Vascularization and Host Response in Bone Tissue Engineering

Sushma Bartaula-Brevik

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This thesis is dedicated to my beloved parents and my husband
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1. Department of Clinical Dentistry
   Faculty of Medicine and Dentistry
   University of Bergen
   Bergen, Norway

2. The Laboratory Animal Facility
   Department of Clinical Medicine
   Faculty of Medicine and Dentistry
   University of Bergen
   Bergen, Norway

Principal supervisor: Professor Kamal Mustafa
Co-supervisors: Professor Anne Isine Bolstad
               Dr. Torbjørn Østvik Pedersen
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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>ANG</td>
<td>Angiopoietin</td>
</tr>
<tr>
<td>ASC</td>
<td>Adipose stem cells</td>
</tr>
<tr>
<td>AV</td>
<td>Arteriovenous</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster of differentiation</td>
</tr>
<tr>
<td>CDH</td>
<td>Cadherin</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary deoxyribonucleic acid</td>
</tr>
<tr>
<td>CO&lt;sub&gt;2&lt;/sub&gt;</td>
<td>Carbon dioxide</td>
</tr>
<tr>
<td>CTRL</td>
<td>Control</td>
</tr>
<tr>
<td>CXCL</td>
<td>C-X-C motif chemokine ligand</td>
</tr>
<tr>
<td>EC</td>
<td>Endothelial cells</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
</tr>
<tr>
<td>EGM</td>
<td>Endothelial cell growth medium</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
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<tr>
<td>EPC</td>
<td>Endothelial progenitor cells</td>
</tr>
<tr>
<td>EPO</td>
<td>Erythropoietin</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
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<tr>
<td>FGF</td>
<td>Fibroblast growth factor</td>
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<tr>
<td>GAPDH</td>
<td>Glyceraldehyde-3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>HA</td>
<td>Hydroxyapatite</td>
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<tr>
<td>HE</td>
<td>Hematoxylin and eosin</td>
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<tr>
<td>HIF</td>
<td>Hypoxia inducible factor</td>
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<tr>
<td>HRE</td>
<td>Hypoxia response elements</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
<td>IGF</td>
<td>Insulin growth factor</td>
</tr>
<tr>
<td>IGFBP</td>
<td>Insulin-like growth factor-binding protein</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>KGF</td>
<td>Keratinocyte growth factor</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MCGS</td>
<td>Mesenchymal cell growth supplement</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix metalloproteinase</td>
</tr>
<tr>
<td>MSCGM</td>
<td>Mesenchymal stem cell growth medium</td>
</tr>
<tr>
<td>MSC</td>
<td>Mesenchymal stem cells</td>
</tr>
<tr>
<td>mTOR</td>
<td>Mammalian target of rapamycin</td>
</tr>
<tr>
<td>MyD</td>
<td>Myeloid differentiation primary response</td>
</tr>
<tr>
<td>N$_2$</td>
<td>Nitrogen</td>
</tr>
<tr>
<td>NF</td>
<td>Nuclear factor</td>
</tr>
<tr>
<td>NIMP</td>
<td>Anti-neutrophil antibody</td>
</tr>
<tr>
<td>NK</td>
<td>Natural killer</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>NOD/SCID</td>
<td>Non-obese diabetic severe combined immunodeficiency mouse</td>
</tr>
<tr>
<td>iNOS/NOS$_2$</td>
<td>Inducible nitric oxide synthase</td>
</tr>
<tr>
<td>NOV</td>
<td>Nephroblastoma overexpressed</td>
</tr>
<tr>
<td>O$_2$</td>
<td>Oxygen</td>
</tr>
<tr>
<td>OCT</td>
<td>Optimum cutting temperature compound</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Real-time reverse transcription polymerase chain reaction</td>
</tr>
<tr>
<td>PDGF</td>
<td>Platelet derived growth factor</td>
</tr>
<tr>
<td>PECAM</td>
<td>Platelet/endothelial cell adhesion molecule</td>
</tr>
<tr>
<td>PGA</td>
<td>Polyglycolic acid</td>
</tr>
<tr>
<td>PGE</td>
<td>Prostaglandin E</td>
</tr>
<tr>
<td>PHD</td>
<td>Prolyl-hydroxylase domain</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphoinositide 3-kinase</td>
</tr>
<tr>
<td>poly(LLA-co-DXO)</td>
<td>poly(L-lactide-co-1,5-dioxepan-2-one)</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
</tr>
<tr>
<td>SERPIN</td>
<td>Serine protease inhibitor</td>
</tr>
<tr>
<td>SMC</td>
<td>Smooth muscle cells</td>
</tr>
<tr>
<td>TCP</td>
<td>Tricalcium phosphate</td>
</tr>
<tr>
<td>TEVG</td>
<td>Tissue-engineered vascular graft</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptors</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor necrosis factor</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>------------------------------------</td>
</tr>
<tr>
<td>TNFR</td>
<td>Tumor necrosis factor receptor</td>
</tr>
<tr>
<td>TPO</td>
<td>Thrombopoietin</td>
</tr>
<tr>
<td>VDJ</td>
<td>Variable, diversity, and joining genes</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>VHL</td>
<td>von Hippel-Lindau</td>
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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. (Submitted manuscript)

II


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 \textit{in vitro} culture conditions through the \textit{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 \textbf{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 \(\varepsilon\)-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 \(\varepsilon\)-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 \textit{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 \textbf{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 $\beta$-tricalcium phosphate/hydroxyapatite ($\beta$TCP/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 inosculcation. 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).
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 biophysical 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 \textit{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 \textit{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).

\textbf{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.
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/NOS₂). 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 E2 (PGE2) by MSC. PGE2 binds to PGE2 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).](image-url)
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 \textit{in vitro} under hypoxic conditions (Study I)

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

3. To evaluate the \textit{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.
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% O₂, 5% CO₂ and 94% N₂ 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,
Accelerator teknik, 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 % CO₂. 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](image_url)

*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 immunodecient (NOD/SCID) mice were used in Studies II and III. They were purchased from Taconic Farms (Bomholtgard 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 suficient 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, Wisconsin, 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.

<table>
<thead>
<tr>
<th>Assay ID</th>
<th>Full name</th>
<th>Gene Symbol</th>
<th>Species</th>
<th>Study</th>
</tr>
</thead>
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<td>GAPDH</td>
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<td>IL-1β</td>
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<td>I</td>
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<td>I</td>
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<tr>
<td>Hs00174103_m1</td>
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<td>IL-8</td>
<td>Human</td>
<td>I</td>
</tr>
<tr>
<td>Hs00961622_m1</td>
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<td>IL-10</td>
<td>Human</td>
<td>I</td>
</tr>
<tr>
<td>Hs00900055_m1</td>
<td>Vascular endothelial growth factor-A</td>
<td>VEGF-A</td>
<td>Human</td>
<td>I</td>
</tr>
<tr>
<td>Hs00966526_m1</td>
<td>Platelet derived growth factor</td>
<td>PDGF</td>
<td>Human</td>
<td>I</td>
</tr>
<tr>
<td>Hs00266645_m1</td>
<td>Fibroblast growth factor</td>
<td>FGF</td>
<td>Human</td>
<td>I</td>
</tr>
<tr>
<td>Hs00375822_m1</td>
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<td>ANG-1</td>
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<tr>
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<tr>
<td>Hs00171022_m1</td>
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<td>CXCL12</td>
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<tr>
<td>VIC MGB</td>
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<td>II,III</td>
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<tr>
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<td>II,III</td>
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<tr>
<td>Mm 00440502_m1</td>
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<td>iNOS (NOS2)</td>
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</tr>
<tr>
<td>Mm 00507836_m1</td>
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<td>HIF-1β (ARNT)</td>
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<tr>
<td>Mm 0044968_m1</td>
<td>Mammalian target of rapamycin</td>
<td>mTOR</td>
<td>Mouse</td>
<td>II,III</td>
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</table>

**PCR Array (Study III)**

Mouse TLR pathway Rt² Profiler PCR arrays (SuperArray Bioscience, Frederick, Maryland, United States) were performed. An Rt² 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 Rt² 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® TGXTM 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.

<table>
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<th>Abbreviation</th>
<th>Full name</th>
<th>Species</th>
<th>Manufacturer</th>
<th>Study</th>
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<td>Abcam</td>
<td>II</td>
</tr>
<tr>
<td>HIF-1α</td>
<td>Hypoxia inducible factor-1α</td>
<td>Mouse</td>
<td>Santa Cruz</td>
<td>III</td>
</tr>
<tr>
<td>HIF-1β(ARNT)</td>
<td>Hypoxia inducible factor-1β</td>
<td>Mouse</td>
<td>Santa Cruz</td>
<td>III</td>
</tr>
</tbody>
</table>

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.

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full name</th>
<th>Species</th>
<th>Manufacturer</th>
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<td>IL-1β</td>
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<td>Abcam</td>
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<td>IL-6</td>
<td>Interleukin-6</td>
<td>Mouse</td>
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<td>II,III</td>
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<tr>
<td>NIMP</td>
<td>Neutrophil antibody</td>
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<td>Abcam</td>
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<td>Cluster of differentiation 11b</td>
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<td>BD</td>
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</tr>
<tr>
<td>CD31</td>
<td>Cluster of differentiation 31</td>
<td>Mouse</td>
<td>BD</td>
<td>III</td>
</tr>
<tr>
<td>HE</td>
<td>Hematoxylin and eosin</td>
<td>Non-specific</td>
<td></td>
<td>II</td>
</tr>
</tbody>
</table>

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.
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™ (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.

![Diagram of tube formation assay]

*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: \( (1 - \frac{\text{number of unattached cells}}{\text{number of seeded cells}}) \times 100 \)

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. NOS2, 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, NOS2 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 NOS2. The expression of NOS2 was up-regulated in a chronic hypoxia rat model where rats were kept under hypoxic conditions for 3 weeks (127, 128). NO generated by NOS2 promotes the accumulation of HIF-1α by decreasing ubiquitination of HIF-1α and downstream gene activation such as VEGF-A (129). NOS2 promotes wound healing, regulates angiogenesis and tissue repair. Incisional and excisional cutaneous wound healing angiogenesis was reduced in NOS2 knockout mice (130). The mRNA expression of NOS2 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 chemotraction (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).

NOS2 knockout mice showed impaired wound healing compared to wild type mice. Increased level of NOS2 could prevent the expansion of Th1 cells and help in immune suppression (155). NOS2 is an important regulator of MSC dependent T cell suppression. MSC from NOS2 knockout mice have shown decreased immunosuppression potential (92, 156). MSC transplantation along with NOS2 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 \textit{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 \textit{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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Hypoxia preconditioning of mesenchymal stem cells accelerates wound healing and vessel formation in vitro

Sushma Bartaula-Brevik\textsuperscript{1*}, Anne Isine Bolstad\textsuperscript{1}, Kamal Mustafa\textsuperscript{1} and Torbjorn O. Pedersen\textsuperscript{1}

\textsuperscript{1}Department of Clinical Dentistry, University of Bergen, Bergen, Norway

* Corresponding author:

Sushma Bartaula-Brevik

Department of Clinical Dentistry

University of Bergen

Årstadveien 19

5009 Bergen

Norway

E-mail: sushma.bartaula@iko.uib.no

Author e-mails:

Anne Isine Bolstad: anne.bolstad@iko.uib.no

Kamal Mustafa: kamal.mustafa@iko.uib.no

Torbjorn O. Pedersen: torbjorn.pedersen@iko.uib.no
Abstract

Hypoxia is one of the factors that trigger the release of inflammatory and vasculogenic cytokines during tissue regeneration. Mesenchymal stem cells (MSC) with and without endothelial cells (EC) were cultured in vitro in normoxic and hypoxic environments. The mRNA expression of inflammatory and vasculogenic cytokines were evaluated at 1, 12, 24 and 48 hours. After 48 hours of incubation in normoxic and hypoxic conditions, supernatants termed as conditioned medium (CM) from each group were collected and analyzed. The protein level of VEGF-A in the CM was determined by ELISA. The effects of the CM from different groups on EC were evaluated using wound healing- and tube formation assays. The mRNA expression of IL-1\(\beta\), IL-6 and IL-8 was up-regulated in both the normoxic and hypoxic co-culture (MSC/EC) group, compared with the mono-culture normoxic group. The VEGF-A protein level was higher in the hypoxic mono- and co-culture group. Wound closure was accelerated by CM from the mono- and co-culture hypoxic groups compared with both normoxic groups. Measurements of tube formation were higher in the hypoxic mono-culture group compared with the normoxic group. The hypoxia preconditioning of the cells accelerated wound healing and vessel formation in vitro.
Introduction

The ability of stem cells to self-renew and differentiate into mature cells of different lineages is regulated by both intrinsic programming and extrinsic input from the stem cell niche or microenvironment [1]. The poor post-implantation survival of transplanted cells limit therapeutic efficacy [2]. Several strategies have been postulated to overcome this challenge, which includes preconditioning of the cells by heat shock, oxidative stress and hypoxia [3]. The oxygen concentration in different tissues and organs varies from 2-9%, whereas bone marrow niches have a lower oxygen concentration at about 1% [4]. This suggests that bone marrow stem cells could favor a hypoxic microenvironment. Several studies have shown that hypoxia induces release of chemokines, cytokines and growth factors involved in cell proliferation, differentiation, migration, apoptosis and angiogenesis [5, 6]. However, the effect of short- and long-term hypoxic environments on survival, proliferation and differentiation of mesenchymal stem cells (MSC) is still controversial [7]. It has been shown that by changing the culture conditions, MSC can be directed towards the endothelial cell lineage [8, 9]. Also, the paracrine effect of implanted MSC promotes vascularization by ingrowth of host microvasculature into tissue-engineered constructs [10].

Different strategies have been suggested to improve vascularization in tissue engineering, which include both pre-vascularized and pre-conditioned constructs. Endothelial cells (EC) have been co-cultured with different cell types, including MSC, adipose stem cells (ASC) and osteoblasts, all with the aim of improving vascularization after being implanted in vivo. It has been shown that the microvasculature in pre-vascularized constructs can interconnect with the host microvasculature, in order to ensure implant survival [11]. Pre-conditioned tissue-engineered constructs can also be generated by incorporating different growth factors, or by changing the physical and chemical properties of the scaffold material [12, 13].
Pre-vascularization of tissue-engineered construct by co-culturing MSC and EC in vitro resulted in microvascular network formation in vivo [14]. Despite of extensive work on co-culture systems, limited effort has been made to address the effect of different culture condition on co-culture. The aim of this study was to evaluate the influence of hypoxia on the paracrine effect of mono- and co-culture during wound healing and vessel formation. Our results show that hypoxia could alter the release of inflammatory and vasculogenic cytokines in mono- and co-culture, and promote wound healing and vessel formation in vitro.

Material and methods

Cell culture

Primary human bone marrow derived MSC and Human umbilical vein endothelial cells were purchased from Lonza (Walkersville, Maryland, United States). MSC were 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). EC were expanded in Endothelial Cell Growth Medium-2 (EGM-2®) BulletKit (Lonza) containing Endothelial Cell Basal Medium and supplemented with Fetal bovine serum (FBS), human epidermal growth factor (hEGF), hydrocortisone, GA-1000, vascular endothelial growth factor (VEGF), human fibroblast growth factor (hFGF-B), insulin-like growth factor (R²-IGF-1), ascorbic acid and heparin. Both cell types were expanded at 37 °C and 5% CO₂. Cells older than passage four were not used for the experiments.

Experimental set up

The cells were seeded in 6-well plates with a seeding density of 200,000 cells/well either MSC alone or MSC/EC in a 5:1 ratio. The cells were allowed to attach overnight. The next day those two groups were further sub-divided into hypoxic and normoxic groups. The four
groups were MSC-NOR (NMSC), MSC/EC-NOR (NMSC/EC), MSC-HYP (HMSC) and MSC/EC-HYP (HMSC/EC). A Modular Incubator Chamber (MIC-101) was used as a hypoxia chamber and the chamber was filled with a tri-gas mixture of 1% O₂, 5% CO₂ and 94% N₂. After 1, 12, 24 and 48 hours the cells were harvested. The cells were trypsinized, and EC were depleted with CD31 Endothelial Cell Dynabeads® (Invitrogen, Carlsbad, California, United States) according to the manufacturer’s instructions. The isolated MSC were stored at -80 °C before RNA isolation. The medium from each group was collected in aliquots, centrifuged and stored at -80°C for further analysis.

**Real-time reverse transcription polymerase chain reaction (RT-PCR)**

Total RNA isolation was done using Maxwell® 16 LEV simplyRNA purification kits (Promega, Madison, Wisconsin, United States). RNA purity and quantification were determined with a Nanodrop Spectrophotometer (ThermoScientific Nano-Drop Technologies, Wilmington, Delaware, USA). 1000ng of RNA was reverse transcribed to cDNA using a cDNA kit (Applied Biosystems, Carlsbad, California, United States). Real-time RT-PCR was performed on a StepOne™ real time RT-PCR system (Applied Biosystems). cDNA corresponding to 10ng mRNA in each reaction was prepared in duplicates for each target gene and the real-time RT-PCR was run under standard cycling conditions. Different angiogenic and inflammatory genes were evaluated (Table 1).
Table 1 List of Taqman® probes used for real time reverse transcription polymerase chain reaction analysis

<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>Assay ID</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>Hs99999905_m1</td>
</tr>
<tr>
<td>IL-1β</td>
<td>Hs01555410_m1</td>
</tr>
<tr>
<td>IL-6</td>
<td>Hs00985639_m1</td>
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<tr>
<td>IL-8</td>
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<td>PDGF</td>
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<tr>
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<td>ANG-1</td>
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<tr>
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<td>Hs01048042_m1</td>
</tr>
<tr>
<td>CXCL12</td>
<td>Hs00171022_m1</td>
</tr>
</tbody>
</table>

Enzyme linked immunosorbent assay

For protein analysis, a commercially available human VEGF-A enzyme-linked immunosorbent assay (ELISA) (R&D Systems, Minneapolis, Minnesota, United States) was used. Cell culture supernatants from the 48h time point were thawed and the VEGF-A concentration was determined according to the manufacturer’s instructions. Optical densities were determined using a FLUOstar OPTIMA microplate reader (BMG LABTECH, Ortenberg, Germany). The values were compared to a known standard curve and the final concentrations are presented in pg/ml.

Wound Healing Assay

Human umbilical vein endothelial cells were cultured in Culture-Insert 24 (80241, ibidi, Martinsried, Germany) at a concentration of 30,000 cells/well in duplicate. The culture inserts were carefully removed after the cells reached confluency. A wound of approximately 500μm width was created by the insert, and the wounded monolayer of cells was washed three times with phosphate buffered saline (PBS) to remove dead cells and debris. The cells were
incubated with conditioned medium from MSC-NOR, MSC/EC-NOR, MSC-HYP, MSC/EC-HYP and positive (EGM-2) and negative (MSCGM) control media for 24 hours. Images were taken at the beginning as a baseline, and at 3, 6, 12, 18 and 24 hours’ time points during the cell migration to close the wound. The images were taken with a Nikon Eclipse Ti microscope (Nikon, Tokyo, Japan) at magnification of 4x in the phase contrast mode. Images obtained from each group were analyzed with a NIS elements AR 3.2 software (NIS elements, Tokyo, Japan). The surface area covered by the wound was measured and exported to a Microsoft Excel (Microsoft Corporation, Redmond, Washington, United States) file before statistical analysis was performed. All the images were normalized with the baseline data.

*Tube Formation Assay*

Growth Factor Reduced Matrigel (BD Biosciences, San Jose, California, United States) was thawed overnight at 4°C, 40μl per well was added to the growth surface of 96 well plates and incubated for 30 min at 37°C to allow the matrigel to solidify. Human umbilical vein endothelial cells were suspended in conditioned medium from MSC-NOR, MSC/EC-NOR, MSC-HYP, MSC/EC-HYP and positive (EGM-2) and negative (MSCGM) control media. The cells were seeded onto the solidified matrigel at a density of 20,000cells/well. After incubating for 6 hours, the formation of tube like structures was observed under the microscope. The images were taken with a Nikon Eclipse Ti microscope (Nikon) at magnification of 4x in the phase contrast mode. The tube formation was quantified using the ImageJ software program (NIH, Bethesda, Maryland, United States) with the Angiogenesis Analyzer plugin [15]. The parameters of tube formation were normalized with control medium (MSCGM).
Results

Inflammatory and angiogenic gene expression

The mRNA expression of pro-inflammatory cytokines Interleukin-1β (IL-1β), Interleukin-6 (IL-6) and Interleukin-8 (IL-8) were evaluated in normoxic and hypoxic culture conditions for 1, 12, 24 and 48 hrs. In normoxia, the expression of IL-1β was exponentially higher in the co-culture group compared with the mono-culture group, whereas in hypoxia, a significant increase was noticed in co-culture group compared with NMSC only at 1hr, and no significant difference between the groups could be found after 12 hrs (Figure 1A). IL-1β expression was down-regulated in HMSC group compared with NMSC group at 12 hrs. The mRNA expression of IL-6 was significantly higher in the co-culture normoxic and both hypoxic groups at 1 hr compared with NMSC (Figure 1B). HMSC showed no significant difference after 12 hrs and HMSC/EC showed no significant difference after 24 hrs, although the expression of IL-6 was still significantly higher in NMSC/EC until 28 hrs (Figure 1B). The mRNA expression of IL-8 was significantly higher in NMSC/EC and HMSC/EC after 1 hr compared to NMSC. At 12 hrs, the expression of IL-8 started to reduce significantly in the HMSC group. In the NMSC/EC group, IL-8 showed continuous up-regulation until 48 hrs. However, IL-8 expression was lower in HMSC/EC group at 24 hrs compared to the NMSC group (Figure 1C). Overall, these results show that the co-culture with EC increased mRNA expression of pro-inflammatory cytokines. Anti-inflammatory cytokine Interleukin-10 (IL-10) was also evaluated but was not expressed by any of the groups.

The mRNA level of VEGF-A was significantly higher expressed in the hypoxic group. The results also showed that the expression of VEGF-A depend on a hypoxic environment, independent of co-seeding with EC (Figure 2A). The mRNA expression of angiopoietin-1 (ANG-1) was down-regulated in all the groups compared with the normoxic mono-culture
group (Figure 2C), whereas angiopoietin-2 (ANG-2) expression was highly up-regulated in the co-culture group irrespective of culture condition (Figure 2D).

The mRNA expression of platelet derived growth factor (PDGF) was down-regulated in the HMSC group at 1 and 12 hrs compared with the NMSC group (Figure 3A). The mRNA expression of fibroblast growth factor (FGF) was higher in HMSC at 1 hr, but the expression was down-regulated after 12 hrs. The NMSC/EC group showed no significant difference compared to NMSC. The HMSC/EC group showed significant down-regulation after 12 hrs compared with the NMSC (Figure 3B). There was no significant difference in the mRNA expression of CXCL-12 between the groups (Figure 3C).

**ELISA**

The protein expression of VEGF-A was evaluated for mono and co-culture groups with and without hypoxia (Figure 2B). VEGF-A expression was higher in the hypoxic group compared with the normoxic group and was statistically significant. Also, the VEGF-A expression on the protein level was independent of the co-culture with EC.

**Wound Healing Assay**

Cell migration towards the wounded area at different time intervals was compared with both positive (EGM-2) and negative (CTRL) controls (Figure 4A). The bar diagram comparing the experimental groups to the NMSC group, show that the wound healing was affected by hypoxia and significantly higher in HMSC and HMSC/EC groups respectively (Figure 4B, 4C, 4D, 4E). After 18 hours of incubation, the wound area was completely closed in both hypoxic groups (Figure 4E).
**Tube Formation Assay**

The formation of tube-like structures was quantified and the overview of the parameters is shown in Figure 5A. Among different parameters of tube formation, four of them was further quantified and normalized with the tube formation in the control group. The four selected parameters were number of nodes, meshes, segments and total length. Results show that hypoxia improved the tube formation and was significantly higher in the HMSC group compared with NMSC. Also, the tube formation was significantly reduced in the NMSC/EC group suggesting that the paracrine effect of EC may have a negative effect on tube formation. (Figure 5B, 5C, 5D, 5E)

**Discussion**

This study shows the effect of a hypoxic microenvironment on the release of inflammatory and angiogenic cytokines, as well as wound healing and angiogenesis, for MSC cultured alone or in co-culture with EC. Under normal oxygen tension, transcriptional activity of hypoxia inducible factor-1 (HIF-1) is ubiquitinated and degraded. However, in hypoxic conditions, HIF-1α gets accumulated and forms a heterodimer with HIF-1β which results in transcription of genes involved in angiogenesis, cell proliferation, cell survival, cell migration and apoptosis [16]. Several studies have demonstrated that the paracrine effects of MSC have regenerative potential [17], and it is known that the conditioned medium (CM) collected from MSC culture *in vitro* contains different cytokines, chemokines and growth factors [18].

It has been shown that MSC cultured in hypoxia release angiogenic factors such as VEGF-A, MCP-1 and angiogenin, which have been detected in the CM [19]. Also the paracrine effect of MSC have been investigated, with results suggesting that MSC grown in normoxia and hypoxia release different soluble factors such as keratinocyte growth factor (KGF), epidermal growth factor (EGF), insulin growth factor (IGF-1), VEGF-A, PDGF, erythropoietin (EPO)
and thrombopoietin (TPO) that increase fibroblast, keratinocyte and endothelial cell migration and increase extracellular matrix, which eventually accelerate wound healing [20-22].

We evaluated the release of different cytokines and angiogenic factors released by mono- and co-culture groups under hypoxic and normoxic conditions. Pro-inflammatory cytokines IL-1β, IL-6 and IL-8 were up-regulated in normoxic co-culture group compared with the NMSC group. However, IL-1β and IL-8 were down-regulated in the HMSC group compared with the NMSC group. Hypoxia preconditioning of MSC could reduce the expression of pro-inflammatory genes, suggesting a potential suppression of the immunoreactivity of MSC. On the contrary, addition of EC increased the short-term expression of pro-inflammatory cytokines in both the normoxia and hypoxia groups, suggesting increased immunoreactivity from co-culturing the two cell types.

VEGF-A gene- and protein expression was higher in the hypoxic groups. Similar result has been reported in other studies [20], and it is known that MSC express significantly more VEGF-A compared with EC in vitro [23]. This suggests that expression of VEGF-A is induced by hypoxia, independent of the presence of EC, although the paracrine effect of MSC has been shown to induce sprouting of EC via VEGF signalling [24].

ANG-1 has been reported as an angiogenic growth factor responsible for a functional and stable vasculature [25]. We observed that hypoxia reduced the expression of ANG-1, whereas the expression of ANG-2 was increased in both the normoxic and hypoxic co-culture groups. It is known that hypoxia induces the expression of ANG-2 in endothelial cells [26], whereas ANG-1 is down-regulated by hypoxia [27]. Hypoxia has been shown to down-regulate the ANG-1/ANG-2 ratio, which could lead to formation of less mature vessels during angiogenesis [28].
Wound healing is a complex process and mediated by the oxygen tension of the surrounding tissue. Hypoxia is a natural process occurring after the formation of a wound. The microenvironment during wound healing attracts and accumulates resident MSC, which migrate to the wounded area. In this hypoxic environment the MSC release paracrine factors that improve healing [21, 22]. Similarly, we have shown that the conditioned medium obtained from hypoxic cultures improved wound healing \textit{in vitro}.

The other important parameter during healing and regeneration is vascularization. MSC release increased levels of vasculogenic cytokines, which in turn improve angiogenesis [29]. The hypoxic conditioned medium from MSC and EC were compared separately, which resulted in reduced vessel formation and decreased VEGF-expression in the EC-group compared with MSC [30]. In our study, the vessel formation was reduced and incomplete luminar structures were detected at a higher density in the NMSC/EC-group. Overall, hypoxia increased the formation of vessel-like structures.

The MSC secretomes enhance the formation of vessels. In previous work from our group, pre-vascularized tissue-engineered constructs could be generated by co-culturing MSC with EC [14]. Therefore, the combination effect of MSC and EC in this context could lead to improved vascularity of the tissue-engineered constructs. Also, MSC and EC co-culture could generate pre-vascularized constructs \textit{in vitro}, whereas the paracrine effect of MSC has shown the potential for increased vascular ingrowth, a feature essential for tissue survival and development. In combination, these elements could result in improved vascularization of tissue-engineered construct and tissue regeneration.
Conclusions
The present study shows that hypoxia preconditioning altered the expression of inflammatory and vasculogenic cytokines released by MSC and MSC/EC. These secretomes from MSC and MSC/EC could accelerate wound healing and also promote angiogenesis in vitro.

Abbreviations
EC; human umbilical vein endothelial cells, MSC; primary human bone marrow derived multipotent stromal cells mesenchymal stem cells, ASC; adipose stem cells, MSCGM; mesenchymal stem cell growth medium, EGM; endothelial cell growth medium, FBS; fetal bovine serum, EGF; epidermal growth factor, FGF; fibroblast growth factor, VEGF; vascular endothelial growth factor, ELISA; enzyme linked immunosorbent assay, CM; conditioned medium, IL; interleukin, ANG; angiopoietin, PDGF; platelet derived growth factor, CXCL; chemokine (C-X-C Motif) ligand, HIF; hypoxia inducible factor, KGF; keratinocyte growth factor, IGF; insulin growth factor, EPO; erythropoietin, TPO; thrombopoietin

Acknowledgments
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Author contributions
Conceived and designed the experiments: SBB, TOP, AIB and KM. Performed the experiments: SBB. Analyzed the data: SBB, TOP, AIB and KM. Contributed reagents, materials and analytical tools: TOP, AIB and KM. Wrote the paper: SBB, TOP, AIB and KM. All authors read and approved the final manuscript.

Competing interests
The authors declare that they have no competing interests.
References:


Figure 1 Relative gene expression of inflammatory cytokines: IL-1β (A), IL-6 (B) and IL-8 (C) comparing NMSC/EC, HMSC and HMSC/EC groups with NMSC at different time points. Data are presented as means ± standard deviation (n=6). * = p<0.05; ** = p<0.01; *** = p<0.001
Figure 2 Relative gene expression of VEGF-A (A), ANG-1 (C) and ANG-2 (D) comparing NMSC/EC, HMSC and HMSC/EC groups with NMSC at different time points. Protein expression of VEGF-A as determined by ELISA (B). Data are presented as means ± standard deviation. * = p<0.05; ** = p<0.01; *** = p<0.001
Figure 3 Relative gene expression of PDGF (A), FGF (B) and CXCL12 (C) comparing NMSC/EC, HMSC and HMSC/EC groups with NMSC at different time points. Data are presented as means ± standard deviation. * = p<0.05; ** = p<0.01; *** = p<0.001
Figure 4 Wound healing assay. The cell migration into the wounded monolayer at different time intervals (A) the percentage of remaining wound in NMSC/EC, HMSC and HMSC/EC compared with NMSC at 3 hours (B), 6 hours (C), 12 hours (D) and 18 hours (E). Data are presented as means ± standard deviation. * = p<0.05; ** = p<0.01; *** = p<0.001 EGM-2 (positive control), CTRL (negative control)
Figure 5 Tube formation assay. The tube formation in different experimental groups at 4x magnification (A) the parameters of tube formation nodes (B), meshes (C), segments (D) and total length (E) in NMSC/EC, HMSC and HMSC/EC groups compared with the NMSC group. Data are presented as means ± standard deviation. * = p<0.05; ** = p<0.01; *** = p<0.001
Leukocyte transmigration into tissue-engineered constructs is influenced by endothelial cells through Toll-like receptor signaling

Sushma Bartaula-Brevik, Torbjorn O Pedersen, Anna L Blois, Panagiota Papadakou, Anna Finne-Wistrand, Ying Xue, Anne Isine Bolstad and Kamal Mustafa

Abstract

Introduction: Inflammation plays a crucial role in tissue regeneration, wound healing, and the success of tissue-engineered constructs. The aim of this study was to investigate the influence of human umbilical vein endothelial cells (ECs) on leukocyte transmigration when co-cultured with primary human bone marrow-derived multipotent stromal cells (MSCs).

Methods: MSCs with and without ECs were cultured in poly (L-lactide-co-1, 5-dioxepan-2-one) (poly (LLA-co-DXO)) scaffolds for 1 week in vitro in a bioreactor system, after which they were implanted subcutaneously in non-obese diabetic/severe combined immunodeficient mice. After 1 and 3 weeks, scaffolds were retrieved, and the mRNA expression of interleukin 1-beta (IL-1β), IL-6, IL-10, hypoxia-inducible factor 1-alpha (HIF-1α), HIF-1β, and mammalian target of rapamycin was examined by real-time reverse transcription-polymerase chain reaction. Furthermore, immunofluorescent staining was performed for IL-1β, IL-6, neutrophils, and CD11b. In addition, Western blotting was done for IL-1β and IL-6. Leukocyte transmigration genes and genes in Toll-like receptor pathways, expressed by MSCs cultured in vitro with or without ECs, were further investigated with a microarray dataset.

Results: In vitro, genes involved in leukocyte transmigration and Toll-like receptor pathways were clearly influenced by the addition of ECs. Platelet/endothelial cell adhesion molecule-1 (PECAM-1) and cadherin-5 (CDH5), both genes involved in leukocyte transmigration, were expressed significantly higher in the MSC/EC group. In vivo, the MSC/EC group showed higher mRNA expression of hypoxia-inducible factors HIF-1α and HIF-1β. The mRNA expression of anti-inflammatory cytokine IL-10 showed no significant difference, whereas the mRNA and protein expression of pro-inflammatory cytokines IL-1β and IL-6 were lower in the MSC/EC group. The quantitative analysis of immunofluorescent staining revealed a significant difference in the number of neutrophils migrating into constructs, with the highest density found in the MSC/EC group. The number of macrophages positive for IL-6 and CD11b was significantly reduced in the MSC/EC group.

Conclusions: The recruitment of leukocytes into tissue-engineered constructs with MSCs is strongly influenced by the addition of ECs via activation of leukocyte transmigration and Toll-like receptor pathways.

*Correspondence: torbjorn.pedersen@iko.uib.no
1Department of Clinical Dentistry, Center for Clinical Dental Research, University of Bergen, Årstadveien 19, N-5009 Bergen, Norway
2Department of Biomedicine, University of Bergen, Jonas Lies vei 91, 5009 Bergen, Norway
Full list of author information is available at the end of the article

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Introduction

Re-establishing the function of lost tissues is the ultimate goal of tissue engineering. The process of regenerating complex tissues is, however, not only dependent on progenitor cells differentiating to specialized parenchymal cells. Development of an adequate blood supply is required to ensure survival of implanted cells as well as development and maintenance of the tissue. Under defined mecha-nochemical culture conditions, primary human bone marrow-derived multipotent stromal cells (MSCs) can differentiate into osteoblasts, chondrocytes, adipocytes, myocytes, and neuronal-like cells [1,2]. The differentiation of MSCs into endothelial cells (ECs) has been shown [3] but is controversial [4]. Pre-vascularization through co-seeding of MSCs and ECs has therefore been performed to generate tissue-engineered constructs with an intrinsic vasculature upon implantation in vivo [5-8].

The influence of ECs on osteogenic differentiation of MSCs has been extensively studied, identifying ECs as an important regulator of MSC commitment to the osteogenic lineage [9-11]. Microarray data have shown that ECs modulate the gene expression profile of MSCs, in particular through the transforming growth factor-beta pathway [12]. In addition, recent work has identified MSCs as appropriate perivascular cells in tissue-engineered constructs containing both ECs and MSCs [13]. The communication between the two cell types is a combination of juxtacrine and paracrine signaling. Vascular assembly has an obvious requirement for direct contact communication, with MSCs regulating EC proliferation, vessel diameter, and maturation of the developing vasculature [6,7]. However, the release of bioactive molecules (cytokines, chemokines, and growth factors) is a significant part of the cellular cross-talk and alters the signal delivered to surrounding tissues after in vivo implantation, thus playing a vital role in the success of the constructs.

Surgical procedures induce acute inflammation that triggers wound healing, repair, and regeneration [14,15]. Also, implantation of cells and biomaterials is likely to result in a combination of acute and chronic inflammatory stimulation to surrounding tissues. In addition, MSCs have been shown to interact with immune cells and modulate their functional activities through the release of anti-inflammatory cytokines [16,17].

In some cases, fibrosis hinders vascularization, which leads to a necrotic core of implanted tissue-engineered constructs. Inflammation and angiogenesis are co-dependent processes in certain pathological processes and in wound healing [18]. A certain level of inflammation is therefore favorable for vascular ingrowth and degradation of the scaffold material and subsequently in achieving the maximal level of regeneration and implant success [19,20].

The vascular endothelium facilitates leukocyte transmigration upon chemotactic signals from damaged or hypoxic tissues. The effects of including a vascular endothelium in a tissue-engineered construct on migration of leukocytes are, however, not well described. We studied the transmigration of leukocytes involved in acute and chronic inflammation into constructs with or without an intrinsic vasculature and the molecular mechanisms behind its modulation.

Materials and methods

In vitro cultivation of cells

Primary human bone marrow-derived MSCs were purchased from StemCell Technologies (Vancouver, BC, Canada). Purity of the cells was confirmed by flow cytometry, which showed that more than 90% of the cells expressed CD29, CD44, CD105, and CD166 and less than 1% expressed CD14, CD34, and CD45. The MSCs were cultured in MesenCult complete medium (StemCell Technologies) in accordance with the instructions of the manufacturer.

Human umbilical vein ECs were purchased from Lonza (Clonetics®, Walkersville, MD, USA). ECs were expanded in EC growth medium (EGM®) (Lonza) containing 500 mL EC basal medium and supplements: fetal bovine serum 10 mL, bovine brain extract [21] 2 mL, human endothelial growth factor 0.5 mL, hydrocortisone 0.5 mL, and GA-1000 0.5 mL. Cells no older than passage five were used, and all cells were cultured at 37°C and 5% CO2.

Production of scaffolds

Poly(l-lactide-co-1,5dioxepan-2-one) [poly(LLA-co-DXO)] scaffolds were prepared as previously described [22,23]. Briefly, porous scaffolds (pore sizes of 90 to 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 a radiation dose of 2.5 Megarad (Mrad) in an inert atmosphere.

Preparation for in vivo implantation

The scaffolds with cells seeded for in vivo implantation were prepared in a similar way as previously described [6,24]. Briefly, scaffolds 12 mm in diameter and 1.5 mm thickness were prewet with MesenCult complete medium (StemCell Technologies) and incubated overnight at 37°C and 5% CO2. Then, 5 × 10^6 cells were seeded per scaffold, either MSCs alone or MSCs/ECs in a 5:1 ratio. To facilitate distribution of cells, an orbital shaker (Eppendorf, Hamburg, Germany) was used, and cells were allowed to attach overnight before scaffolds were transferred to separate modified spinner flasks (Wheaton Science, Millville, NJ, USA) for 1 week in a dynamic culture system with 50
rotations per minute. After 1 week in vitro, 6-mm discs were punctured with a dermal skin puncher and the scaffolds were implanted in vivo.

Animal experiments
The animal experiments were performed after approval from the Norwegian Animal Research Authority and conducted according to the European Convention for the Protection of Vertebrates used for Scientific Purposes (local approval number 3029). Non-obese diabetic/severe combined immunodeficient (NOD/SCID) mice (n = 15) purchased from Taconic Farms (Bomholtgård Breeding and Research Center, Ry, Denmark) were used in this study. The animals were kept in sterile polystyrene cages containing wood shavings in a climate-controlled environment with 12 dark/ light cycles and fed with standard rodent chow and water ad libitum. The animals were 6 to 8 weeks at the time of the surgery. An intramuscular injection of 20 μL of Rompun (Xylazin) (20 mg/mL) (Bayer Health Care, Leverkusen, Germany) and Narketan (Vétoquinol, Lure, France) in 1:2 ratios was performed to anesthetize the animals. On the back of the mice, a 2.5-cm incision was made providing sufficient space for subcutaneous implantation of scaffolds. A scaffold for each experimental group (MSC or MSC/EC) was placed in the mouse, and six mice were used for each time point. For the 3 weeks of implantation, an additional six empty scaffolds were implanted in three mice. Wounds were closed with Vetbond™ Tissue Adhesive (n-butyl cyanoacrylate) (3M™, St. Paul, MN, USA). After 1 or 3 weeks of implantation, animals were euthanized with deep isoflurane (Schering Plough, Kenilworth, NJ, USA) anesthesia followed by cervical dislocation, after which the implanted scaffolds were carefully dissected and retrieved. The samples were then cut into two halves, and each half was further processed for real-time reverse transcription-polymerase chain reaction (RT-PCR) analysis or histological embedding.

Real-time reverse transcription-polymerase chain reaction
RNA was extracted from the scaffolds with an E.Z. N.A. Total RNA Kit (Omega Bio-Tek, Norcross, GA, USA) after being snap-frozen in liquid nitrogen. Quantifications and determination of RNA purity were performed with a NanoDrop Spectrophotometer (ThermoScientific NanoDrop Technologies, Wilmington, DE, USA). A high-capacity cDNA Archive Kit (Applied Biosystems, Carlsbad, CA, USA) was used for the reverse transcription reaction. Total RNA (1,000 ng) was mixed with nuclease-free water, reverse transcriptase buffer, random primers, deoxyribonucleotide triphosphate (dNTP), and MultiScribe reverse transcriptase. Standard enzyme and cycling conditions were applied for 2 minutes at 50°C and 20 seconds at 95°C, followed by 1 second at 95°C and 20 seconds at 60°C per cycle (40 cycles). cDNA corresponding to 10 ng mRNA in each reaction was prepared in duplicates since the standard deviation between the duplicates was minimal for each target gene. Real-time RT-PCR was performed on a StepOnePlus™ real-time PCR system (Applied Biosystems). Mouse-specific TaqMan gene expression assays (Applied Biosystem) were used (Table 1). Data analysis was performed with a comparative threshold cycle (Ct) method with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as endogenous control [25].

Histological staining
The remaining part of the sample was immediately embedded in optimal cutting temperature compound (Tissue-Tek O.C.T., Sakura Finetek, Tokyo, Japan) and kept at −80°C. Cryosectioning was done with Leica CM 3050S (Leica Microsystems, Wetzlar, Germany) at −24°C with 8-μm-thick sections. Sections from the middle part of the scaffolds were used for immunostaining. Samples intended for paraffin sectioning were fixed in 4% paraformaldehyde. Sections from the middle part of the samples were deparaffinized and stained with hematoxylin and eosin. Sections obtained after 3 weeks of implantation were incubated with primary antibodies in blocking buffer overnight at 4°C and with secondary antibodies for 2 hours the following morning. Nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI) (1:1,000) for 2 minutes, and the slides were mounted with Prolong® Gold Antifade Reagent (Invitrogen, Carlsbad, CA, USA) before imaging. Exposure time and fluorescence intensities were normalized to appropriate control images.

Rabbit polyclonal anti-mouse interleukin-1-beta (IL-1β) antibody (Abcam, Cambridge, UK), rat monoclonal anti-mouse neutrophil antibody (NIMP) (Abcam), rabbit polyclonal anti-mouse IL-6 antibody (Abcam), and rat monoclonal anti-mouse CD11b antibody (BD Biosciences, San Jose, CA, USA) were used as primary antibodies.

Table 1 List of TaqMan probes used for quantitative real-time reverse transcription-polymerase chain reaction analysis

| Gene symbol |  | Lot number |
|-------------|  |           |
| GAPDH       | VIC MGB | 4352339E |
| TNFa        | Mm 00443260_g1 | 1172346 |
| IL1β        | Mm 00434228_m1 | 1172142 |
| IL6         | Mm 00446190_m1 | 1169749 |
| IL4         | Mm 00445259_m1 | 1173207 |
| IL10        | Mm 00439614_m1 | 1172560 |
| iNOS        | Mm 00440502_m1 | 1171338 |
| HIF1a       | Mm 00468869_m1 | P121207-003A02 |
| HIF1B (ARN) | Mm 00507836_m1 | P110804-009B06 |
| mTOR        | Mm 0044968_m1 | P110708-007D02 |
Goat anti-rabbit fluorescein isothiocyanate (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and Alexa Fluor 546 goat anti-rat (Invitrogen) were used as secondary antibodies. Double staining with IL-1β and NIMP antibodies was performed to identify the number of IL-1β-positive cells and the number of neutrophils, respectively. Double staining with IL-6 and CD11b antibodies was performed to co-localize macrophages or monocyte-derived cells and their production of IL-6.

Quantification of immunostaining
On each slide, five sections from one scaffold were mounted. Each section on the slides was divided onto five measuring grids starting from top to bottom in vertical direction. Five sections on each slide (average for the mouse) and five measuring grids in each section (average for the section) were used for the image quantification. The images were taken with Zeiss AxiosVision 4.8.1 (Carl Zeiss, Toronto, ON, Canada) at magnification of 10x, and the files were exported as JPEG standard. The measuring grids in each section were noted carefully to avoid any overlap while taking the images. In each section, the areas with no cells were excluded. NIS elements AR 3.2 software (NIS elements, Tokyo, Japan) was used for quantifications. First, the threshold was defined for each channel: red, green, and blue. Next, all the channels were simultaneously referred before counting the cells to avoid misinterpretation. The blue channel was referred continuously to visualize the nuclei, and then the counting was done in the red and green channels separately. Finally, co-localized cells were counted for red and green fluorescent staining together. The number of counted cells per measuring grid was exported to a Microsoft Excel (Microsoft Corporation, Redmond, WA, USA) file before statistical analysis was performed.

Western blotting
Protein extraction was performed in accordance with the protocol of Chomczynski [26]. Briefly, protein precipitation from the organic phase was prepared by adding isopropanol. Precipitate was washed with ethanol and dissolved in 0.5% sodium dodecyl sulfate solution. Quantifications and determination of protein purity were performed with a NanoDrop Spectrophotometer (ThermoScientific NanoDrop Technologies). Total protein (30 μg) was mixed with 4X Laemmli sample buffer (Bio-Rad Laboratories, Hercules, CA, USA) and loaded on 4% to 15% Mini-PROTEAN TGX™ Precast Gel (Bio-Rad Laboratories) for electrophoresis. Transfer was done with polyvinylidene fluoride (PVDF) transfer membranes (TRANS-Blot™ Turbo™ System, Bio-Rad Laboratories). The membranes were blocked overnight at 4°C followed by primary antibodies—rabbit polyclonal anti-mouse IL-1β (Abcam), rabbit polyclonal anti-mouse IL-6 (Abcam), and rabbit polyclonal anti-mouse GAPDH (Santa Cruz Biotechnology) in blocking buffer overnight at 4°C—and with secondary antibody: horseradish peroxidase-conjugated goat anti-rabbit IgG (Bio-Rad Laboratories) for 1 hour. Immunoblotting bands were visualized by Immun-Star™ WesternC™ Chemiluminescence Kits, and a Gel Doc™ EZ System (Bio-Rad Laboratories) was used for imaging and protein-band assay.

Microarray
A microarray study of the gene expression profiles of MSCs co-cultured with ECs in vitro was previously conducted by our research group and reported recently [12]. From this study, a microarray data set was obtained and processed further. Data analysis was performed by J-Express 2009 software (MolMine, Hafsfjord, Norway) [27]. The significance analysis of microarrays (SAM) method was used. The data sets were submitted to the Database for Annotation, Visualization, and Integrated Discovery (DAVID) [28] as separate sets of inflammatory related genes, and pathways were determined by Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway mapping. The data are publically available at the National Center for Biotechnology Information with Gene Expression Omnibus accession number GSE63099.

Statistical analysis
The significance level was set to P value of less than 0.05 for all statistical analysis, with n = 6 for each group and time point. SPSS Statistics 21 (IBM, Armonk, NY, USA) was applied for statistical processing and analysis. Two groups (MSC and MSC/EC) were compared with the independent samples t test, whereas a multiple comparison one-way analysis of variance was performed to compare three experimental groups (MSC, MSC/EC, and empty scaffold).

Results
Gene ontology analysis
The DAVID pathway database [28] was used to explore and view functionally related genes. Multiple genes in the Toll-like receptor signaling pathway (Figure 1A) and the leukocyte transendothelial migration pathway (Figure 1B) had been influenced by the ECs. The over-represented genes in the respective pathways are presented in Table 2.

Cell migration to the construct
Hematoxylin-and-eosin staining showed the overall relationship between implanted scaffold and the local cells. One- and three-week samples reflected the recruitment of inflammatory cells in relation to the scaffold and progression over time (Figure 2C). Quantification of DAPI staining was done to show the difference in total cells migrating into the construct during the experimental
Figure 1 Genes involved in the leukocyte transendothelial migration and Toll-like receptor signaling pathways. The over-represented gene lists were submitted to the Database for Annotation, Visualization, and Integrated Discovery (DAVID) [28], and 24 genes were involved in the Toll-like receptor signaling pathway (A), whereas seven genes were involved in the leukocyte transendothelial migration pathway (B). Upregulated genes are labeled with a red star, and downregulated genes are labeled with a black star.
period, which was significantly higher after 3 weeks compared with 1 week (Figure 2D and 2E) \((P < 0.001)\). Three representative areas of \(200 \times 200 \mu m^2\) per picture, five pictures per section, and five sections per mouse were used for quantification.

**Table 2** Up- and downregulated genes from microarray gene ontology analysis comparing MSC (control) and MSC/EC (test)

<table>
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<th>PROBE_ID</th>
<th>Gene symbol</th>
<th>Fold change</th>
<th>FDR[]</th>
<th>PROBE_ID</th>
<th>Gene symbol</th>
<th>Fold change</th>
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<td></td>
<td></td>
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EC, endothelial cell; FDR[], false discovery rate; MSC, multipotent stromal cell.

Real-time reverse transcription-polymerase chain reaction

RT-PCR for mouse-specific genes was performed on in vivo samples to evaluate the mRNA expression of selected biomarkers important for recruitment of acute and chronic inflammatory cells as well as genes differentially expressed under hypoxic conditions. After 1 week of implantation, most of the target genes evaluated showed no significant differences between the experimental groups. However, IL-1β was significantly downregulated in the MSC/EC group \((P < 0.001)\) (Figure 3).

After 3 weeks of implantation, significant upregulations of pro-inflammatory biomarkers were found, compared with scaffolds implanted without cells. The mRNA expression of IL-1β and IL-6 was higher for the MSC group compared with MSC/EC constructs (Figure 4). There was similar expression of IL-10, an anti-inflammatory marker, for all of the groups. However, the mRNA expression of nitric oxide synthase 2 (NOS2) was significantly upregulated in both the MSC and the MSC/EC groups compared with control scaffolds implanted without cells. Hypoxia-inducible factor 1 alpha (HIF-1α), HIF-1β, and mammalian target of rapamycin (mTOR) expression was upregulated in both groups compared with the control. There was also a significant upregulation of these genes in the MSC/EC group compared with the MSC group.

Immunofluorescent staining and Western blotting

To investigate the association between inflammatory cytokines and migrating leukocytes, we used immunofluorescence double staining on samples retrieved after 3 weeks of implantation (Figure 5A). A remarkable overexpression of neutrophil-positive staining \((P < 0.001)\) was found in constructs implanted with vascular ECs. IL-1β-positive cells were fewer in the co-culture group than in the mono-culture group, although a statistical difference could not be found (Figure 5B). On the other hand, IL-1β-positive cells were significantly more in the MSC and the MSC/EC groups compared with control scaffolds implanted without cells \((P < 0.001)\) (Figure 5B).

Double staining for IL-6 and CD11b was performed as shown in Figure 5A. IL-6- and CD11b-positive cells were systematically quantified (Figure 6B). There was a significantly lower expression in the tissue-engineered constructs that included ECs compared with monocyte culture. CD11b-positive multinucleated giant
cells were observed in close relation to the surface of the scaffolds, contributing to their degradation process (Figure 6B). Giant cells co-stained with CD11b and IL-6 are shown in Figure 5C. IL-6- and CD11b-positive cells were significantly more in the MSC and MSC/EC groups compared with control scaffolds implanted without cells (Figure 6B) \(P<0.001\). Western blotting also revealed that the protein levels of IL-1\(\beta\) (Figure 5C) and IL-6 (Figure 6C) were lower in the co-culture group compared with the mono-culture group.
Figure 3 Real-time reverse transcription-polymerase chain reaction (RT-PCR) for mouse-specific genes after 1 week of implantation in vivo. Relative gene expression of tumor necrosis factor alpha (TNFα) (A), interleukin-1-beta (IL-1β) (B), IL-6 (C), IL-10 (D), nitric oxide synthase 2 (NOS2) (E), mammalian target of rapamycin (mTOR) (F), hypoxia-inducible factor 1 alpha (HIF-1α) (G), and HIF-1β (H) comparing multipotent stromal cell (MSC) and MSC/endothelial cell (MSC/EC) constructs. Data are presented as mean ± standard deviation (n = 6). *P < 0.05; **P < 0.01; ***P < 0.001. GAPDH, glyceraldehyde 3-phosphate dehydrogenase.
Discussion
The aim of this study was to investigate the early inflammatory response to implantation of tissue-engineered constructs containing human cells, and we therefore selected a moderately immunocompromised animal model. A xenograft model with implantation of human MSCs.
and ECs in NOD/SCID mice was applied. These animals are unable to perform VDJ recombination and subsequent production of antibodies but produce monocyte-derived cells and neutrophils, so that an early innate immune response can be induced [29]. However, analysis of additional inflammatory cells and cytokines associated with the adaptive immune response is one of the limitations of this study. Analysis of the inflammatory response therefore focused on the aforementioned neutrophils and monocyte-derived cells during the first 3 weeks after implantation, and the emphasis was on the transmigrations of these cells, which are events of central importance for creating a favorable microenvironment for tissue regeneration.

Tissue-engineered constructs with or without seeded vascular ECs were evaluated for their effect on transmigration of acute and chronic inflammatory cells following subcutaneous implantation. A two-dimensional culture model in vitro was performed to identify pathways for the observed modulation of the inflammatory stimuli, via a microarray gene ontology analysis.

At the site of inflammation, neutrophils are the first line of defense and are later replaced by monocyte-derived cells, T cells, and B cells. The number of neutrophils after 3 weeks of implantation was significantly higher on the constructs co-seeded with MSCs and ECs compared with constructs seeded with MSCs only. In addition, a reduced number of CD11b-positive cells were found for constructs comprising MSCs and ECs. The CD11b-positive cells were observed in close proximity to the surface of the scaffolds, and most cells were large and multinucleated. Biomaterials in tissue engineering are generally considered temporary structural support and delivery devices for bioactive molecules or stem cells or both. The rate of biomaterial degradation is an
important event developing in parallel with deposition of new extracellular matrix proteins that are intended to gradually replace the degrading material. The release of degradation products influences the pH in the local environment, potentially prolonging inflammation and influencing tissue repair, and polymer degradation is in part a result of a foreign-body giant-cell reaction [30]. Interestingly, the multinucleated giant cells in the MSC/EC group were generally larger, whereas in the MSC group an increased number of cells were observed, but these were smaller cells with fewer nuclei. These observations were not quantified, and longer observation time would be of further interest to evaluate whether the rate of degradation was influenced by the altered morphology of the multinucleated giant cells. Cross-sectional time points also have limitations in describing the development of acute and chronic inflammation but are useful in observing phase shifts between experimental groups.

Various cytokines are involved in attracting leukocytes from the systemic circulation to the site of a tissue defect. We performed analysis of gene expression on selected inflammatory biomarkers after 1 and 3 weeks of implantation. In the comparison of the MSC and MSC/EC groups, most target genes evaluated after 1 week were not significantly different, except IL-1β, which was clearly downregulated ($P < 0.001$) in the MSC/EC group. This downregulation was also found after 3 weeks ($P < 0.001$), and IL-1β expression was also strongly upregulated in the MSC group compared with the empty control scaffolds ($P < 0.001$). Also, immunostaining and Western blotting for IL-1β showed a downregulation in the MSC/EC group after 3 weeks of implantation, but this was not statistically significant. The expression of IL-1β was, however, inversely related to the number of transmigrated neutrophils detected at the same time point, which was strongly increased with the implantation of ECs. Additional time

![Figure 6](http://stemcellres.com/content/5/6/143)
points could have provided more information about the long-term effect of the release of IL-1β on neutrophil recruitment to the constructs. However, reliable information about the long-term immune response could not be obtained with the animal model selected in this study.

Analysis of human-specific gene and protein expression would also have been of interest to evaluate the inflammatory stimuli delivered directly by the implanted cells. Hematoxylin-and-eosin staining was used to study migration of murine cells into constructs, showing that the scaffolds were not rejected by the host cells. Multiple inflammatory biomarkers were influenced on the mRNA level after 3 weeks of implantation. IL-6 is a cytokine which exhibits both pro- and anti-inflammatory properties, and IL-6 trans-signaling helps to switch from neutrophils to monocytes by activating different chemokines [31]. IL-6 produced by MSCs can inhibit T-cell proliferation [32], and IL-6-dependent prostaglandin E2 (PGE2) secretion by MSCs can modulate the immune response by reducing the local inflammation [33]. In addition, MSCs can switch immune profiles from Th1 to Th2 through production of IL-6 [33].

The mRNA expression of IL-6 was significantly upregulated in both the MSC and the MSC/EC groups compared with the empty control scaffolds (P <0.001), and the expression in the MSC group was higher than in the MSC/EC group (P <0.05). The results were confirmed with immunostaining for IL-6-positive cells and Western blotting.

Overall, the expression of pro-inflammatory cytokines was reduced by adding ECs to the constructs. However, the expression was still higher than in the control group. Previous results have shown that implantations of human cells in this experimental animal model have generated an increased vascular density, and this could be suggested as a consequence of the release of inflammatory cytokines [6].

Cytokines, chemokines, and growth factors are released from a site of injury, attracting native MSCs [34]. Immunomodulatory properties of MSCs are influenced by local recruitment of inflammatory cells which secret pro-inflammatory cytokines interferon-gamma, tumor necrosis factor alpha (TNFs), IL-1α, and IL-1β. These cytokines induce upregulations of inducible nitric oxide synthase (NOS2) and chemokines, resulting in accumulation of T cells, B cells, and other immune cells responding to implanted MSCs. Increased levels of nitrous oxide can also suppress immune cell function [35,36], and the observed upregulated expression of genes encoding NOS2 could lead to upregulated expression of NOS2 and thus contribute to leukocyte recruitment and function.

Several target genes expressed during tissue hypoxia were upregulated in both experimental groups compared with the control, and the highest expression was detected in the MSC/EC group. Tissue hypoxia is directly related to inflammation, and inflamed resident and immune cells are highly metabolic and their oxygen consumption is very high. These cells migrate from a high oxygen tension in the blood stream to a hypoxic environment, resulting in production of HIFs. HIFs also exhibit both pro- and anti-inflammatory properties [37,38] and regulate the microenvironment at the site of inflammation. Cells from the myeloid lineage are also regulated by HIFs, and knockdown of HIF genes results in the impairment of both acute and chronic inflammatory processes [38]. In the present work, upregulated expression of HIF genes could be correlated to an increased acute inflammation. Measurements of tissue oxygen tension would be of interest to further explore these findings.

MSCs express various genes in the Toll-like receptor pathway, recruiting cells to sites of injury by activating both the innate and adaptive immune system. TLR4 and TLR3 and their ligands have the capability to induce nuclear factor-κB activity as well as the production of IL-6, IL-8, and CXCL10 [39]. It has been postulated that MSCs could also be attracted to a site by similar mechanisms. MSCs express different Toll-like receptors which directly affect the cell homing of other MSCs. TLR4-primed MSCs regulate pro-inflammatory cytokines, whereas TLR3-primed MSCs regulate anti-inflammatory cytokines. The production of IL-6 and IL-8 is a downstream mechanism of Toll-like receptors [40,41]. Our gene ontology data detected a total of 24 over-represented genes in the Toll-like receptor pathway after co-culture with ECs, modulating the production of inflammatory cytokines, and with both TLR3 and TLR4 upregulated in the MSC/EC group. The pro-inflammatory cytokine IL-6 was downregulated by fivefold, whereas IL-8—a cytokine acting as both a potent chemoattractant and an inducer of neutrophil activation—was upregulated by 10-fold in the MSC/EC group [42]. The CXCL12/CXCR4 axis is a key factor for homing neutrophils within the bone marrow and blood and has a major role in leukocyte trafficking in both homeostasis and inflammation [43]. The CXCL12/CXCR4 axis was downregulated in the MSC/EC group, and the influence of ECs on Toll-like receptor signaling and CXCL12/CXCR4 axis signaling presents possible pathways for activation of leukocyte transendothelial migration.

Conclusions

The addition of vascular ECs to tissue-engineered constructs clearly influenced transmigration of leukocytes involved in both acute and chronic inflammation. After 3 weeks of implantation, the number of neutrophils was significantly increased, whereas the number of monocyte-derived cells was decreased, suggesting a phase shift in the inflammatory response with the presence of ECs.
Evaluation of gene and protein expression showed altered expression of inflammatory cytokines, and gene ontology analysis revealed multiple genes in the leukocyte transmigration and Toll-like receptor pathways regulated by ECs.

Abbreviations
DAPPI: 4′,6-diamidino-2-phenylindole; DATDB: Database for Annotation, Visualization, and Integrated Discovery; EC: endothelial cell; GAPDH: glyceraldehyde 3-phosphate dehydrogenase; HIF: hypoxia-inducible factor; IL: interleukin; MSC: multipotent stromal cell; NIMP: monoclonal anti-neutrophil antibody; NOD/SCID: non-obese diabetic/severe combined immunodeficient; NO2: nitric oxide synthase 2; poly(L-LA-co-DX): poly(l-lactide-co-1,5-dioxepan-2-one); RT-PCR: reverse transcription-polymerase chain reaction.

Competing interests
The authors declare that they have no competing interests.

Authors’ contributions
TOP, ALB, YX, and KM conceived and designed experiments. SB-B, TOP, ALB, PP, and YX performed experiments. SB-B, TOP, PP, YX, and KM analyzed data. AFW and KM contributed reagents, scaffold production, materials, and analytical tools. SB-B, TOP, ALB, and KM wrote the manuscript. All authors read and approved the final manuscript.

Authors’ information
SB-B and TOP are shared first authors.

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Author details
1. Department of Biomedical Engineering, University of Bergen, Bergen, Norway. 2. Department of Clinical Dentistry, Center for Dental Clinical Research, University of Bergen, Bergen, Norway. 3. Centre for Cancer Biomarkers, Department of Clinical Medicine, Section for Pathology, University of Bergen, Bergen, Norway. 4. Children's Hospital Boston, Vascular Biology Department, Harvard Medical School, Boston, MA, USA. 5. Department of Fiber and Polymer Technology, KTH Royal Institute of Technology, Teknikringen 42, SE-100 44 Stockholm, Sweden. 6. Department of Clinical Dentistry - Periodontics, University of Bergen, Bergen, Norway.

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Angiogenic and Immunomodulatory Properties of Endothelial and Mesenchymal Stem Cells

Sushma Bartaula-Brevik, BDS,1 Torbjorn O. Pedersen, DDS, PhD,1 Anna Finne-Wistrand, PhD,2 Anne Isine Bolstad, DDS, PhD,1 and Kamal Mustafa, DDS, PhD1

It has been suggested that the effect of implanted cells on the local environment is important when selecting the appropriate cell type for tissue regeneration. Our aim was to compare the local tissue response to implanted human mesenchymal stem cells (MSC) and human umbilical vein endothelial cells (EC). MSC and EC were cultured in poly(l-lactide-co-1,5-dioxepan-2-one) scaffolds for 1 week in a bioreactor system, after which they were implanted subcutaneously in NOD/SCID mice. After 3 weeks, scaffolds were retrieved, and the mRNA expression of selected genes involved in hypoxia and inflammation was examined by real-time reverse transcription polymerase chain reaction and correlated with immunofluorescent staining for corresponding proteins. The Toll-like receptor signaling pathway was examined by superarray hybridization. The expression of 53 angiogenesis-related proteins was investigated by a proteome profiler angiogenesis antibody array kit. Vasculization was quantified using immunohistochemistry for CD31. The expression of hypoxia-inducible factors and biomarkers for angiogenesis was more strongly upregulated in response to implanted EC than to MSC, suggesting a higher sensitivity to low oxygen tension among EC. Hypoxic signaling was increased after implantation of EC compared with MSC, leading to a prolonged acute inflammatory phase that promoted ingrowth of vascular cells and establishment of the circulation. Inflammatory cytokines were also differently expressed at the gene and protein levels in the two experimental groups, resulting in altered recruitment of acute and chronic inflammatory cells. The end result of these differences was increased vessel formation within the constructs in the EC group.

Introduction

For vascular tissue engineering, as well as in regeneration of parenchymal tissue, such as muscle or bone, extensive efforts have been made to learn how to generate functional vascular supply for implanted cells. These efforts have been made based on the premise that implanted cells cannot survive, differentiate, and regenerate lost tissue without an immediate functional blood supply. In situations where the circulation has been compromised, cell therapy has been explored with the aim of re-establishing circulation to regenerate the damaged tissue. Hematopoietic stem cells have demonstrated cardiomyogenic potential after implantation in ischemic cardiac tissue,1 and endothelial progenitor cells have been the subjects of extensive research efforts for their potential in cardiovascular regeneration.2 Bone marrow mesenchymal stem cells (MSC) are the most widely applied cells in cell therapy due to their availability and differentiation potential. The interaction between MSC and vascular cells has been extensively explored, and MSC have diverse roles in the vascularization of tissue through either direct contact or indirect signaling. The autocrine and paracrine effects of MSC initiate release of cytokines, growth factors, and extracellular matrix proteins.3–5 In an attempt to generate functional vessels that can be connected with the local circulation after implantation, coculture systems have been used with vascular cells grown with supporting cells, such as smooth muscle cells (SMC) or MSC.5–7

The proliferation and maturation of endothelial cells (EC) and surrounding matrix depend on local oxygen supply,8,9 and the crosstalk between EC and immune cells, which
results in release of cytokines and chemokines. The direct contribution of implanted vascular or MSC to tissue regeneration is not well described in most studies, although both cell types contribute to development and repair of the majority of the body tissues. Several authors have suggested that the favorable effect of implanting cells or bioactive molecules on regeneration in a damaged area is as much the result of creating a favorable microenvironment for cell migration and proliferation as it is direct deposition of extracellular matrix components by the implanted cells.

It is clear, however, that the basis for healthy tissue is a functional circulation, which in turn applies to both parenchymal and vascular tissue engineering. All implanted cells are exposed to a hypoxic environment after implantation due to the acute inflammation following the surgical procedure, as well as the initial absence of blood vessels. The cellular response to hypoxia is therefore a key in facilitating an adequate postoperative inflammatory reaction and the establishment of a functional blood supply. These cellular events are closely connected to each other and crucial for ensuring vital and healthy tissue regeneration.

Based on this, our hypothesis was that MSC and EC respond differently to the hypoxic environment created when cells are implanted in vivo and that the inflammatory response as well as the establishment of the blood supply are different between the two cell types. The aims of the study therefore were first to compare the effect of implanting MSC and EC on the expression of inflammatory cytokines and the migration of acute and chronic inflammatory cells and second to compare the effect of implanted cells on expression of vascular factors and development of the circulation.

Materials and Methods

Fabrication of scaffolds

Poly(l-Lactide-co-1,5-dioxepan-2-one) [poly (LLA-co-DXO)] was synthesized, and scaffolds were prepared as previously described. Briefly, a solvent casting/particulate leaching method was used to produce poly (LLA-co-DXO) porous scaffolds with pore sizes of 90–500 μm, 12 mm diameter, and 1.5 mm thick. The sterilization of scaffolds was carried out in a pulsed electron accelerator operating at 6.5 MeV (Mikrotron, Accelerator teknik, The Royal Institute of Technology, Stockholm, Sweden), with radiation of 2.5 Mrad dose in an inert atmosphere.

Cell culture

Primary human MSC were purchased from StemCell Technologies. Flow cytometry was done to confirm the purity of the cells, >90% of the cells expressed CD29, CD44, CD105, and CD166 and <1% expressed CD14, CD34, and CD45. The MSC were cultured in MesenCult® complete medium (StemCell Technologies) following the manufacturer’s instructions.

Human umbilical vein EC were purchased from Lonza (Clonetics®). They were expanded in endothelial cell growth medium (EGM®) (Lonza) containing 500 mL endothelial cell basal medium and supplements, 10 mL fetal bovine serum, 2 mL bovine brain extract, 0.5 mL human endothelial growth factor, 0.5 mL hydrocortisone, and 0.5 mL GA-1000. The MSC and EC were cultured at 37°C and 5% CO₂ and were used between passages 2 and 5.

In vivo implantation

The scaffolds were preseeded with cells before in vivo implantation and were treated as previously described. Briefly, the scaffolds were divided into two groups: MSC and EC, pretreated with respective complete medium and incubated overnight at 37°C and 5% CO₂. In total, 5 × 10⁵ cells were seeded per scaffold in each group. An orbital shaker (Eppendorf®) was used to facilitate homogeneous distribution of cells. The scaffolds loaded with cells were kept in an incubator overnight to allow cell attachment. The following day, scaffolds were transferred to separate modified spinner flasks (Wheaton Science) for 1 week in a dynamic culture system with 50 rpm. After 1-week in vitro culture, 6-mm discs were punctured from the center of the scaffold with a dermal skin puncher and then implanted in vivo. For evaluating seeding efficiency of MSC and EC, the cells were seeded in scaffold as described above, and after 1 h, the scaffolds were removed from the wells, and unattached cells were counted by an Automated Cell Counter (Invitrogen). Cell seeding efficiency was calculated by the following equation:

\[
\text{Cell seeding efficiency} = \left(1 - \frac{\text{number of unattached cells}}{\text{number of seeded cells}}\right) \times 100
\]

The animal experiments were approved by the Norwegian Animal Research Authority and conducted according to the European Convention for the Protection of Vertebrates used for Scientific Purposes (local approval no. 3029). Nonobese diabetic/severe combined immunodeficient (NOD/SCID) mice (n = 15) were purchased from Taconic Farms (Bomholtgård Breeding and Research Center). The animals were kept in polystyrene cages containing wood shavings in a climate-controlled environment with 12-h dark–12-h light cycles and fed with standard rodent chow and water ad libitum. Animals were 6–8 weeks old at the time of surgery. The animals were anesthetized with an intramuscular injection of 20 mL of Rompun (xylazine) (20 mg/mL) (Bayer Healthcare) and Narketan (ketamine) (Vétoquinol) in 1:2 ratio. On the back of the mice, a 2.5-cm incision was made, providing sufficient space for subcutaneous implantation of scaffolds. A scaffold for each experimental group (MSC or EC) was placed in the mouse, with a total of six mice used for each time point. Wounds were closed with Vetbond™ Tissue Adhesive (n-butyl cyanoacrylate) (3M™). After 3-week implantation, animals were euthanized with deep isofluran (Schering Plough) anesthesia, followed by cervical dislocation, after which the implanted scaffolds were carefully dissected and retrieved. The samples were then divided and further processed either for real-time reverse transcription polymerase chain reaction (RT–PCR) analysis and western blotting or for histological embedding.

Real-time RT–PCR

E.Z.N.A.® Total RNA Kit (Omega Bio-Tek) was used to isolate the RNA from the samples. Quantifications and determination of RNA purity were performed with a NanoDrop Spectrophotometer (Thermo Scientific NanoDrop Technologies). A high-capacity cDNA Archive Kit (Applied Biosystems) was used for the reverse transcription reaction. Total RNA (1000 ng) was mixed with nuclease-free water,
reverse transcriptase buffer, random primers, dNTP, and MultiScribe reverse transcriptase. Standard enzyme and cycling conditions were applied for 2 min at 50°C and 20 s at 95°C, followed by 1 s at 95°C and 20 s at 60°C per cycle (40 cycles). cDNA corresponding to 10 ng mRNA in each reaction was prepared in duplicate since the standard deviation between the duplicates was minimal for each target gene. Real-time RT–PCR was performed on a StepOnePlus™ Real-Time PCR System (Applied Biosystems). Mouse-specific TaqMan® gene expression assays (Applied Biosystems) were used to evaluate postimplanted constructs. The preimplanted constructs were also assessed to evaluate baseline expression of inflammatory and hypoxic markers. For this, human-specific TaqMan gene expression assays (Applied Biosystems) were used. Data analysis was performed with a comparative Ct method, with GAPDH as endogenous control.17

Superarray analysis was performed for the mouse Toll-like receptor (TLR) pathway Rt² Profiler PCR Arrays (SuperArray Bioscience). Rt² PCR Array First Strand Kit (SuperArray Bioscience) was used for cDNA synthesis. PCR was done on a StepOnePlus Real-Time PCR System (Applied Biosystems) with Rt² Real-Time SyBR Green/Rox PCR Mix (SuperArray Bioscience).

Immunofluorescent staining

The remaining half of the sample was immediately embedded in optimal cutting temperature compound (Tissue-Tek® O.C.T.; Sakura Finetek) and kept at −80°C. Eight-micrometer-thick cryosectioning was done with Leica CM3050 S (Leica Microsystems) at −24°C. Sections were obtained from the middle part of the scaffolds and were used for immunostaining. Sections from 3-week samples were incubated with primary antibodies in blocking buffer overnight at 4°C and with secondary antibodies for 2 h the following day. The nuclei were counterstained with DAPI (1:1000) for 2 min, and the slides were mounted with ProLong® Gold Antifade Reagent (Invitrogen) before imaging. Rabbit polyclonal anti-mouse interleukin (IL)-1β (Abcam), rat monoclonal anti-mouse neutrophil (NIMP) (Abcam), rabbit polyclonal anti-mouse IL-6 (Abcam), rat monoclonal anti-mouse CD11b (BD Biosciences), and rat monoclonal anti-mouse CD31 (BD Biosciences) were used as primary antibodies. Goat anti-rabbit FITC (Santa Cruz Biotechnology) and Alexa Fluor 546 goat anti-rat (Invitrogen) were used as secondary antibodies. Double staining with IL-1β and NIMP was performed to identify the number of IL-1β-positive cells and the number of neutrophils, respectively. Double staining with IL-6 and CD11b was performed to colocalize macrophages or monocyte-derived cells and their production of IL-6. CD31 staining was performed to allow determination of the total area fraction of vessels.

Quantification of immunostaining

Five sections from one scaffold were mounted on each slide. Each section on the slides was divided into five measuring grids starting from top to bottom in the vertical direction. Five sections on each slide (average for the mouse) and five measuring grids in each section (average for the section) were used for image quantification. Images were taken with a Zeiss AxioVision 4.8.1 at 10× magnification, and the files were exported as JPEG standard. The measuring grids in each section were noted carefully to avoid any overlap while taking the images. In each section, the areas with no cells were excluded. NIS Elements AR 3.2 software was used for quantifications. First, the threshold was defined for each channel: red, green, and blue. Next, to avoid misinterpretation, all the channels were simultaneously referred before counting the cells. The blue channel was referred continuously to visualize the nuclei, and then, the counting was done in the red and green channels separately. Finally, colocalized cells were counted for red and green fluorescent staining together. The number of counted cells was exported to a Microsoft Excel (Microsoft Corporation) file before statistical analysis was performed. For quantification of CD31 immunostaining, images were taken at 20× magnification, and the total area fraction of vessel was quantified.

Western blotting

Protein extraction was performed following the protocol of Chomczynski.18 Briefly, isopropanol was added to the organic phase (extracted during RNA isolation) for protein precipitation. Precipitate was washed with ethanol and dissolved in 0.5% sodium dodecyl sulfate (SDS) solution. NanoDrop Spectrophotometer (Thermo Scientific NanoDrop Technologies) was used to quantify and determine protein purity. A total of 30 µg protein was mixed with 4× Laemmli sample buffer (Bio-Rad Laboratories) and loaded on 4–15% Mini-PROTEAN® TGX™ Precast Gel (Bio-Rad) for electrophoresis. Transfer was done with PVDF transfer membranes (TRANS-Blot® Turbo™ System; Bio-Rad). The membranes were blocked overnight at 4°C and incubated the next day with primary antibodies (Santa Cruz): anti-mouse hypoxia-inducible factor 1-alpha (HIF-1α), anti-mouse aryl hydrocarbon receptor nuclear translocator (ARNT), and anti-mouse GAPDH in blocking buffer overnight at 4°C and the following day with secondary antibody: horseradish peroxidase-conjugated goat anti-rabbit IgG (Bio-Rad) 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.

Proteome profiling

The Mouse Angiogenesis Array (R&D Systems, Inc.) was used to detect the expression of 53 angiogenesis-related proteins in the experimental groups. These Proteome Profiler Arrays were handled according to the manufacturer’s protocol. Briefly, the protein extraction was done, and 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 chemiluminescence detection reagents. The Gel Doc EZ System (Bio-Rad) was used for imaging. The mean spot pixel density was quantified using image software analysis.

Statistical analyses

SPSS Statistics 21 (IBM) was applied for statistical processing and analysis. Two groups, MSC and EC, were compared with the independent samples t-test. The significance level was set to p < 0.05 for all statistical analyses, with n = 6 for each group.
Results

Alteration in the TLR pathway

The evaluation of genes related to the mouse TLR pathway showed that 49 genes were downregulated and 35 genes were upregulated, with a fold change more than one in the EC group compared with the MSC group (Fig. 1A, B).

Release of inflammatory cytokines

Inflammation was evaluated through the release of pro- and anti-inflammatory cytokines. RT–PCR (human-specific genes) of preimplanted constructs showed higher mRNA expression of the genes encoding IL-1β and IL-10 in the MSC group, whereas the expression of the gene IL-6 was similar in both groups (not shown). RT–PCR for mouse-specific genes was performed after 3 weeks of implantation. Decreased mRNA expression of the genes encoding the proinflammatory cytokines, IL-1β and IL-6, was observed in EC constructs ($p < 0.05$) (Fig. 2B, C). However, the mRNA expression of the gene encoding the anti-inflammatory cytokine IL-10 was upregulated in EC constructs compared with MSC constructs ($p < 0.01$) (Fig. 2D).

Inflammatory cell recruitment

The acute and chronic inflammatory cell recruitment was evaluated by immunofluorescence staining and systematic

FIG. 1. Alteration in the Toll-like receptor (TLR) signaling pathway. (A) RT² Profiler TLR signaling polymerase chain reaction (PCR) array following 3 weeks of implantation in vivo. Heat maps representing the relative expression levels of all genes involved in the mouse TLR signaling pathway. Increased and decreased fold changes in endothelial cell (EC) constructs compared with mesenchymal stem cell (MSC) constructs are represented by red and green squares, respectively. (B) Gene layout of heat maps and data are presented as *$p < 0.05$, **$p < 0.01$. Color images available online at www.liebertpub.com/tea

FIG. 2. Real-time reverse transcription (RT)–PCR for mouse-specific genes after 3 weeks of implantation in vivo. Relative gene expression of the genes encoding the inflammatory cytokines (A) tumor necrosis factor-alpha (TNFa), (B) interleukin-1-beta (IL-1β), (C) IL-6, and (D) IL-10 comparing MSC and EC constructs. Data presented as mean ± standard deviation ($n = 6$). *$p < 0.05$, **$p < 0.01$. 
quantification. Cells stained positive with antineutrophil antibody were found scattered all over the constructs, predominantly in the EC group (Fig. 3A) \((p < 0.01)\). Fewer IL-1\(\beta\)-positive cells were seen in the EC group than in the MSC group \((p < 0.01)\) (Fig. 3C). Double staining for monocyte-derived cells (CD11b) and IL-6 showed fewer positive cells in the EC group, but this difference was not statistically significant (Fig. 3C). Cells stained positive with CD11b and IL-6 were found in close proximity to the scaffold and may have aided in the degradation process (Fig. 3B).

**Cellular response to a hypoxic environment**

To evaluate the response to hypoxia after implantation, the mRNA expression of the genes encoding HIF-1\(\alpha\) and ARNT was analyzed. Both markers were significantly upregulated in EC constructs after 3 weeks of implantation \((p < 0.01)\). On the contrary, western blotting showed lower protein level of HIF-1\(\alpha\) in EC constructs, while the protein level of ARNT was higher in EC constructs compared with MSC constructs (Fig. 4E). The mRNA expression of the gene encoding mTOR was significantly upregulated in the EC group (Fig. 4D). The mRNA expression of the genes encoding human HIF-1\(\alpha\) and ARNT was significantly higher in the EC group before implantation compared to the MSC group.

**Angiogenic properties of MSC and EC**

To evaluate the angiogenic properties of the cells after 3 weeks of implantation, proteome profiling was done (Fig. 5A). The histogram generated after the mean spot pixel density quantification revealed that of the 53 related proteins for angiogenesis, 10 proteins were highly regulated. The proteins related to vascular development were higher in the EC group compared with the MSC group (Fig. 5B). In addition, the total area fraction of vessel formation was quantified to determine the influence of implanted cells on establishment of the vasculature. The EC constructs had a higher density of blood vessel formation, as evaluated by CD31 immunostaining, and the total vessel area fraction was significantly higher \((p < 0.01)\) (Fig. 5C).
In cell therapy, the release of inflammatory cytokines facilitates vascularization and recruitment of leukocytes in the first phase after implantation. If the balance between pro- and anti-inflammatory cytokines is favorable, this leads to tissue organization and regeneration. The aims of this study were to evaluate and compare the initiation of angiogenesis and early inflammation after implantation of cell/scaffold constructs using two different cell types. The cells were grown under the same culture conditions before implantation. The seeding efficiency of the two cell types in the scaffold was similar. After implantation of the cells, host tissue response was evaluated. The NOD/SCID mouse model was chosen as it allows human cells to grow and differentiate, and previous studies have shown that these...
animals can produce monocyte-derived cells and neutrophils.21 This moderately immunocompromised murine model thus also allows investigation of the early innate immune response to implantation. Response of the implanted MSC and EC to hypoxia was investigated, as was their effect on the local microenvironment, subsequent recruitment of acute and chronic inflammatory cells, and establishment of blood vessels in the area of the implant. The mouse-specific markers were used to evaluate the host response toward the implanted cells.

MSC have been reported to express TLR that may play a role in immune regulation. TLR priming of MSC results in two active phenotypes, MSC1 and MSC2. TLR4-primed MSC (MSC1) expresses proinflammatory cytokines, whereas TLR3-primed MSC (MSC2) regulates anti-inflammatory cytokines.22 TLR activation in MSC could produce inflammatory mediators, such as IL-1β, IL-6, IL-8/CXCL8, and CCL5, resulting in recruitment of inflammatory cells.23 TLR and its ligands may also induce the proliferation and differentiation of MSC.24 TLR ligand activation on EC regulates the expression of TLR1, TLR3, and TLR4 and downstream production of IL-6.25 The superarray analysis in the present study for the mouse TLR pathway demonstrated downregulation of the genes encoding TLR1, TLR2, TLR3, TLR4, and TLR8 in the EC group compared with the MSC group (p < 0.05). The downstream production of TLR was evaluated by looking at mRNA expression of the genes encoding IL-1β, IL-6, and IL-10. Macrophages are subdivided into M1 and M2, according to their ability to produce different cytokines. M1 releases proinflammatory cytokines in contrast to M2, which releases anti-inflammatory cytokines.26 The mRNA expression of genes encoding proinflammatory markers, such as IL-1β and IL-6, was significantly downregulated in the EC group compared with the MSC group (p < 0.05), and EC contributed to the anti-inflammatory effects via upregulated mRNA expression of the IL-10 gene (p < 0.01). These findings suggest that EC can switch the cytokine expression of macrophages to the anti-inflammatory M2 phenotype.

Copolymer scaffolds provide temporary support in tissue engineering constructs as they degrade over time. A foreign-body giant cell reaction mediates degradation of the scaffold along with buildup of new extracellular matrix protein and recruitment of granulocytes, MSC, and monocyte-derived cells.27 Sung et al. found no significant difference in inflammatory cell recruitment by different copolymers after 2–4 weeks of implantation in vivo. Furthermore, they showed that inflammatory cell recruitment had a positive correlation with angiogenesis.28 Similarly, we demonstrated the recruitment of inflammatory cells adjacent to the scaffold after 3 weeks’ implantation. Neutrophils are the first line of defense in acute inflammation. After 3 weeks’ implantation, we observed that neutrophil transmigration to the EC constructs was significantly higher than in MSC constructs (p < 0.01). Our previous study also found that coculture of MSC with EC resulted in more leukocyte transmigration to the construct via the TLR pathway.29 Furthermore, IL-1β-positive monocyte-derived cells were decreased in the EC group relative to the MSC group (p < 0.01), while the number of IL-6- and CD11b-positive cells was higher in the MSC group. Multinucleated giant cells were found in proximity to the scaffold, aiding in degradation. Neutrophils and CD11b-positive cells have been shown to increase angiogenesis, and the differing inflammatory cell recruitment seen between the experimental groups may also have contributed to the difference in vascularization.

Tissue ischemia is one cause of failure of tissue-engineered constructs. The inflamed microenvironment is highly metabolic and has higher oxygen consumption, leading to low oxygen tension. Additionally, hypoxia also mediates acute and chronic inflammation. Hypoxia in the surrounding environment activates the release of HIF, a transcription factor for angiogenesis.32–34 The preimplanted EC group showed upregulation of human hypoxia-related genes. The paracrine effect of these cells could have enhanced the host microenvironment to promote angiogenesis. The mRNA expression of the HIF-1α and HIF-1β genes was significantly upregulated in the EC group, p < 0.05 and p < 0.001, respectively. The upregulation in HIF genes promotes angiogenesis. In addition, HIF-1α protein levels were higher in the MSC group. The initiation of angiogenesis leads to higher oxygen saturation in the construct area, which could have resulted in lower HIF-1α protein expression in EC constructs. However, it has been reported that HIF-1β is less sensitive to change in oxygen tension.35 In the present study, we found that mRNA expression and the protein level of HIF-1β were upregulated in the EC group compared to the MSC group.

NOS2/NOS has also been reported as a hypoxia-inducible gene. It regulates vascular homeostasis and inflammation and may also promote HIF-1α stabilization and activate downstream gene expression.36,37 We observed upregulation of the NOS2 gene in the EC group compared with the MSC group, promoting angiogenesis. The mRNA expression of the gene encoding mTOR was higher in the EC constructs, which also leads to vascularization of the constructs. The mTOR pathway regulates cell proliferation, adhesion, migration, metabolism, and survival, and mTOR modulates angiogenic factors by activating either HIF-1α or NOS2.38

Proteome profiling was performed to compare the angiogenic factors released by EC and MSC constructs after 3 weeks’ implantation. Among all differentially regulated proteins, osteopontin, nephroblastoma overexpressed (NOV), and insulin-like growth factor-binding protein-3 (IGFBP-3) highly upregulated in EC constructs. Osteopontin is a potent angiogenic factor, which promotes proliferation, migration, and capillary formation of EC.39 NOV is highly expressed in SMC of the arterial vessel wall and supports cell adhesion, migration, and survival of EC, resulting in neovascularization.40 IGFBP-3 has diverse roles in angiogenesis. It regulates proangiogenic molecules and induces capillary formation in vivo.41 In 1999, Dawson et al. identified a serine protease inhibitor, SERPIN F1, as an antiangiogenic factor.42 SERPIN F1 was increased following MSC implantation in the present work. The release of proangiogenic proteins was higher than antiangiogenic factors after implantation of EC, which was reflected in the increased area of vessel formation.

**Conclusions**

Implantation of EC resulted in downregulation of TLR and biomarkers associated with acute inflammation. Significantly higher numbers of neutrophils were present after 3 weeks’ implantation, which was associated with hypoxia-induced signaling. In addition, increased vascular ingrowth...
was found after implantation of EC. These findings suggest that implantation of EC stimulates hypoxic signaling pathways, which leads to a prolonged acute inflammatory phase compared with implantation of MSC. This promotes ingrowth of vascular cells and establishment of the circulation.

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Disclosure Statement

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Address correspondence to:
Kamal Mustafa, DDS, PhD
Department of Clinical Dentistry
University of Bergen
Ardstaveien 19
5009 Bergen
Norway

E-mail: kamal.mustafa@iko.uib.no

Sushma Bartaula-Brevik, BDS
Department of Clinical Dentistry
University of Bergen
Ardstaveien 19
5009 Bergen
Norway

E-mail: sushma.bartaula@iko.uib.no

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