Introduction

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INTRODUCTION

Ischemia is defined as a restriction, or inadequacy, in local blood supply. The most common causes of ischemia are acute arterial thrombus formation, stenosis as seen in atherosclerosis, vasoconstriction and embolisms. Consequent to the insufficient blood supply, a shortage of oxygen and nutrients, as well as a build-up of metabolic waste occurs. These events can be experienced by cardiovascular patients suffering from ischemia as angina pectoris in myocardial ischemia, or intermittent claudication in peripheral artery disease.

In the Netherlands the overall lethality of cardiovascular diseases dropped from 1076 deaths in 1980 to 496 deaths in 2008 (per 100,000 people), resembling a 54% reduction in the last 28 years. Despite the reduction of mortality by the medical advantages made in the last decades, the aging population and increased occurrence of obesity and diabetes did not only cause an increase in the occurrence of heart failure but also in chronic ischemic cardiovascular pathologies, such as poor perfusion of the heart and the legs. Apparently, the endogenous adaptation of blood vessels is not always sufficient for providing the tissue’s requirements. New approaches to stimulate vascularization of ischemic tissues are desired. Furthermore, new avenues in tissue engineering are hampered by poor perfusion of the transplanted graft. Stimulation of neovascularization is pivotal for the survival of these transplants.

Ischemia always results in an inadequate delivery of oxygen to meet the demands of a tissue, i.e. hypoxia. The resulting drop in the partial oxygen pressure in a tissue leads to a reduction in mitochondrial respiration and oxidative metabolism and an increase in glycolytic activity in order to cope with the energy demand. For brief episodes of hypoxia this metabolic switch is capable of maintaining tissue viability. However, prolonged exposure to hypoxia will result in cellular dysfunction and ultimately cell and tissue death.

Pharmacological and surgical interventions are until now the only options to alleviate severe and widespread ischemia, but they have reached a limit in their effects. An alternative approach may be to stimulate the body’s own ability to improve the vascularization of a tissue, such as also occurs during development and in the formation of a granulation tissue as part of wound healing. In the adult this process is stimulated by hypoxia and inflammation. Better understanding of the mechanism of this vascularization process may identify and overcome bottle-necks in the body’s own revascularization system.
The human body is equipped with a defensive oxygen-sensing machinery, which initiates adaptations to minimize hypoxic injury, promotes survival in a low oxygen environment and contributes to restoring cellular oxygen delivery. This system, which is for a larger part controlled by the transcription factor hypoxia-inducible factor (HIF), is a major driving force for the induction of angiogenic growth factors and neovascularization, and also controls red blood cell production to increase blood oxygen carrying capacity. Neovascularization in the adult encompasses sprouting and intussusceptive angiogenesis, and requires adaptation of the proximal arterial vascular bed, also called arteriogenesis, for adequate perfusion of the new vessels. The scope of this thesis is on the contribution of endothelial progenitor cells and chronic hypoxia on angiogenesis. Therefore, we will first introduce the neovascularization process and the role and contribution of hypoxia and circulating progenitor cells in angiogenesis.

Neovascularization

The formation of new microvessels can occur by different mechanisms (Fig. 1). During embryogenesis the blood islands fuse into a vascular network, a process that was originally indicated as vasculogenesis. The vascular network subsequently expands usually by sprouting angiogenesis, and sometimes by non-sprouting splitting of pre-existing vessels (intussusception). Proper perfusion of the new vascular bed requires adaptation of the proximal vessels (arterioles and arteries), which occurs by structural widening of the vessels.

In the adult, angiogenesis is observed in the female reproduction organs, in wound repair and in many pathological conditions including chronic inflammation, tissue ischemia, and cancer. In sprouting angiogenesis, angiogenic growth factors and cytokines activate endothelial cells in pre-existing blood vessels through their corresponding receptors. The prime angiogenic factor is vascular endothelial growth factor A (VEGF-A), which is highly induced by hypoxia and acts on endothelial cells via the VEGF receptors KDR (VEGFR2) and Flt-1 (VEGFR1). These activated endothelial cells start to produce proteases (described in Chapter 2) which break down the surrounding matrix, thereby facilitating the migration and proliferation of endothelial cells towards the angiogenic stimulus (Fig. 1). The massive sprouting response induced by VEGF is controlled by the induction of DLL4, which activates Notch signaling and limits sprouting. By this response a differentiation occurs between the sprouting cells on the tip of a sprout (tip cells) and the stalk cells, which contribute to the size of the new endothelial tube. After subsequent deposition of a new basal membrane, the new capillary sprouts become stabilized by interaction
with pericytes. Simultaneously, the sprouting tubules connect to parent blood vessels and form loops with a full-fledged vessel lumen, which start to supply a poorly perfused area with oxygen and nutrients.

Vasculogenesis was first believed to occur only during embryonic development, in

Fig 1. The three processes of adult neovascularization: adult vasculogenesis, angiogenesis and arteriogenesis.

During adult vasculogenesis, developing blood vessels are supported, or created de novo, by circulating endothelial colony-forming cells (ECFC) which differentiate into true endothelial cells and align into a functional endothelial layer. The perivascular colony-forming unit endothelial cells (CFU-EC) support the ECFC and resident endothelial cells by the production and release of a large number of pro-angiogenic growth factors and cytokines. In angiogenesis, capillaries are made from the pre-existing vasculature. Activated endothelial cells start to produce proteases which break down the surrounding matrix, thereby facilitating the migration and proliferation of endothelial cells towards the angiogenic stimulus. The capillary sprouts become stabilized by the interaction with pericytes. In the third process, arteriogenesis, the diameter of existing arterial vessels increases and maturation of pre-existing arterioles to mature collaterals occurs. Arteriogenesis is mainly driven by increased blood flow and the thereby induced increased wall shear stress on the endothelial cells and their effects on the underlying smooth muscle cells.
which endothelial progenitor cells (angioblasts) migrate and differentiate in response to local cues to form new blood vessels\textsuperscript{31}. However, after the identification of so-called endothelial progenitor cells (EPC) by Asahara et al. it was proposed that these cells contribute to neovascularization in the adult via a process that has many similarities with vasculogenesis (Fig. 1)\textsuperscript{32-38}. As will become clear in the section on EPC and a number of the chapters of this thesis (Chapter 3, 4 and 5), the original concept of EPC has changed considerably and nowadays involves a population of monocyte-like cells with endothelial characteristics that support angiogenesis by providing angiogenesis-stimulating factors, and a small population of endothelial colony-forming cells (Fig. 2, Table 1).

The above-mentioned angiogenic processes are an absolute requirement for the appropriate delivery of oxygen and nutrients to the tissue. However, without a proper connection to the supplying arterial bed, these processes are ineffective. In order to successfully restore tissue perfusion, angiogenesis and vasculogenesis need to coincide with arteriogenesis, which refers to an increase in the diameter of existing arterial vessels and maturation of pre-existing microvessels to mature collaterals (Fig. 1)\textsuperscript{39}. Arteriogenesis is linked to elevated flow. The increased shear stress on the endothelial surface induces a cellular response that facilitate the arteriogenesis process, while sustained elevated blood pressure increases radial wall stress and additional smooth muscle cell adaptation. In physiological conditions the vessel increases in diameter until the stress is normalized, and a new balance is created between newly formed microvessels and the functionally connected supplying arterioles and arteries\textsuperscript{40}.

**ENDOTHELIAL PROGENITOR CELLS**

In addition to angiogenesis, it is the current believe that vasculogenesis can also contribute to neovascularization in adult life. This process, which is driven by the recruitment and incorporation of endothelial progenitor cells in developing blood vessels, was first described in 1997\textsuperscript{32, 41}. Endothelial progenitor cells gained a lot of scientific interest because of their potential as a therapeutic vehicle for angiogenesis and reendothelialization, as well as the usage as biomarker in a variety of pathological conditions\textsuperscript{42-44}.

The \textit{in vitro} enumeration of colony-forming units of supposed endothelial progenitor cells (CFU-EC) has been widely used to quantify EPC circulating in human peripheral blood. Hill \textit{et al.} observed a correlation between the number of CFU-EC and a subjects’ combined Framingham risk factor score\textsuperscript{45}, which suggested that endo-
The loss of functional possible circulating progenitor cells may affect the progression of cardiovascular disease. Since this initial observation, small variations of the EPC culture assay have been reported. A reduction in number or function of EPC (i.e., EPC dysfunction) was observed in patients with chronic congestive heart failure, acute lung injury, chronic obstructive pulmonary disease, diabetes, and rheumatoid arthritis. These findings provided a means to study EPC numbers as a prognostic tool, whereby they reflected the course of a disease, or their causal contribution to the etiology of vascular diseases.

At the onset of the study described in this thesis, EPC were described as bone marrow-derived circulating cells positive for CD34, AC133 (CD133), and VEGFR2 (vascular endothelial growth factor receptor 2, or KDR), which displayed shared characteristics with hematopoietic stem cells and the embryonic hemangioblast, which can give rise to all blood cell lineages and vascular cells. The observation of endothelial outgrowth from CD34-enriched cell fractions was interpreted as constituting evidence for the presence of circulating angioblasts, one of the two products of the hemangioblast from which endothelial cells are derived (the other being the hematopoietic stem cell). In vitro, the uptake of acetylated low-density lipoprotein (Ac-LDL), Ulex europaeus lectin binding, together with endothelial marker expression were commonly used to define these cells.

The strategy of stimulating neovascularization by transplanting these EPC has been widely explored in preclinical and clinical settings. Initially, a number of preclinical studies on EPC transplantation demonstrated a positive therapeutic efficacy. However, during the course of the studies of this thesis it has become clear that the actual incorporation efficacy of EPC into the endothelial lining of developing vessels is limited. In contrast, a significant number of reports documented a perivascular localization of transplanted EPC. These cells stained positive for angiogenic growth factors and chemokines. It was therefore claimed that the capacity of EPC to promote neovascularization was mainly accomplished by paracrine effects (see also Chapter 3).

However, most clinical trials on the use of EPC for neovascularization showed minor beneficial effects, or only temporal or no improvement at all, whereas other trials showed adverse side effects, such as an increased risk of in-stent stenosis and possibly myocardial ischemia. Since there is no evidence of long-term engraftment of cultured EPC in the endothelial lining, many investigators speculated that the paracrine effects of EPC were responsible for the modest effect seen in
patients\textsuperscript{100-102}. The variety in clinical outcome of progenitor cell transplantation most likely depended on choice of cell population, dosage, timing of injection, and route of administration\textsuperscript{103}.

**EPC identification, origin and characterization**

When the studies of this thesis were in their final stage, two different types of EPC were recognized, being the early-outgrowth or myeloid endothelial progenitor cells with pro-angiogenic properties, but no potential to differentiate into endothelial cells, and the ECFC with true endothelial properties (Fig. 2, Table 1)(see also Chapter 3). The identification of these different cell types became established during the progression of this study, which resulted in the examination of different types of cells, as described in Chapter 4 and 5. In the next paragraphs, we will describe the fun-

![Fig 2. Schematic representation of the two cultures of ‘endothelial’ progenitor cells.](image)

Culture of colony-forming unit – endothelial cells (CFU-EC, >99\% CD45+) includes a 3 to 7-day process wherein blood or bone marrow mononuclear cells give rise to EPC colonies. These colonies are mainly comprised of CD4\+ T cells and CD14\+ monocytes admixed with B cells, myeloid progenitor cells, natural killer cells and platelet fragments. The cluster of round cells sits on top of the spindle-shaped adherent macrophages which emigrate from the base of the cell cluster. Over time, the cluster of cells disappears, leaving the macrophages that display features of endothelial cells. Endothelial colony-forming cells (ECFC) are derived as late-outgrowth colonies from adherent MNC cultured for 7–21 days in endothelial conditions. These colonies display a cobblestone morphology, which closely resemble mature differentiated endothelial cells. The ECFC are derived from a circulating fraction of CD34\+CD45- cells that do not express the leukocyte markers CD45, or CD14, or the stem cell marker CD133.
<table>
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<tr>
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<th>CIRCULATING EC</th>
<th>CIRCULATING EPC</th>
<th>CFU-EC</th>
<th>ECFC</th>
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<td>CD34</td>
<td>+/- 60</td>
<td>+ 55, 60, 88</td>
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<td>45</td>
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<td>CD146</td>
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<td>+/- 45, 92</td>
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<td>+/- -91</td>
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<td>AcLDL binding</td>
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<tr>
<td>CD11c</td>
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<td>-</td>
<td>+ 92, 93</td>
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<td>-435</td>
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<td>+/- 436</td>
<td>+/- 91, 96</td>
<td>+ 91, 96, 103, 107</td>
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<tr>
<td>In vivo vessel formation</td>
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<td>-91, 106</td>
<td>+ 91, 106</td>
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<td>Incorporation EC monolayer</td>
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<td>+/- 03, 07</td>
<td>+/- 96</td>
<td>96</td>
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<td>Production growth factors</td>
<td>-</td>
<td>+/- 03, 05</td>
<td>+ 93, 96, 104, 437</td>
<td>+/- 96</td>
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<tr>
<td>Proliferation</td>
<td>-87</td>
<td>438</td>
<td>+ 90, 93, 96</td>
<td>+ 87, 90, 96, 103, 107</td>
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Table 1 Characterization of EPC, CFU-EC and ECFC
damental research, which eventually led to a better understanding of the nature and characterization of the different types of EPC, and their contribution to neovascularization. The mobilization, homing and incorporation of EPC into developing blood vessels are described in more detail in Chapter 3.

After the initial identification of EPC in 1997, Lin et al. were among the first to investigate more closely the origin of circulating endothelial cells and endothelial outgrowth of cultured peripheral blood mononuclear cells. By studying patients transplanted with gender-mismatched bone marrow, they were able to demonstrate that circulating endothelial cells and early-outgrowth endothelial cells (after 9 days of culture), were predominantly of recipient origin, suggesting a source other than bone marrow. These cells displayed a limited proliferative capacity. Remarkably, one month after continuing the same cultures, late-outgrowth endothelial cells were seen with a far greater proliferative rate. These cells were mostly derived from transplantable marrow-derived cells. Although the 'starting' cell of the late outgrowth endothelial cultures was not identified at that time, the delayed outgrowth but high proliferative capacity of these marrow-derived circulating cells was interpreted as the best approximation of circulating angioblasts. Others confirmed these initial experiments, although no consensus was achieved on the nomenclature of these late-outgrowth endothelial cells, which resulted in aliases like multi-potent adult progenitor cells (MAPC), blood-outgrowth endothelial cells (BOEC), or endothelial colony-forming cells (ECFC).

Gulati et al. continued this important line of research on the lineage relationship between early EPC and late-outgrowth endothelial cells. They demonstrated that the vast majority of early EPC were derived from a CD14+ (the surface endotoxin receptor) subpopulation of cells, whereas the late-outgrowth cells developed exclusively from a CD14- cell population. Earlier results already hinted at a role for myeloid cells in the generation of these early EPC, since endothelial cells could be generated from bone marrow-derived cells of the myeloid lineage of patients with chronic myeloid leukaemia. These bone marrow-derived cells formed clusters of cells with spindle-shaped progeny that expressed a panel of proteins including CD34, CD31, CD144, E-selectin, factor VIII, and VWF, and the cells ingested acetylated LDL. Later on, it was shown that the majority of these early EPC expressed monocyte/macrophage markers such as CD14, Mac-1 and CD11c. Differential labelling experiments showed that early EPC did not serve as a precursor for the late-outgrowth endothelial cells. Furthermore, the early EPC and late-outgrowth EC displayed differences in morphology (spindle-shaped vs. cobblestone), proliferation rates, and survival be-
havior (Fig. 2). Unexpectedly, both cell types equally contributed to neovascularization in mouse model of hind limb ischemia\textsuperscript{70}. The late-outgrowth EC produced more nitric oxide, incorporated more readily into a human umbilical vein endothelial cell monolayer, and formed capillary tubes better than early EPC. In contrast, early EPC secreted angiogenic cytokines, such as VEGF, hepatocyte growth factor (HGF), G-CSF, GM-CSF, and IL-8, more so than late-outgrowth EC, suggesting a paracrine contribution to vascularization\textsuperscript{70, 71}. In agreement, Rohde \textit{et al.} demonstrated that culturing monocytes, being the predominant CD14\textsuperscript{+} cell fraction, under angiogenic conditions displayed a commonly accepted EPC phenotype, including Ac-LDL uptake and lectin binding, CD31/CD105/CD144 reactivity, and formation of cord-like structures\textsuperscript{106}. This indicated that the commonly used endothelial marker definition was not sufficient to distinguish early EPC from monocytes. Strikingly, even primary monocytes already expressed most tested endothelial genes and proteins at equal or even higher levels than their supposed EPC progeny\textsuperscript{106}. Although the early EPC are derived from a subpopulation of CD14\textsuperscript{+} cells, it remains unknown which cell in this fraction is responsible for colony formation. Therefore, the early EPC, or CFU-EC, can only be identified by its progeny.

Similar to other progenitor cells, EPC should be defined by their clonogenic and proliferative potential, as well as their ability to give rise to functional progeny, as was reasoned by Ingram \textit{et al.}\textsuperscript{85} They discovered a yet unidentified hierarchy of EPC. Single-cell plating of either cord blood or peripheral blood-derived endothelial colony-forming cells (ECFC) generated high proliferative potential ECFC (HPP-ECFC) and low proliferative potential ECFC (LPP-ECFC), as well as endothelial cell clusters with virtually no clonogenic capacity. Even in vessel wall-derived endothelial cell cultures a hierarchy of progenitor cells was identified, with high proliferative and low proliferative potential\textsuperscript{107}. This latter fact suggested that not all vessel wall endothelial cells are mature, terminally differentiated cells which lack the potential to undergo massive proliferation and rejuvenate the endothelial lining.

As mentioned earlier, the majority of studies to date identified and enumerated EPC via flow cytometric analysis, by the coexpression of CD34, AC133, and VEGFR2\textsuperscript{55, 60, 62, 108}. As these markers are also expressed on hematopoietic stem and progenitor cells\textsuperscript{87, 109-112}, Case \textit{et al.} isolated CD34\textsuperscript{+}CD133\textsuperscript{+}VEGFR2\textsuperscript{+} cells from cord blood and G-CSF-mobilized peripheral blood and subjected these suggested EPC to hematopoietic and endothelial cell clonogenic assays to study their progeny. In adult peripheral blood and cord blood this cell fraction was extremely rare (84 and 210 per 10\textsuperscript{6} MNC, respectively). Immunoselection demonstrated that CD34\textsuperscript{+}CD133\textsuperscript{+}VEGFR2\textsuperscript{+} cells did
not form either CFU-EC or ECFC, but developed into hematopoietic progenitor cells with high and low proliferative potential, which were all positive for CD45+, but devoid of vessel forming activity. In contrast, the CD34⁺CD45⁻ cells did give rise to ECFC, with robust proliferation and vessel-forming capacity.

In a subsequent study, subtractive CFU-EC analyses were performed to determine the impact of the various blood cell types during colony formation. It was found that CFU-EC (>99% CD45⁺) were mainly comprised of T cells and monocytes admixed with B cells and natural killer cells (Fig. 2). A combination of purified T cells and purified monocytes alone was sufficient to form CFU-EC. Furthermore, depletion of either monocytes or T cells completely abrogated CFU-EC formation. In detail, CD4⁺ T cells were shown to be indispensable for the induction of colony formation. T cell receptor-MHC class II interaction between CD4⁺ T cells and monocytes was essential for formation of CFU-EC. Upon activation T cells produced paracrine factors which induced monocyte differentiation and colony formation. In agreement with Case et al., highly enriched CD34⁺ did not yield CFU-EC, nor did CD34-depletion interfere with CFU-EC formation. In addition, soluble factors derived from the CFU-EC supported vascular network formation in an in vitro tube formation assay. Although the activated T cells and monocytes produce a number of proangiogenic and inflammatory factors, like MMP-2 and -9, uPA, Ang-2, FGF1, IL-8, IFN-γ and TNF-α, the exact soluble factor was not identified which induced colony formation. Since the CFU-EC are derived from a subset of monocytes, and do not give rise to actual endothelial cells, it was hypothesized that the number of CFU-EC more likely reflects the ability of inflammatory cells to participate in neovascularization at sites where vascular branching and lumen widening occurs.

Because of the controversial nature of EPC, and the recent discovery of CFU-EC and ECFC, Yoder et al. compared the nature and function of these two cell types. CFU-EC were descendents of HSC that retained some myeloid progenitor activity with no ability to form secondary colonies. Furthermore, CFU-EC differentiated into phagocytic macrophages and not EC, which supports the initial evidence of CD14⁺ monocytes giving rise to CFU-EC. In contrast, the rare circulating ECFC are clonally distinct from CFU-EC and displayed robust proliferative potential. With respect to their in vitro behavior in the various experimental and commercially available culture assays, they showed that non-adherent cells in the ECFC assay differentiated towards CFU-EC, whereas the adherent cells in the CFU-EC developed into ECFC. In vivo, CFU-EC did not form chimeric vessels in a xenograft model of blood vessel formation, but they were detected. ECFC, on the other hand, did form chimeric
vessels which were perfused, indicating anastomoses with the resident circulation.\textsuperscript{81}

Timmermans \textit{et al.}\textsuperscript{65} extended on the work of Case \textit{et al.}\textsuperscript{66} and made a detailed dissection of the phenotype of EPC precursors with emphasis on the markers CD34, CD133, CD45 and VEGFR2, as mentioned before markers for putative circulating endothelial precursor cells. In agreement with others, the ECFC (in that article indicated as endothelial-outgrowth cells, or EOC) did not express the leukocyte markers CD45, CD14, or CD133. The EOC could be expanded for more than 60 population doublings, and generated vascular tubes in a matrigel assay. FACS analysis demonstrated that EOC-generating CD34\textsuperscript{+}CD45\textsuperscript{-} cells expressed VEGFR2 but not CD133, whereas CD34\textsuperscript{+}CD45\textsuperscript{+} HPC expressed CD133 as expected, but not VEGFR2. Conversely, CD34\textsuperscript{+}CD45\textsuperscript{-} HPC did give rise to monocytic cells with characteristics of early EPC. Of importance, the monocyte-to-macrophage marker CD163 was specifically upregulated in CD34\textsuperscript{+}CD45\textsuperscript{-} cultures, but not in CD34\textsuperscript{+}CD45\textsuperscript{-} EOC cultures. This work clearly discriminated between the hematopoietic origin of CFU-EC and non-hematopoietic EOC. However, it still remains a question how to discriminate circulating mature EC from EC precursors, since they both express VEGFR2 and CD34.

**MESENCHYMAL STROMAL CELLS**

The multi-potentiality, intrinsic ability to home to injured sites, and demonstrated stimulatory effect on neovascularization, may make mesenchymal stromal cells suitable candidates for vascular regenerative therapies.\textsuperscript{114-116, 117-126} Mesenchymal stromal cells (MSC), also referred to as mesenchymal stem cells, were recognized by Friedenstein \textit{et al.}\textsuperscript{127}, as a population of adherent, bone marrow-derived cells that could differentiate into bone, cartilage, and adipose tissue \textit{in vitro},\textsuperscript{128} and provided the supportive micro-environmental niche for hematopoietic stem cell engraftment.\textsuperscript{118, 129, 130} MSC are defined as progenitor cells with a high degree of plasticity, capable of giving rise to a number of unique, differentiated mesenchymal and non-mesenchymal cell types.\textsuperscript{114, 131-144} Given the lack of one specific MSC marker, MSC are characterized by a panel of positive and negative (mainly hematopoietic and endothelial) surface markers, which was proposed by the Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy.\textsuperscript{145} The minimal criteria for defining MSC include positive staining for CD105, CD73, CD90, and negative for CD45, CD34, CD14 or CD11b, CD79a or CD19, and HLA-DR. However, such a marker set is only specific in a particular context or is redundantly expressed by other cell types. Consequently, MSC are defined on an operational basis, i.e. functional assays using cultured cells.
To address the persistent inconsistency between nomenclature and biologic properties of MSC, and to clarify the terminology, in 2005 the International Society for Cellular Therapy (ISCT) suggested that the fibroblast-like plastic-adherent cells, regardless of the tissue from which they are isolated, be termed multipotent mesenchymal stromal cells\textsuperscript{146}. The term mesenchymal stem cell is used only for cells that meet specified stem cell criteria. The use of the acronym MSC in the forthcoming paragraphs will refer to these multipotent mesenchymal stromal cells.

**Sources of MSC and multi-potency**

Concerning the \textit{in vivo} localization, MSC most likely exist in virtually all tissues, where they represent a minor fraction of cells\textsuperscript{147-165}. Recently, a large number of publications have suggested a perivascular niche for MSC; correlating these cells with pericytes\textsuperscript{115, 166-173}. This intriguing model proposes that MSC stabilize blood vessels and contribute to tissue and immune system homeostasis under physiological conditions and assume a more active role in repair of focal tissue injury\textsuperscript{174, 175}. With disruption of a blood vessel in or near injured tissue, the MSC responds to loss of contact with endothelial cells and the basement membrane, divides, and secretes a broad spectrum of bioactive factors that function to protect and repair or regenerate the injured tissue. Initial \textit{in vitro} testing has demonstrated the multilineage differentiation capacity of perivascular MSC, and ability to migrate towards injured tissue\textsuperscript{166, 167, 176}.

Although the above-mentioned model is without doubt very attractive, further research is necessary to demonstrate that all MSC are pericytes\textsuperscript{158, 175, 176}. Of note, the molecular differences verified in tissue-specific MSC may reflect functional activities influenced by distinct niches and should be considered when developing clinical protocols involving MSC from different sources\textsuperscript{177-179}.

**MSC and neovascularization**

A positive role of paracrine factors produced by MSC on neovascularization has been described by a number of groups\textsuperscript{180-186}. It was shown that MSC from various tissues produced factors like VEGF, TGF-\(\beta\)-1, MMP-2, IL-6, bFGF, HGF, SDF-1, and PlGF that have known pro-angiogenic and anti-apoptotic effects on endothelial cells\textsuperscript{120, 134, 185, 187-198}.

In addition, a limited number of papers describe the differentiation of MSC into endothelial(-like) cells capable of forming capillary tubes\textsuperscript{180, 199-205}. Considering the heterogeneous nature of MSC cultures and limited phenotypic characterization presented in these reports, this matter remains debatable. MSC cultures ‘contamina-
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et with both mature endothelial cells and endothelial progenitor cells might explain these observations\textsuperscript{201, 206, 207}, as well as endothelial mimicry displayed by differentiating MSC. However, this does not exclude MSC from enhancing angiogenesis and thereby contributing to tissue survival. Ghajar \textit{et al.} demonstrated the absolute requirement of endothelial cells for mesenchymal stromal cells to form polarized, tubular structures that were intimately associated with mesenchymal cells. This MSC-stimulated capillary formation relied solely on membrane-type matrix metalloproteinases (MT-MMP)\textsuperscript{208}.

\textit{In vivo}, transplanted MSC have been shown to augment both arteriogenesis and angiogenesis, thereby restoring tissue perfusion\textsuperscript{182, 184, 190, 191, 203, 209-216}. Unfortunately, poor cell viability associated with transplantation has limited the reparative capacity of these cells\textsuperscript{217}. An interesting possibility currently being explored is to enhance MSC survival and the angiogenic effects of transplanted cells by modifying them \textit{ex vivo}\textsuperscript{218-220}. Transplantation studies using preconditioned MSC, or MSC transfected with plasmids encoding VEGF, HGF, SDF-1\textalpha, heme oxygenase 1, or a combination of these factors, demonstrated marked improvements in MSC survival (via PI3K/Akt), greater vascular density and a reduction in infarct size and fibrosis\textsuperscript{194, 221-230}.

Hypoxia, one of the driving forces of neovascularization, facilitated MSC pro-angiogenic properties by chemoattracting MSC\textsuperscript{231}, enhancing mobilization into peripheral blood\textsuperscript{232} and potentiating MSC growth factor production\textsuperscript{194, 212, 233-236}. In addition, hypoxia controlled proliferation and differentiation of MSC towards endothelial-like cells, vascular permeability and subsequent tube formation\textsuperscript{180, 196, 234, 237-241}. Initial \textit{in vivo} testing has demonstrated that preculturing MSC under hypoxic conditions (i.e. hypoxic preconditioning) prior to transplantation improved their tissue regenerative potential in various models of ischemic injury; proposing this method as a novel strategy for cell-based therapies\textsuperscript{242-244}.

\textbf{Hypoxia}

Hypoxia, defined as insufficient, or inadequate, oxygen delivery occurs in a variety of physiological and pathological conditions, including high altitude, strenuous exercise, impaired gas exchange, stroke, myocardial infarction, inflammation and in solid tumors that outgrow their existing vasculature\textsuperscript{245-247}. Molecular oxygen (O\textsubscript{2}) is required for aerobic metabolism to maintain intracellular bioenergetics and to serve as the ultimate electron acceptor in oxidative phosphorylation. However, the need for O\textsubscript{2} as metabolic substrate is opposed by the inherent risk of oxidative damage to macromolecules by reactive oxygen species. Because of this dual character of O\textsubscript{2}
as an essential but potentially toxic molecule, its concentration within cells is maintained within a narrow range that optimally balances supply and demand.\(^{248}\)

The distribution of oxygen within the human body does not display an uniform pattern, since different cells have diverse energy requirements, operate in different environments, and are normally exposed to a varying range of oxygen concentrations.\(^{249}\) Ambient oxygen is 21% O\(_2\) (~150 mm Hg), but most mammalian tissue is exposed to a significantly lower level of oxygen; arterial oxygen content is 10 - 14% O\(_2\) (75-100 mmHg), resting skeletal muscles and ventricular myocardium 4 - 6.5% O\(_2\) (30-50 mmHg), whereas the osteogenic niche of bone marrow maintains a very low oxygen concentration (< 1% O\(_2\)).\(^{250-258}\) Consequent to the wide range in tissue oxygen concentration, the hypoxic setpoint varies from one tissue to another.\(^{259-264}\) Prolonged exposure of tissue to hypoxia will result in cellular dysfunction and ultimately cell death.\(^{5-7, 265, 266}\)

The human body is equipped with a highly efficient cardiovascular and respiratory system, which in combination with erythrocytes facilitates transport of oxygen. This network is essential in providing all cells in the body with adequate oxygen levels since the estimated diffusion distance of O\(_2\) is a mere 145 \(\mu\)m.\(^{267-269}\)

A second line of defense consists of an oxygen-sensing mechanism, which initiates adaptations to minimize hypoxic injury, promotes survival in a low oxygen environment and induces genes to restore cellular oxygen delivery. In 1992, hypoxia-inducible factor (HIF) was identified as the key transcriptional factor linking oxygen concentration to adaptation processes at the cellular and systemic level.\(^{270-272}\) The heterodimer HIF consists of an oxygen-sensitive HIF-\(\alpha\) subunit and a stable HIF-\(\beta\) subunit, and can act as a transcription factor for many genes (Fig. 3). HIF regulates erythropoiesis, glycolysis, autophagy (degradation and recycling of cellular components to restore ATP levels), and angiogenesis, with important consequences for cardiovascular (patho)physiology. Importantly, hypoxia is the major driving force for neovascularization. Hypoxia mediates the induction of various angiogenic molecules like vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF), thereby promoting endothelial cell survival, proliferation, and migration.\(^{263, 278-282}\)

Overall, the expression of more than seventy genes is regulated at the transcriptional level by HIF as determined by various criteria, including induction of gene expression following hypoxic exposure, presence of a functionally essential HIF
binding site, and the effect of HIF gain-of-function or loss-of-function on target gene expression\textsuperscript{283-286}. However, whole-genome array analysis implies that this number will be substantially larger, possibly up to 2000 genes that are directly or indirectly regulated by hypoxia\textsuperscript{249, 259, 287-290}.

**Hypoxia-inducible factors**

![Diagram of HIF-1 signaling during normoxia and hypoxia.](image)

**Fig 3. HIF-1 signaling during normoxia and hypoxia.**

(R) Adequate oxygen delivery, or normoxia, results in the hydroxylation of proline residues P402 and/or P564 of HIF-1α by the family of prolyl hydroxylases (PHD), which facilitates the binding of Von Hippel-Lindau protein (VHL). As a component of the E3 ubiquitin ligase complex, VHL induces poly-ubiquitination of HIF-1α and degradation by the proteasome complex. A second level of control over HIF-1α activity is established by factor inhibiting HIF-1 (FIH-1). During normoxia, FIH-1 hydroxylates a conserved asparaginyl residue N803 inside the C-terminal transactivation domain. The resulting steric clash of this domain interferes with the binding of the cofactors p300 and CBP and thereby inhibits gene transcription. (L) In a hypoxic episode, the lack of oxygen abrogates the function of the PHD and FIH-1. HIF-1α migrates to the nucleus where it binds its partner molecule HIF-1β. By attracting the transcription cofactors p300/CBP a gene transcription program is initiated which controls a number of essential adaptations to minimize hypoxic injury, promotes survival in a low oxygen environment and induces genes to restore cellular oxygen delivery.
HIF is a heterodimeric, redox-sensitive protein composed of a stable HIF-1β (ARNT) and labile HIF-α protein\textsuperscript{291, 292}. Unlike the HIF-α subunit, HIF-1β is constitutively expressed and insensitive to O\textsubscript{2} changes\textsuperscript{293}.

During episodes of hypoxia, the HIF-α subunit is stabilized, translocates to the nucleus where it binds HIF-1β\textsuperscript{294, 295}. This transcriptionally active complex recruits co-activators and binds to hypoxia-responsive elements (HRE) in the promoter or enhancer regions of genes involved in metabolism, inflammation, erythropoiesis and angiogenesis (Fig. 3)\textsuperscript{294, 296, 297}. Deletion analysis and mutagenesis originally identified a sequence (5’-CTACGTGCT-3’) that, when modified, resulted in loss of oxygen-dependent HRE activity\textsuperscript{298-300}.

Over the last decade a total of three different HIF-α proteins (HIF-1α, HIF-2α and HIF-3α) have been identified that are similarly regulated by O\textsubscript{2} availability (Fig. 4). All HIF proteins are member of the basic helix-loop-helix polypeptide family, containing two different PAS domains and two dedicated transactivation domains (N-terminal transactivation domain or ‘NTAD’ and C-terminal transactivation domain or ‘CTAD’), which activate transcription when bound to DNA. The HIF PAS domains play a vital role in heterodimer formation, DNA binding, and HIF stabilization via heat shock protein 90 association (Fig. 4)\textsuperscript{284, 285, 294, 301-310}. For review about the function of the different HIF domains see Scheuermann et al.\textsuperscript{311}

HIF-1α is ubiquitously expressed, and acts as the master regulator of oxygen homeostasis in almost every cell type, whereas HIF-2α expression is more restricted to the vascular endothelium, liver parenchyma, lung type II pneumocytes, kidney epithelial cells, tumor cells, and neural crest-derived sympathetic ganglia\textsuperscript{312-320}. Knockout studies in mice demonstrated the undisputable role of HIF-1α in O\textsubscript{2} homeostasis. Loss of HIF-1α resulted in early embryonic lethality caused by defective vascularization of the embryo and yolk sac, cardiac malformations and mesenchymal cell death\textsuperscript{5, 321-323}. Comparable to HIF-1α knockout, genetic ablation of its counterpart HIF-1β resulted in embryonic lethality caused by defective angiogenesis of the yolk sac and branchial arches and a stunted development\textsuperscript{324}.

Targeted disruption (knockdown) of HIF-2α, also referred to as endothelial PAS domain protein 1 (ePAS1), resulted in defective vascular remodeling, fetal catecholamine production, altered lung maturation, and anemia\textsuperscript{314, 325-327}. However, these findings largely depended on the genetic background. Complete HIF-2α knockout led to severe vascular defects in the yolk sac and embryo, and ultimately lethality of
the embryo. Of importance was the finding that HIF-2α overexpression was not able to rescue cells lacking HIF-1α from hypoxia-induced cell death. Additional experiments on the non-redundancy between both two HIF-α proteins revealed that although HIF-1α and HIF-2α have many activated genes in common, depending on cell type and the duration of hypoxia, differences exist with respect to the induction of specific genes. This apparent selectivity of particular genes for either HIF-1α or HIF-2α resides not in DNA binding or dimerization domains, but in sequences lying c-terminal of the HRE.

With respect to angiogenesis, endothelial cell-specific knock-out of HIF-1α was used to determine its role in neovascularization. Knockout of HIF-1α did not interfere with embryonic development and vascularization, indicating that HIF-1α-/- in cells other than endothelial cells is responsible for lethality and defective vascularization. However, adult mice lacking HIF-1α in endothelial cells displayed delayed wound healing and tumorigenesis, as well as defective cell growth and tube formation in response to hypoxia, which was thought to arise from the loss of VEGFR2 expression.

Alternatively, endothelium-specific overexpression of HIF-2α cDNA in HIF-2α-/- mice restored normal vascular development at all stages examined and allowed HIF-2α-/- embryos to survive at a frequency comparable to that of HIF-2α+/- embryos. Skuli et al. identified a number of pathways which might be interfered by vascular endothelial cell-specific ablation of HIF-2α, such as decreased expression of fibronectins, integrins, endothelin B receptor (ET-B), Ang-2 and DLL.

Takeda et al. used a mouse model to study the regulation of HIF-2α on mature angiogenesis. Adenovirus-mediated delivery of the HIF-2α gene significantly induced the expression of VEGF, Flt-1, Flk-1, and Tie2 mRNA at wound sites and promoted adult neovascularization. This clearly indicated that normal vessel formation is extremely sensitive to HIF-2α expression levels. Furthermore, simultaneous dominant-negative interference with both HIF-1α and HIF-2α specifically in endothelial cells resulted in more severe cardiovascular defects. Besides from impaired cardiac development, blood vessel formation was seriously disrupted, which culminated in early embryonic death.

To date, the regulation of HIF-3α expression is unique. A number of HIF-3α splice variants have been identified, which lack the trans-activation domain, the oxygen-dependent degradation domain, or both. It is suggested that these splice variants...
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thereby function as competitive inhibitors over the other two HIF-α proteins\textsuperscript{347-354}. Furthermore, transcriptional regulation of HIF-3α rose significantly in line with the duration of hypoxia, which contrasted the nearly static mRNA levels of HIF-1α and HIF-2α during hypoxia\textsuperscript{293}. This mechanism supposedly provided negative feedback gene regulation in adaptive responses to hypoxia\textsuperscript{353}.

Prolyl hydroxylases and FIH-1

HIF-α protein levels are, for the larger part, regulated at the post-translational level by the family of prolyl hydroxylases (PHD), consisting of 3 members (PHD-1, PHD-2 and PHD-3)\textsuperscript{284}. For their biological activity PHD require iron, 2-oxoglutarate, ascorbate, and above all oxygen\textsuperscript{355, 356}. In the presence of oxygen HIF-α is rapidly hydroxylated and degradated (within minutes). Hydroxylation of P402 (in the N-terminal ODDD) and/or P564 (in the C-terminal ODDD) facilitates the binding of Von Hippel-Lindau protein, a component of the E3 ubiquitin ligase complex\textsuperscript{357-361}, which induces poly-ubiquitination of HIF-α and degradation by the proteasome complex (Fig. 3)\textsuperscript{362-365}. In addition, the rate of HIF-1α degradation depends on the duration of hypoxia\textsuperscript{366}.

Fig 4. Domain structures of the HIF transcription factors.

The three HIF-α paralogues, HIF-1α, HIF-2α and HIF-3α, and common binding partner HIF-1β (ARNT), all contain bHLH (basic helix-loop-helix) and PAS (Per/ARNT/Sim) domains. The three HIF-α isoforms all contain oxygen-dependent degradation domains (ODDD), as well as a N-terminal and C-terminal trans-activation domain (NTAD and CTAD). HIF-1α and HIF-2α each contain an oxygen-regulated CTAD. Numbers to the right of the HIF transcription factors resemble the amino-acid length. For HIF-1α and HIF-2α the position is given of proline and asparagine hydroxylation by the PHD and FIH-1.
Knock-out studies in mice were performed to elucidate the relative contributions of the three individual PHD members to HIF-α hydroxylation. PHD-2 was shown to be both essential for viability, as well as being the most active family member, having the highest affinity for HIF-1α. In vitro experiments confirmed these initial findings and demonstrated that PHD-2 contributed the most to HIF-1α hydroxylation in normoxic cells, thereby setting normoxic HIF-1α levels. Of note, PHD-1 and -3 had a higher affinity for HIF-2α. Furthermore, conditional inactivation of PHD-2 was sufficient to activate a subset of HIF target genes.

Whereas single PHD-1-/- and PHD-3-/- manifested no symptoms of impaired HIF regulation, a double knockout (PHD-1-/- + PHD-3-/-) did develop polycythemia, caused by elevated plasma levels of EPO. The E3 ligases Siah 1 and 2 might be responsible for the differential regulation of the PHD family members, since these factors controlled the stability and availability of PHD-1 and PHD-3 during episodes of hypoxia.

Because PHD-3, and to a minor extent PHD-2, are hypoxia-inducible, a negative feedback mechanism is provided in which the HIF response can be dampened during prolonged hypoxia. The resulting increase in PHD abundance may partially compensate for their reduced hydroxylase activity. Furthermore, PHD induction under hypoxia may prepare cells to quickly clear HIF-α if they are subsequently exposed to higher oxygen levels, for example in reperfusion after ischemia (as seen after myocardial infarction). The same pathway is also triggered in hypoxic preconditioning, in which a sublethal hypoxic insult protects tissue against subsequent lethal hypoxia or ischemic insults.

A second level of control over HIF-α activity is established by factor inhibiting HIF-1 (FIH-1). Similar to the PHD, FIH-1 is an iron- and 2-oxoglutarate-dependent dioxygenase. FIH-1 is capable of hydroxylating a conserved asparaginyl residue (Asn803) within the C-terminal transactivation domain of HIF-α proteins exclusively during normoxic episodes. The resulting steric clash of this domain prevents binding of the cofactors p300 and CBP (Crep-binding protein) and thereby gene transcription. FIH-1 remains active at lower oxygen concentrations than the PHD and thereby suppresses the activity of HIF-α proteins that escape degradation during moderate hypoxia. In addition, relative resistance of HIF-1 and -2α to FIH-1-mediated inactivation appears to control HIF selectivity. Although interaction between HIF-α and its cofactors p300 and CBP is essential for the full repertoire of hypoxia-induced target genes, a number of studies have shown that, in the...
absence of FIH-1 or p300 binding, some target genes are still HIF-inducible\textsuperscript{391, 393-395}. Differences in the requirement for the CTAD relative to the NTAD by these genes presumably caused this discrepancy. It is therefore suggested that the oxygen-sensitive attenuator FIH-1 is a central factor in setting the differential gene expression repertoire dictated by the cellular oxygen tension.

**Alternative hypoxia-regulated pathways**

Although HIF is a key transcription factor directly regulating hypoxia-adaptive processes, other transcription factors and pathways have been described, which either interfere with the HIF pathway, or activate certain genes by themselves during low oxygen tension. In a number of conditions, the inflammatory mediator NF-κB, which is induced during hypoxia, modulates HIF-1α transcriptionally\textsuperscript{396-400}, and, depending on tissue type, acts as a survival signal\textsuperscript{401}. Recently, signalling through mammalian target of rapamycin (mTOR) and signalling through activation of the unfolded protein response (i.e. endoplasmatic reticulum stress) have been implicated as two additional O\textsubscript{2}-sensitive pathways regulating the above-mentioned process\textsuperscript{275}. Furthermore, the pleiotropic transcription factor AP-1 assists HIF-1 in exerting its full transcriptional activity during hypoxia\textsuperscript{402-405}.

Activation of the tumor suppressor protein p53 during hypoxia is to be expected, given its sensitivity to most types of stress within cells\textsuperscript{406-408}. However, during hypoxia p53 did not induce the same set of genes as a typical p53-activating stimulus\textsuperscript{409, 410}. BNIP3L was identified as a key p53 target gene activated under hypoxia that is responsible for p53-mediated apoptosis following hypoxic stress\textsuperscript{411}. Mechanistically, p53 inhibited HIF-α activity by disrupting HIF/CBP interaction\textsuperscript{412}. In addition, p53 targeted HIF-1α for oxygen-independent degradation by recruiting it to MDM2, a component of E3 ubiquitin ligase complex\textsuperscript{413}. Interestingly, recent data suggested that a specific microRNA (miR-107) can modify the p53 regulation of HIF by suppressing the expression of HIF-1β\textsuperscript{414}.

ERK5 (also known as BMK1) is an example of an oxygen-independent negative regulator of HIF-1α stability\textsuperscript{415}. Once activated, ERK5 reduces HIF-1α to undetectable levels in a PHD- and ODDD-independent manner, resulting in decreased endothelial cell migration and inhibition of *in vitro* angiogenesis\textsuperscript{415}.

**Short-term and prolonged hypoxia**

Although the general molecular machinery behind oxygen sensing and hypoxia is rapidly being resolved, cardiovascular studies to date have partly neglected the strong
time-dependency of hypoxia-regulated gene expression\textsuperscript{416}. The concepts of short-term and prolonged (chronic) hypoxia were first described by Chaplin \textit{et al.}, who differentiated between diffusion-limited, chronic hypoxic tumor cells and short-term hypoxic cells, as a result of transient changes in blood flow caused by radiation treatment and the abnormal tumor vasculature\textsuperscript{417, 418}. These two types of cells differed in their radio-sensitivity, genetic stability as well as metastatic potential\textsuperscript{419-424}.

Concerning hypoxia after myocardial infarction, prolonged activation of the HIF pathway can be both adaptive as well as pathological. Immediately after ischemia the initial protective response is a shift from aerobic to glycolytic metabolism as mediated by HIF-1\textgreek{a}. This is beneficial in short-term, but insufficient to meet long-term needs. Long-term protective effects include: HIF-2\textgreek{a}-mediated induction of angiogenesis and augmentation of mitochondrial respiration and mitochondrial biogenesis to maximize energy production from the available oxygen content\textsuperscript{425}. To compensate for produced ROS, the heart induces expression of antioxidant enzymes that eliminate ROS\textsuperscript{426-428}.

In advanced ischemic heart disease, this above-mentioned system appeared to be imbalanced. Lei \textit{et al.} generated mice with cardiac myocyte-specific deletion of the VHL protein, resulting in chronic activation of the HIF pathway during normoxia. These mice developed severe progressive heart failure and perished prematurely. Nearly 50\% of VHL-null hearts showed malignant cardiac tumors with features of rhabdomyosarcoma and the capacity to metastasize\textsuperscript{429}. A study on human chronic heart failure supported these findings, suggesting the detrimental effect of a chronically activated HIF system\textsuperscript{430}. Furthermore, prolonged HIF signalling potentially contributed to the pathogenesis of endothelial dysfunction, characterized by decreased vasorelaxation, increased thrombosis, increased inflammation, and altered angiogenic potential, which are all factors intimately associated with progression and severity of cardiovascular disease\textsuperscript{431}. In cancer biology, a similar condition of prolonged hypoxia resulted in continued upregulation of hypoxia-induced genes, which might induce or predispose individuals to malignancy\textsuperscript{432}, and acted upon the aggressive phenotype of tumor cells\textsuperscript{433}.

When the studies of this thesis were conducted, Ginouves \textit{et al.} reported a thorough study on the effect of short-term and prolonged hypoxia on the different factors of the HIF oxygen sensing system \textit{in vitro}\textsuperscript{434}. Whereas short-term hypoxia inhibited the PHD, leading to HIF-\textgreek{a} stabilisation, chronic (prolonged) hypoxia induced HIF-1 and -2\textgreek{a} ‘desensitization’ \textit{in vitro} and \textit{in vivo}. A decrease in HIF-\textgreek{a} protein levels was
seen as early as 4 hours after exposure to hypoxia. Blocking the ubiquitin ligase pathway demonstrated that during chronic hypoxia HIF-α levels declined because of increased protein instability. Chronic hypoxia augmented PHD expression (mainly PHD-2 and PHD-3), but also overactivated the three PHD isoforms. This overactivation appeared to be mediated by an increase in O₂ availability consequent to the inhibition of mitochondrial respiration. By inducing PHD expression and activity a feedback mechanism was initiated which protected cells against necrotic cell death and adapted them to chronic hypoxia. Under certain conditions, and in specific cell types, a number of mechanisms have been postulated to control the prolonged (chronic) hypoxic response: autocrine upregulation of HIF-α activity by HIF-α synthesis, inhibition of HIF-α by hypoxia-induced dominant-negative HIF inhibitors, degradation of HIF-α by hypoxia-induced PHD synthesis, and succinate-induced PHD inhibition. However, little is known about the endothelial response during chronic hypoxia.
SCOPE OF THE THESIS
The contribution of endothelial progenitor cells to angiogenesis and the specific adaptations of endothelial cells to hypoxia in order to facilitate angiogenesis are two promising approaches for improving neovascularization. In the field of tissue engineering as well as the development of new cardiovascular treatment strategies, a better understanding of these two factors is essential to initiate more successful (pre)clinical studies on restoring efficient perfusion.

AIM OF THE STUDY
The aim of the present study was two-fold. First, at the onset of the studies we wondered how EPC could stimulate angiogenesis. As the insight in the nature and complexity matured during our studies, we moved from evaluating ‘classical’ EPC towards investigating more specific subpopulations, in particular endothelial colony forming cells (ECFC). Second, as hypoxia is mainly studied in the acute phase of tissue repair we were interested how endothelial cells responded to chronic hypoxia. To this end, we designed a hypoxia chamber in which endothelial cells could be cultured and studied for many weeks under defined oxygen concentrations without interruption by oxygen fluctuations due to medium renewal.

CHAPTERS OF THIS THESIS
Sprouting angiogenesis always starts with the activation of endothelial cells and subsequent production of proteases to create the necessary space for new blood vessels to grow (Chapter 1). The functions of these matrix proteases in angiogenesis are manifold. Chapter 2 describes not only the ‘classical’ role of proteases, being the degradation of the basement membrane, cell invasion and lumen formation, but also highlights novel insights in the role of proteases in neovascularization. In Chapter 2 of this thesis current knowledge on different families of proteases is reviewed, which fine-tune proteolytic activities on the leading edge of migrating and invading endothelial cells. Furthermore, it describes how these proteases are involved in modification of growth factors and receptors, generation of matrix fragments with anti-angiogenic properties (matrikines), and the recruitment of bone marrow-derived progenitor cells, enforcing angiogenesis.

In addition to sprouting angiogenesis, it is generally believed that vasculogenesis can also contribute to neovascularization in the adult. This process, which is reminiscent of embryonic vessel formation, is driven by the recruitment of bone marrow-derived endothelial progenitor cells (EPC) and the subsequent integration of these
cells in the growing vessels. On the basis of different cell culture and characterization protocols at least two different populations of EPC have become recognized during the time course of the investigations of this thesis, being the early-outgrowth cells and endothelial colony-forming cells (indicated as blood-outgrowth endothelial cells or BOEC). Chapter 3 surveys the characteristics of these different EPC phenotypes and current insights into the molecular mechanisms that are involved in mobilization and recruitment of EPC to areas of neovascularization. In particular the role of proteases and receptors regulating the liberation of EPC from the bone marrow and retention into the newly developing vasculature is discussed.

Whereas the early-outgrowth endothelial cells are most likely derived from a sub-population of monocytes, and support the angiogenic process predominantly in a paracrine fashion. The endothelial colony-forming cells are confined to the small CD34+CD45- progenitor cell fraction, display characteristics of mature endothelial cells and are able to incorporate into the endothelial lining of new blood vessels and actually forms endothelial tubes by themselves.

The CD34+CD45+ progenitor cell population contains cells that can give rise to early-outgrowth EPC. In Chapter 4 the effect of freshly isolated CD34+ cells and cultured early-outgrowth EPC on human neovascularization is investigated. A 3-dimensional in vitro tube formation model is used to dissect the different mechanisms involved, being migration, proliferation, differentiation, stimulation of and participation in capillary formation.

Because of the high proliferative capacity and the ability to form capillary tubes themselves, blood-outgrowth endothelial cells (BOEC/ECFC) are a potential candidate for tissue engineering applications. BOEC can be isolated from bone marrow and peripheral blood, each method with its own specific advantages and disadvantages. Cord blood BOEC have been reported as more proliferative than peripheral blood BOEC, to that date a thorough characterization and comparison had not been made. In Chapter 5, the angiogenesis-related characteristics of BOEC expanded from cord and peripheral blood are described.

For all applications, the behaviour of expanded progenitor cells will be related to the ability to function and survive as standard mature endothelium and contribute to the formation of mature, stabilized blood vessels. Single transplantation of endothelial progenitor cells may not be sufficient to promote the formation of functional blood vessels. Mesenchymal stromal cells may circumvent this problem, since they have
been described to affect angiogenesis by physical stabilization of maturing vessels as well as by the secretion of angiogenic growth factors. MSC also possess immune suppressive properties, which would make co-transplantation of EPC and MSC feasible for cell-based therapies in perfusion disorders. The ability of human MSC of fetal origin to activate endothelial cells towards a pro-angiogenic state, and identification of soluble growth factors secreted by MSC, is reported in Chapter 6.

Apart from the abovementioned research on progenitor cells and their potential for cell-based neovascularization therapies, more knowledge is urgently required on the endothelial cells own contribution to angiogenesis during a deficit of perfusion and oxygenation. Hypoxia, or inadequate oxygen delivery, initiates a cascade of adaptive processes in endothelial cells, which aim to cope with hypoxia and to restore blood supply. Chapter 7 describes the transcriptional responses of endothelial cells during short-term and prolonged hypoxia. In general, the time-dependency of the hypoxic response has received rather little attention, which is surprising, since a disorder like heart ischemia often involves a prolonged period of hypoxia. The research presented in Chapter 7 will shed more light on the effects of prolonged hypoxia on the genomic adaptation of endothelial cells. It may provide cues how to improve the (largely) insufficient adult neovascularization process, as seen after tissue infarction or transplantation of large grafts.

In Chapter 8, the findings in this thesis are summarized and discussed in the context of recent developments, in an attempt to extend our insight in the multi-factorial process of neovascularization.
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