Scaffolds (Studies II and III)

3.2.1 Fabrication of scaffolds

Poly (L-Lactide-*co*-1, 5dioxepan-2-one) [poly (LLA-co-DXO)] scaffolds were used in **Studies II** and **III** and were prepared as previously described (99, 100). Briefly, porous scaffolds with a dimension of 12 mm diameter, 1.5 mm thickness and pore sizes of 90-500 µm were produced from co-polymer poly (LLA-co-DXO) by a solvent-casting particulate-leaching method. The sterilization of scaffolds was carried out in a pulsed electron accelerator operating at 6.5 MeV (Mikrotron,

Acceleratorteknik, The Royal Institute of Technology, Stockholm, Sweden) with radiation dose of 2.5 Mrad in an inert atmosphere.

3.2.2 Preparation of scaffolds

The scaffolds were pre-seeded with cells before *in vivo* implantation and were treated as previously described (54, 101). Briefly, the scaffolds were pre-wet with complete medium and incubated overnight at 37 °C and 5 % CO_2. The next day, cells were seeded and allowed to attach overnight. An orbital shaker (Eppendorf®, Hamburg, Germany) was used in order to facilitate homogeneous distribution of cells. The following day, scaffolds were transferred to separate modified spinner flasks (Wheaton Science, Millville, New Jersey, United States) for 1 week in a dynamic culture system with 50 rotations per minute. After 1 week culture *in vitro*, 6 mm discs were punched from the center of the scaffold with a dermal skin punch and then implanted *in vivo* (Fig. 12).

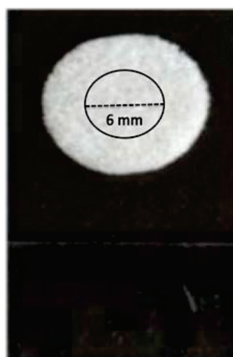


Figure 12. A macroscopic image of the poly (LLA-co-DXO) scaffold before cell seeding and implantation in vivo.

3.3 Animal Procedures (Subcutaneous mouse model) (Studies II and III)

Non-obese diabetic/severe combined immunodeficient (NOD/SCID) mice were used in **Studies II** and **III**. They were purchased from Taconic Farms (Bomholtgård Breeding and Research Center, Ry, Denmark). On the back of the mice an incision was made and subcutaneous pouches were created with blunt dissection which provided sufficient space for implantation of the scaffolds. Wounds were closed with Vetbond™ Tissue Adhesive (n-butyl cyanoacrylate) (3M™, St. Paul, Minnesota, United States). After 3 weeks' implantation, animals were euthanized with deep Isoflurane (Schering Plough, Kenilworth, New Jersey, United States) anesthesia followed by cervical dislocation and cell/scaffold constructs were dissected and retrieved. The samples were then divided and further processed for analysis.

3.4 Gene Profiling

3.4.1 Real-time RT-PCR (Studies I-III)

An E.Z.N.A.® Total RNA Kit (Omega Bio-Tek, Norcross, Georgia, United States) was used to isolate the RNA from the samples (**Study II** and **III**). In **Study I**, a Maxwell® 16 LEV simplyRNA Cells Kit (Promega Corporation, Madison, Winconsin, United States) was used for RNA isolation. A NanoDrop Spectrophotometer (ThermoScientific NanoDrop Technologies, Wilmington, Delaware, United States) was used to quantify and evaluate RNA purity. A total of 1000 ng of the total RNA was reverse transcribed to cDNA using a cDNA kit (Applied Biosystems, Carlsbad, California, United States). Real-time reverse transcription polymerase chain reaction (Real-time RT-PCR) was performed on a StepOnePlus™ real time PCR system (Applied Biosystems). TaqMan® gene expression assays (Applied Biosystem) were applied in all studies. GAPDH was used as endogenous control and data analysis was performed with a comparative Ct method (102).

Table 1. Overview of gene expression assays.

Assay ID	*Full name*	*Gene Symbol*	*Species*	*Study*
Hs99999905_m1	Glyceraldehyde 3-phosphate dehydrogenase	GAPDH	Human	I
Hs01555410_m1	Interleukin-1β	IL-1β	Human	I
Hs00985639_m1	Interleukin-6	IL-6	Human	I
Hs00174103_m1	Interleukin-8	IL-8	Human	I
Hs00961622_m1	Interleukin-10	IL-10	Human	I
Hs00900055_m1	Vascular endothelial growth factor-A	VEGF-A	Human	I
Hs00966526_m1	Platelet derived growth factor	PDGF	Human	I
Hs00266645_m1	Fibroblast growth factor	FGF	Human	I
Hs00375822_m1	Angiopoietin-1	ANG-1	Human	I
Hs01048042_m1	Angiopoietin-2	ANG-2	Human	I
Hs00171022_m1	Chemokine ligand 12	CXCL12	Human	I
VIC MGB	Glyceraldehyde 3-phosphate dehydrogenase	GAPDH	Mouse	II,III
Mm 00443260_g1	Tumor necrosis factor α	TNFα	Mouse	II,III
Mm 00434228_m1	Interleukin-1β	IL-1β	Mouse	II,III
Mm 00446190_m1	Interleukin-6	IL-6	Mouse	II,III
Mm 00445259_m1	Interleukin-4	IL-4	Mouse	II,III
Mm 00439614_m1	Interleukin-10	IL-10	Mouse	II,III
Mm 00440502_m1	Inducible nitric oxide synthase 2	iNOS (NOS_2)	Mouse	II,III
Mm 00468869_m1	Hypoxia inducible factor-1α	HIF-1α	Mouse	II,III
Mm 00507836_m1	Hypoxia inducible factor-1β	HIF-1β (ARNT)	Mouse	II,III
Mm 0044968_m1	Mammalian target of rapamycin	mTOR	Mouse	II,III

PCR Array (Study III)

Mouse TLR pathway Rt^2 Profiler PCR arrays (SuperArray Bioscience, Frederick, Maryland, United States) were performed. An Rt^2 PCR array First Strand Kit (SuperArray Bioscience) was used for cDNA synthesis. PCR array was done on a StepOnePlus™ real-time PCR system (Applied Biosystem) with Rt^2 Real-time SyBR Green/Rox PCR mix (SuperArray Bioscience).

3.4.2 **Microarray (Study II)**

The gene expression profile of MSC co-cultured with EC *in vitro* was previously published by our group (103) and the microarray data obtained from this study was further analyzed by J-Express 2009 software (104). A set of inflammatory genes were submitted to the DAVID database and the pathways were determined by KEGG pathway mapping (105).

3.5 Protein Isolation (Studies II and III)

The organic phase during RNA isolation was preserved at -80°C. Protein isolation was performed following the protocol of Chomczynski (106). Isopropanol was added to the organic phase and a precipitate was formed. The precipitate was washed with ethanol for five minutes and the process was repeated three times. After washing, the precipitate was dissolved in 0.5% sodium dodecyl sulphate (SDS) solution. Quantifications and determination of protein purity was performed with a NanoDrop Spectrophotometer (ThermoScientific NanoDrop Technologies).

3.6 Western Blotting (Studies II and III)

After measuring the protein concentration, 30 μg of total protein was mixed with 4X Laemmli sample buffer (Bio-Rad laboratories, Hercules, California, United States) and loaded on 4-15 % Mini-PROTEAN® TGX™ Precast Gel (Bio-Rad) for electrophoresis and transferred to PVDF transfer membranes (TRANS-Blot® Turbo™ System, Bio-Rad). The membranes were blocked in 5% BSA/TBST and incubated with primary antibodies (in 5% skimmed milk/TBST) as presented in Table 2 at 4°C overnight. After three washes with TBST, the membranes were incubated with appropriate secondary antibody (in 5% skimmed milk/TBST) as listed in Table 2 for 1 h. Immunoblotting bands were visualized by Immun-Star™ WesternC™ Chemiluminescence Kits and a Gel Doc™ EZ System (Bio-Rad) was used for imaging and protein-band assay.

Table 2. Overview of western blotting.

Abbreviation	*Full name*	*Species*	*Manufacturer*	*Study*
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase	Mouse	Santa Cruz	II,III
IL-1β	Interleukin-1β	Mouse	Abcam	II
IL-6	Interleukin-6	Mouse	Abcam	II
HIF-1α	Hypoxia inducible factor-1α	Mouse	Santa Cruz	III
HIF-1β(ARNT)	Hypoxia inducible factor-1β	Mouse	Santa Cruz	III

3.7 Proteome Profiling (Study III)

The Mouse Angiogenesis Array Kit (R&D Systems, Inc., Minneapolis, Minnesota, United States) was used to detect the expression of 53 angiogenesis related proteins. The Proteome Profiler Array was performed according to the manufacturer's protocol. Briefly, 100 μg of total protein was mixed with a cocktail of biotinylated detection antibodies and then incubated with a nitrocellulose membrane spotted with capture antibodies in duplicate. Protein-detection antibodies bound to the capture antibody were detected using Streptavidin-HRP and chemiluminescent detection reagents. The Gel Doc™ EZ System (Bio-Rad) was used for imaging. The mean spot pixel density was quantified using image software analysis.

3.8 ELISA (Study I)

The enzyme-linked immunosorbent assay kit (ELISA) (R&D Systems, Minneapolis, Minnesota, United States) was used to measure the concentration of VEGF-A in the samples, following the manufacturer’s instructions. Optical densities were determined using FLUOstar OPTIMA microplate reader (BMG LABTECH, Ortenberg, Germany).

3.9 Tissue staining and imaging (Studies II and III)

3.9.1 Tissue Preparation

The samples intended for cryosectioning were immediately embedded in optimal cutting temperature compound (Tissue-Tek® O.C.T., Sakura Finetek, Tokyo, Japan) and kept at -80°C. Samples intended for paraffin sectioning were fixed in 4% paraformaldehyde. The frozen samples were sectioned with a Leica CM 3050S (Leica Microsystems, Wetzlar, Germany) at -24°C with 8 μm thick sections, whereas paraffin- embedded samples were sectioned with a microtome (Leica).

3.9.2 Histological staining

Tissue staining was done as described in paper 2 and 3. The overview of the staining is presented in Table 3.

Table 3. Overview of histological staining.

Abbreviation	*Full name*	*Species*	*Manufacturer*	*Study*
IL-1β	Interleukin-1β	Mouse	Abcam	II,III
IL-6	Interleukin-6	Mouse	Abcam	II,III
NIMP	Neutrophil antibody	Mouse	Abcam	II,III
CD11b	Cluster of differentiation 11b	Mouse	BD	II,III
CD31	Cluster of differentiation 31	Mouse	BD	III
HE	Hematoxylin and eosin	Non-specific		II

3.9.3 Quantification of immunostaining

In Studies II and III, 5 sections from one scaffold were mounted on each slide. Each section on the slides was divided onto 5 measuring grids starting from top to bottom in the vertical direction (Fig. 13). The 5 sections on each slide (average for the mouse) and 5 measuring grids in each section (average for the section) were used for image quantification. Images were made with a Zeiss Axiovision 4.8.1 (Toronto, Ontario, Canada) and the files were exported as JPEG standard.

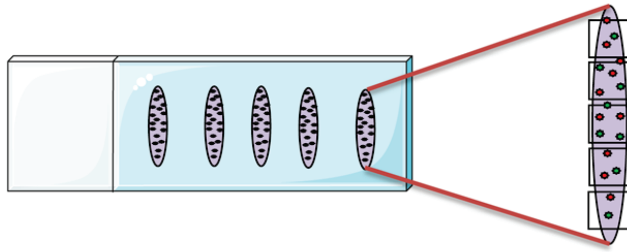


Figure 13. Schematic representation showing measuring grids for quantification.

3.10 Wound healing assay (Study I)

Approximately 30,000 EC/well were seeded in Culture-Insert 24 (80241, ibidi, Martinsried, Germany) until they were confluent. The inserts were removed and a monolayer wound was created. The cells were incubated with conditioned medium from hypoxic and normoxic groups and compared to EGM as positive control and vehicle media as negative control. Images were made at different time intervals (3, 6, 12, 18 and 24 h) with a Nikon Eclipse Ti microscope (Nikon, Tokyo, Japan) at 4x magnification using phase contrast mode. The surface area of the wound was calculated with NIS elements AR 3.2 software (NIS elements, Tokyo, Japan). The overview of wound healing assay is illustrated in Fig. 14.

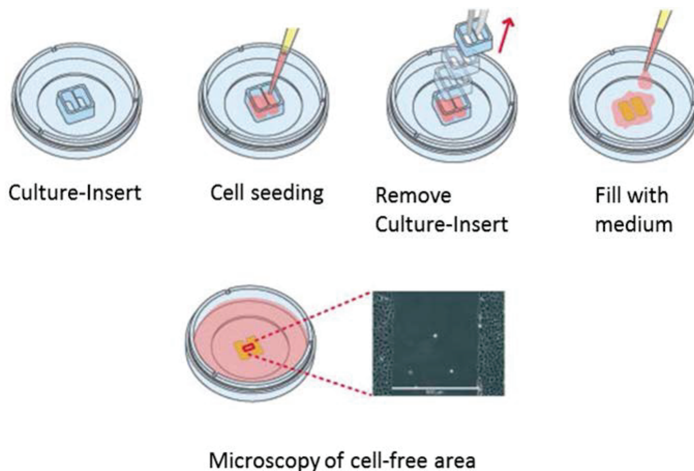


Figure 14. Steps in the wound healing assay (modified from ibidi.com).

3.11 Tube formation assay (Study I)

Growth Factor Reduced Matrigel^{TM} (BD Biosciences, San Jose, California, United States) was added to 96 well-plates and incubated to solidify. EC were suspended in the conditioned medium from hypoxic and normoxic groups and compared to EGM and vehicle media. The EC were seeded onto the solidified matrigel. After 6 h, tube formation was observed and images were made. The tube formation was quantified with the ImageJ software program (NIH, Bethesda, Maryland, United States) with the Angiogenesis Analyzer plugin (107). The schematic representation of the tube formation assay is shown in Fig. 15.

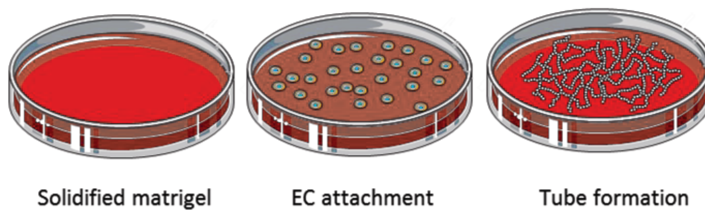


Figure 15. Steps in the tube formation assay.

3.12 Statistical Analysis (Studies I-III)

The significance level was set to $p<0.05$ for all statistical analysis. SPSS Statistics 21 (IBM, Armonk, New York, USA) was applied for statistical processing and analysis. Two groups were compared with the independent samples t-test, whereas a multiple comparison one-way ANOVA was performed to compare three or more experimental groups.

4. RESULTS AND SUMMARY OF PAPERS

4.1 Hypoxia preconditioning of MSC (Study I)

4.1.1 Hypoxia increased VEGF expression

Study I evaluated the effect of hypoxic culture conditions on MSC. The influence of hypoxia was assessed in both mono- and co-culture groups. The gene expression of VEGF-A was significantly higher in the hypoxic groups compared to the normoxic groups after 12 h incubation. After 48 h incubation, the protein level was relatively higher in hypoxic groups compared to the normoxic groups and the difference was statistically significant.

4.1.2 Hypoxia accelerated wound healing

The conditioned medium from hypoxic groups (HMSC and HMSC/EC) showed accelerated wound healing compared to normoxic groups *in vitro*. The wounded area was completely closed by 12 h in the positive control group. The cell-free area was completely filled with migrated cells within 18 h in HMSC and HMSC/EC groups. All groups were normalized to baseline data at time point 0 h.

4.1.3 Hypoxia increased vessel formation

The EC started to form tube-like structures within 6 h in the positive control medium. The tube formation in different experimental groups was normalized with vehicle medium as controls. The tube-like structures were quantified for various parameters such as number of nodes, meshes, segments and total lengths (108). The hypoxic groups exhibited more tube-like structures compared to their normoxic counterparts.

4.2 Host cell migration into the construct

The seeding efficiency of both MSC and EC in the scaffold was evaluated *in vitro* using the following equation (109).

Seeding efficiency of the cells: $\left(1 - \frac{\text{number of unattached cells}}{\text{number of seeded cells}}\right) \times 100$

Both MSC and EC showed similar seeding efficiency (**Study III**). The native mouse cell infiltration into the constructs was evaluated at 1 and 3 weeks *in vivo*. The total number of migrating cells into the construct was higher at 3 weeks. The HE staining showed recruitment of inflammatory cells in relation to the scaffold and their increased progression over time (**Study II**).

4.3 Leukocyte transmigration into the pre-vascularized construct (Study II)

Study II assessed the influence of EC on leukocyte transmigration. The DAVID pathway database (105) showed that multiple genes related to the TLR signaling pathway and the leukocyte transendothelial migration pathway were differentially regulated in the co-culture group compared to the mono-culture group *in vitro*. Further, RT-PCR of mouse specific genes was performed for *in vivo* week 1 and 3 samples for selected biomarkers related to hypoxia and inflammation. Among all the selected markers, only IL-1β was significantly ($p<0.001$) down-regulated in the MSC/EC group compared to the MSC group after 1 week of implantation. After 3 weeks' implantation all the markers were highly up-regulated in the MSC and MSC/EC groups compared to scaffolds implanted without cells. IL-1β ($p<0.001$) and IL-6 ($p<0.05$) were significantly down-regulated in the MSC/EC group compared to the MSC group. NOS_2, mTOR, HIF-1α and HIF-1β were highly up-regulated in the MSC/EC group compared to the MSC group.

After 3 weeks' implantation the scaffolds were retrieved and immunofluorescence double staining and western blotting was performed to evaluate the association between inflammatory cytokines and migrating inflammatory cells. The number of

neutrophils was markedly higher in the co-culture constructs ($p<0.001$). However, IL-1β positive cells were fewer in the MSC/EC group compared to the MSC group. Both neutrophils and IL-1β positive cells were present in significantly higher numbers in mono- and co-culture constructs compared with construct implanted without cells. Monocyte derived cells were co-stained with IL-6 and CD11b. The number of co-stained cells was significantly higher in MSC and MSC/EC constructs compared with control construct implanted without cells. IL-6 and CD11b positive cells were significantly less in the group with added EC compared to MSC alone. Protein expression of IL-1β and IL-6 were lower in the co-culture group compared to the monoculture group.

4.4 Effects of MSC and EC on host response and vascularization (Study III)

The mouse TLR pathway was evaluated after 3 weeks' implantation. Out of 84 genes, 49 were down-regulated in the EC group compared to the MSC group. Additionally, RT-PCR for pro- and anti-inflammatory cytokines was performed. The gene expression of IL-1β and IL-6 were significantly lower in the EC group compared to the MSC group. On the other hand, IL-10 expression was higher in the EC group compared to the MSC group. To investigate the recruitment of acute and chronic inflammatory cells in relation to the scaffold, double staining was performed. The number of neutrophils was markedly higher in EC constructs compared to MSC constructs, although IL-1β positive cells were more numerous in MSC constructs. Double stained cells with CD11b and IL-6 were fewer in EC compared to MSC constructs.

The mRNA of hypoxia related genes were investigated to evaluate the response to hypoxia after 3 weeks' implantation of MSC and EC constructs. The gene expression of HIF-1α, HIF-1β, NOS_2 and mTOR were significantly higher in the EC group compared to the MSC group. However, protein expression of HIF-1α was lower in the EC group compared to the MSC group whereas the expression of HIF-1β was higher in the EC group compared to the MSC group. The increase in hypoxia related

genes could lead to increased angiogenesis. To assess the angiogenic property, angiogenic proteome profiling was performed. Out of 53 proteins, 10 were highly regulated. The proteins related to vascular growth were increased in EC constructs compared to MSC constructs. Additionally, the total vessel area fraction was quantified to evaluate the influence of implanted cells on vascularization. The CD31 immunostaining of the vessels showed that EC constructs had a higher blood vessel density compared to MSC constructs.

5. DISCUSSION

The field of tissue engineering has grown greatly in the past few decades. Advances in tissue engineering have improved the treatment modalities of different diseases. However, vascularization of tissue-engineered constructs remains a challenge in the field of regenerative medicine. Inflammation, vascularization and regeneration are interrelated processes with major roles in tissue engineering. MSC are widely studied for their regenerative potential, as are EC for vascular regeneration. Taking into account the potential of both the cell types, an effort has been made by our group to grow both the cells together, and this co-culture system has been used to generate pre-vascularized constructs (54). After implantation of the tissue-engineered construct, implanted cells experience hypoxia until native vascularization to the construct is achieved and regeneration is achieved (110).

5.1 Hypoxia accelerates wound healing

Hypoxic conditions release cytokines and chemokines to maintain homeostasis in the tissue. With hypoxia, oxygen tension decreases resulting in accumulation of transcription factor HIF, which leads in turn to transcription of genes related to angiogenesis, cell proliferation, cell migration, cell survival and apoptosis (111, 112). Hypoxia also mediates inflammation and the inflammatory microenvironment is highly metabolic with increased oxygen consumption as a result (113, 114). The paracrine effects of MSC have been widely studied in the inflammatory environment, and it has been shown that they can induce wound healing and tissue regeneration (115). The conditioned medium resulting from hypoxic preconditioning of MSC expressed different cytokines and extracellular matrix proteins components than from non-hypoxic cells (116, 117). Hypoxia preconditioning of cells derived from other sources than bone marrow was found to influence the expression of cytokines and chemokines promoting wound healing and angiogenesis. In a distraction osteogenesis mice model, application of hypoxic conditioned medium demonstrated improved bone healing compared to normoxic conditioned medium (118). The *in vitro* results

presented in **Study I**, demonstrated that the conditioned medium from hypoxic culture groups accelerated wound healing. These finding are in accordance with a previous study where hypoxic conditioned medium from MSC improved wound healing in a murine model *in vivo* (119).

5.2 Hypoxia regulates angiogenesis

Vascularization is the most essential factor for survival of the implanted tissue-engineered construct. VEGF-A is a pro-angiogenic factor, expressed by MSC under normal culture conditions, and the expression is dependent on differentiation of MSC (120). It has been shown that MSC express more VEGF-A than do EC *in vitro* under normoxic conditions (24). Further, the conditioned medium obtained from MSC under hypoxic conditions was enriched with different cytokines and chemokines than from MSC under normoxic conditions (121). In order to examine the effect of hypoxia on the implanted cells in **Study II** and **Study III**, an *in vitro* experiment was first performed (**Study I**), where the cells were exposed to short-term hypoxic condition. With a normal oxygen concentration, the HIF-1α is ubiquitinated and degraded. However, during hypoxia, HIF-1α accumulates and forms a heterodimer with HIF-1β resulting in transcription of angiogenic genes such as VEGF-A (111, 122). After hypoxia, we observed that the expression of VEGF-A was higher in HMSC and HMSC/EC groups and the expression was independent of the addition of EC. Several studies have shown that hypoxic preconditioning of MSC resulted in increased VEGF-A expression (119). The hypoxic preconditioning of MSC activated the PI3K-AKT pathway and increased phosphorylation of Akt, which is known to regulate angiogenesis (121). **Study I** demonstrated that the preconditioning of MSC with hypoxia improved angiogenesis.

mTOR, a central regulator of cell proliferation, cell metabolism and angiogenesis, modulates the expression of VEGF-A and NO (123). A study done in human osteoblasts showed that cell proliferation and angiogenesis was suppressed via inhibition of the PI3K/AKT/mTOR pathway (124). Endothelial sprout formation was promoted under hypoxia but after addition of mTOR inhibitor, angiogenesis was

reduced, whereas overexpression of mTOR restored hypoxia and induced angiogenesis (125). The mRNA expression of mTOR was higher in MSC/EC and EC constructs in the studies presented here (**Studies II** and **III**). The generation of pre-vascularized constructs with MSC and EC co-culture *in vitro* before implantation *in vivo* (**Study II**) and the paracrine factors released by MSC under a hypoxic environment during healing may improve inosculation of pre-vascularized constructs with the surrounding microenvironment.

In **Studies II** and **III**, tissue hypoxia after implantation of the construct was observed. Hypoxia inducible factors were more highly expressed in constructs with EC. The up-regulation of HIF genes are known to increase angiogenesis. The initiation of angiogenesis resulted in lower HIF-1α protein level in the EC group in **Study III**. The HIF-1α concentration is directly dependent on the oxygen concentration, whereas HIF-1β is less sensitive to oxygen saturation (126). Hypoxia induces the expression of NOS_2. The expression of NOS_2 was up-regulated in a chronic hypoxia rat model where rats were kept under hypoxic conditions for 3 weeks (127, 128). NO generated by NOS_2 promotes the accumulation of HIF-1α by decreasing ubiquitination of HIF-1α and downstream gene activation such as VEGF-A (129). NOS_2 promotes wound healing, regulates angiogenesis and tissue repair. Incisional and excisional cutaneous wound healing angiogenesis was reduced in NOS_2 knockout mice (130). The mRNA expression of NOS_2 was higher in MSC/EC and EC constructs compared to MSC constructs, inducing angiogenesis. We also observed that expression of angiogenic markers was increased in MSC constructs compared to constructs without cells. Tissue hypoxia after implantation may have promoted angiogenic gene expression from MSC, as we have shown that MSC under hypoxic conditions enhance wound healing and angiogenesis compared to MSC under normoxia (**Study I**). Different angiogenic proteins were analyzed to compare the angiogenic activity of MSC and EC *in vivo* (**Study III**). The release of pro-angiogenic proteins were comparatively higher in EC constructs compared to MSC constructs, whereas the release of anti-angiogenic factor was higher in MSC constructs. CD31 immunostaining of histological samples after 3 weeks' *in vivo* implantation showed increased vessel

formation in the EC group and this result correlates with the increased expression of pro-angiogenic factors by EC.

5.3 Inflammatory reaction after implantation of tissue-engineered constructs

The release of inflammatory cytokines promotes vascularization and regeneration. The favorable balance between pro- and anti- inflammatory cytokines induces tissue organization and regeneration (131, 132). *In vivo* **Studies II** and **III** were carried out in order to investigate the early host response after implantation of tissue-engineered constructs with human cells. A moderately immunocompromised murine model was chosen. NOD/SCID mice are unable to perform VDJ (variable, diversity, and joining genes) recombination and subsequent antibody production but can produce monocyte-derived cells and neutrophils, and thus are able to induce the early immune response (133). The seeding efficacy of the MSC and EC was similar on the scaffolds (**Study III**). A gradual increase in the number of host (mouse) cells infiltrating into the construct was noticed from 1 to 3 weeks of implantation *in vivo* (**Study II**). Similar observations were illustrated from day 1 to day 28 in a PriMatrix implant subcutaneous mice model (134).

5.3.1 EC altered the TLR expression of MSC

It has been shown that MSC express various genes related to the TLR pathway and may have a role in immunomodulation. TLR priming of MSC results in two active phenotypes, MSC1 and MSC2. Two TLR are mainly involved in this process, TLR4 and TLR3. TLR4-primed MSC (MSC1) and TLR3-primed MSC (MSC2) regulate pro-and anti-inflammatory cytokines respectively (135). TLR activation in MSC can induce NF-κβ activity and downstream production of IL-1β, IL-6, IL-8/CXCL8, CXCL10 and CCL5, which results in recruitment of inflammatory cells (136-138). TLR ligand activation with lipopolysaccharide (LPS) of EC resulted in the expression of TLR1, TLR3 and TLR4 and downstream production of IL-6 (139). A microarray gene ontology analysis of a two-dimensional culture model *in vitro* identified 24

over-represented genes in the TLR pathway after co-culture with EC (**Study II**). In the MSC/EC group both TLR3 and TLR4 were up-regulated which ultimately modulated the production of inflammatory cytokines. The pro-inflammatory cytokine IL-6 was down-regulated but IL-8, a chemoattractant and neutrophil activator (140), was highly up-regulated in the MSC/EC group. The SuperArray analysis for the mouse TLR pathway after 3 weeks' implantation *in vivo* showed down-regulation of TLR1, TLR2, TLR3, TLR4 and TLR8 in the EC constructs compared to the MSC constructs (**Study III**). The down-stream production of TLR was evaluated through the release of inflammatory cytokines.

5.3.2 Release of inflammatory cytokines and inflammatory cell recruitment

Copolymer scaffolds are biodegradable and serve as a temporary framework for tissue-engineered constructs (100). Scaffold degradation occurs in parallel with deposition of new matrix protein and recruitment of granulocytes, stem cells and monocyte-derived cells (141). This is an essential process of a foreign body giant cell reaction. Neutrophils are the first line of defense in acute inflammation and are later followed by monocyte-derived cells, T-cells and B-cells (64, 142, 143). Macrophages are broadly sub-divided into M1 and M2 phenotypes and they release pro-and anti-inflammatory cytokines, respectively (144). Pro- and anti-inflammatory cytokines were evaluated after 1 week (**Study II**) and 3 weeks' (**Studies II** and **III**) implantation *in vivo*. A study done with different co-polymers showed no significant difference in inflammatory cell infiltration into the constructs after 2-4 weeks of implantation and also correlated inflammatory cells infiltration with angiogenesis (145). After 1 week, among all target genes only IL-1β, a pro-inflammatory cytokine was significantly down-regulated in MSC/EC constructs (146). Gene- and protein expression of IL-1β was down-regulated in MSC/EC (**Study II**) and EC (**Study III**) constructs. The implantation of EC resulted in less IL-1β positive multinucleated cells. Despite decreased IL-1β expression, the number of neutrophils was exponentially higher in constructs with EC.

IL-6 has been shown to have a dual function, with activation of IL-6 trans- and classic signaling resulting in pro- and anti-inflammatory function of IL-6, respectively. Trans-signaling regulates switching of neutrophils to monocytes by regulating the different chemokines involved in neutrophil and monocyte chemo-attraction (147-149). IL-6 can regulate the immunomodulatory property of MSC by inhibiting both dendritic cell differentiation and T-cell activation (150). MSC from IL-6 knockout mice were less efficient in resolving local swelling in an arthritic mouse model. Additionally, MSC from wild type could suppress T-cell proliferation and could switch from Th1 to Th2 lymphocytes (151). The infiltration of monocyte-derived cells, co-stained with IL-6 and CD11b, was decreased in constructs with EC. The CD11b and IL-6 positive cells were seen in close proximity to the implanted scaffolds and degradation of the material was observed. Even though most of the cells were large and multinucleated, it was noted that MSC/EC constructs had fewer but larger multinucleated giant cells associated. On the other hand, MSC constructs were infiltrated with a greater number of smaller multinucleated cells (**Study II**). This specific pattern was not quantified. A longer *in vivo* experimental observation time would have helped to define the relation of these large cells in to the observed degradation profile of the scaffold.

In the presence of EC, expression of the anti-inflammatory marker IL-10 was increased (**Studies II** and **III**). IL-10 inhibits the production of chemokines and pro-inflammatory cytokines. It also regulates the extracellular matrix, fibroblast and EPC and plays a vital role in tissue regeneration (152, 153). In IL-10 knockout mice, the survival and mobilization of EPC was reduced after myocardial infarction but after administration of IL-10 the number of EPC was increased and neovascularization was achieved (154).

NOS_2 knockout mice showed impaired wound healing compared to wild type mice. Increased level of NOS_2 could prevent the expansion of Th1 cells and help in immune suppression (155). NOS_2 is an important regulator of MSC dependent T cell suppression. MSC from NOS_2 knockout mice have shown decreased immunosuppression potential (92, 156). MSC transplantation along with NOS_2 in

fibrotic livers in mice resulted in resolution of fibrosis with improved liver function and MSC survival compared to transplantation of MSC alone (157). NOS_2 gene expression was comparatively higher in MSC/EC and EC constructs. Taken together, the above findings suggest that the presence of EC in the tissue-engineered construct can improve regeneration by switching the cytokine expression of macrophages from the M1 to M2 phenotype.

6. CONCLUDING REMARKS

The results of this series of studies suggest the following conclusions:

- The secretomes released after hypoxic preconditioning of MSC and MSC/EC accelerated wound healing and promoted tube formation *in vitro*. Vasculogenic markers were up-regulated in the hypoxic groups compared to the normoxic groups.

- The tissue-engineered constructs containing both MSC and EC recruited an increased number of neutrophils and decreased number of monocyte-derived cells, which suggests a phase shift in the inflammatory response. Leukocyte transmigration into the tissue-engineered construct was greatly influenced by the addition of EC to the constructs. Mono-and co-culture constructs induced an active immunogenic reaction compared to the control scaffold without cells.

- EC influenced the hypoxic pathway and promoted vascularization *in vivo.* Implantation of EC induced higher vascular density compared to implantation of MSC.

- Implantation of MSC and EC altered the release of inflammatory cytokines. On addition of EC, up-regulation of anti-inflammatory cytokines and down-regulation of pro-inflammatory cytokines were observed, suggesting resolution of inflammation and initiation of tissue regeneration.

7. FUTURE PERSPECTIVES

The present studies highlighted the vasculogenic and immunomodulatory properties of MSC and EC. Further research is warranted to generate a large vascularized graft with long-lasting blood vessels for clinical applications. In depth research is needed to explore the inter-relationship between MSC and other cells, which communicate with MSC during the healing process.

By tracking the implanted human cells, we can identify the post-implantation survival of the cells and their integration with host tissues.

The results of these studies show alteration in recruitment of inflammatory cells after addition of EC. The role of co-culture and the paracrine effect of EC on MSC in neutrophil quiescence and in the phase shift from M1 to M2 need further investigation.

The results of these studies also demonstrate the importance of EC in regenerating vascularization, and future studies might include obtaining EPC and MSC from the same donor to generate pre-vascularized construct for clinical use.

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