Modulation of vascular remodeling : a role for the immune system, growth factors, and transcriptional regulation

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General introduction

In peripheral arterial occlusive disease (PAOD), the formation of an atherosclerotic lesion eventually results in a significant stenosis of a major artery thereby disrupting blood flow in peripheral arteries towards lower limb tissue. This causes ischemia which results in the main presenting symptom of PAOD, namely intermittent claudication. Patients with PAOD often suffer from intermittent claudication with a progressing decrease of walking distance. In patients with moderate PAOD, treatment varies from initial exercise training towards more invasive revascularization approaches such as stenting, angioplasty and peripheral artery bypass surgery [1, 2].

Unfortunately, there is a substantial number of patients that suffer from severe PAOD, with rest pain, ulceration or gangrene and these patients are given a diagnosis of 'critical limb ischemia', which is associated with a poor prognosis and high rates of amputation and mortality. Co-morbidities such as diabetes mellitus, hypercholesterolemia, hypertension and subsequent extensive atherosclerotic lesion formation throughout the cardiovascular system further complicates disease and treatment in this patient category. Especially, diabetes mellitus is demonstrated to complicate and aggravate revascularization [3]. Despite state-of-the-art revascularization treatment options and optimal control of co-morbidities these 'no option' patients remain at high risk for limb amputation. It is these patients that require new therapeutic applications to augment neovascularization and prevent them from limb amputation. Therefore, a wide range of studies have been, and still are, conducted to optimize and stimulate revascularization through two vital postnatal compensatory mechanisms, known as arterio- and angiogenesis.

By arteriogenesis, or collateral artery growth, pre-existing arterioles are recruited to become collateral arteries "bypass" an occluded major artery, thereby restoring the disrupted blood flow. In angiogenesis endothelial cell sprouting augments capillary formation and subsequently local blood supply in ischemic tissues (Figure 1). Further mechanistic details of arterio- and angiogenesis are discussed below. Identification of new methods controlling the stimulation of these two vital compensatory mechanisms may lead to the discovery of new promising therapeutic targets for the 'no option' patients suffering from critical limb ischemia.

Revascularization by arterio- and angiogenesis requires vessel growth and vessel remodeling which requires complex involvement of growth factors, cytokines, and multiple cell types, including endothelial cells, fibroblasts, vascular smooth muscle cells, and inflammatory cells such as monocytes, macrophages, or T cells.

The bone marrow is a reservoir for these multiple cell types and serves as a rich source for these potent effector cells in many pre-clinical and clinical revascularization studies. Therefore, a wide range of pre-clinical and clinical studies focused on the stimulation of blood vessel growth by cellular therapies, such as infusion of bone marrow cells [4-6] or pre-stimulated monocytes [7], immune modulation [8] or application of vascular growth factors [9-12].

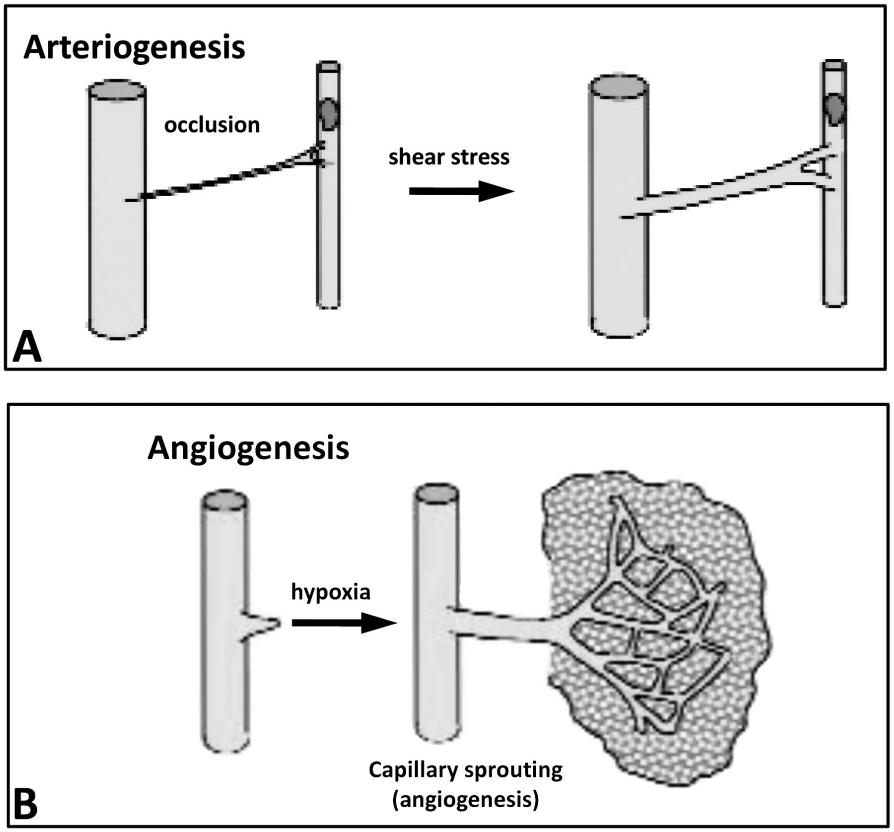

Figure 1. Schematic illustration of; **A.** shear stress driven arteriogenesis, occlusion of a main artery results increased blood flow in collateral arterioles. Concomitant increase of shear stress induces endothelial cell activation and a subsequent initiation of an inflammatory cascade facilitating growth of collateral arterioles into mature collateral arteries. **B.** hypoxia induced capillary sprouting, hypoxia induces production of hypoxia inducible factors, such as vascular endothelial growth factor (VEGF). This induces sprouting of endothelial cells into the hypoxic area towards a VEGF gradient, which finally results in expansion of endothelial capillary network. *(illustrations adapted from: Carmeliet, Nature Medicine, 2000) See color figure on page 210.*

Pre-clinical [12] and clinical studies [9, 10] using vascular growth factors by either direct infusion or by gene therapy induced expression gave mixed results[11], and more information is required about the cellular and molecular mechanisms as well as about the safety concerns of this method.

With the discovery of the contributory role of circulating progenitor cells in neovascularization [13-15], a lot of pre-clinical and clinical studies focused on the application of these bone marrow derived progenitor cells. The application of autologous bone marrow cells to stimulate vessel growth seems a promising therapeutic option.

Several clinical studies have attempted to demonstrate that implantation of bone marrow derived cells improves major amputation free survival rates in patients with critical limb ischemia [4-6, 16]. The recent studies from Idei et al and Walter et al demonstrate improved wound healing and increased amputation free survival after bone marrow application.
Another cell based approach is the infusion of bone marrow derived leukocyte subsets that were pre-conditioned [7]. A great variety of leukocyte subsets have been identified to play a contributory role in blood vessel growth. Specific stimulation of leukocyte subsets and modulation of the immune response provides a wide array of therapeutic options.
Although there are multiple potential options for therapeutic stimulation of blood vessel growth, it should be realized that blood vessel growth and atherosclerosis share common principles and therefore great homology exists between patients with peripheral arterial obstructive disease and coronary artery disease. Stimulation of blood vessel growth may induce vascular remodeling during atherosclerotic lesion formation and could thereby result in adverse events, which is discussed as the Janus phenomenon of vascular remodeling by Epstein et al [17]. Expansion of our current knowledge is required to gain insight in cellular and molecular mechanisms that are involved in neovascularization, to optimize revascularization therapies for patients with critical limb ischemia. This thesis covers several aspects of molecular and cellular mechanisms that are involved in neovascularization.
The **first part** of this thesis focuses on the contributory role of several immune cell subsets in arteriogenesis. In the **second part** of the thesis several aspects in regulation of angiogenesis are covered.

Arteriogenesis
Collateral artery growth has been studied over the past decades as an essential compensating mechanism to treat ischemic disease. From the 1960s on Wolfgang Schaper investigated cellular and molecular mechanisms of collateral artery growth [18]. In 1996 Schaper introduced the concept of arteriogenesis as shear stress driven development of collateral arteries from pre-existing collateral arterioles [19, 20].

By the occlusion of a major artery a steep pressure gradient is created by a drop in blood pressure distal to the occlusion, which results in a redistribution of blood flow via a network of pre-existing collateral arterioles. The increased blood flow in the pre-existing collateral arterioles increases fluid shear stress to the vascular wall (Figure 2). Pre-existing collateral arterioles have an approximate diameter of 10μm, which rapidly increases under control of increased shear stress, which induces vascular remodeling of the arteriolar wall. Finally, the arterioles remodel into mature collateral arteries that restore distal blood flow. This requires remodeling in all layers of the arterial wall ranging

from intima, media to adventitia and occurs by means of an inflammatory process. Thus multiple cell types are involved in the complex regulation of this vascular remodeling process: endothelial cells, smooth muscle cells, adventitial fibroblast, inflammatory cells of the innate immune system, monocytes/macrophages, NK cells, and inflammatory cells of the acquired immune system, like T-cells.

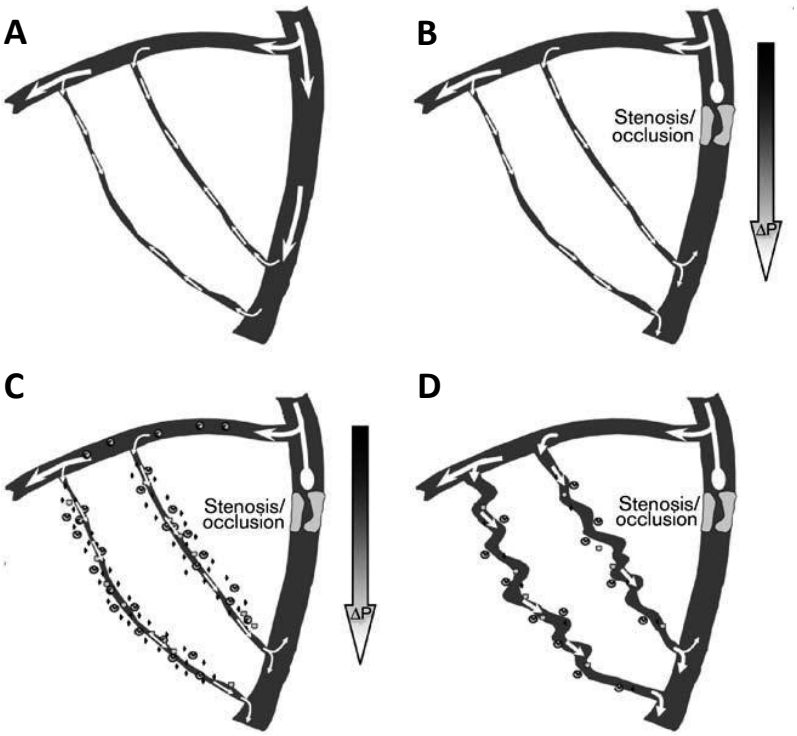

Figure 2 A. Physiologic situation, blood flow is bi-directional in pre-existing collateral arterioles. **B.** Occlusion of a main artery induces redistribution of blood flow along the steep pressure gradient, recruiting the collateral network. **C.** Increased shear stress activates the collateral endothelium, which in turn secretes chemokines, such as MCP-1, and express increased numbers of adhesion molecules on the cell surface. Circulating monocytes and other leukocyte subsets are attracted and invade the vessel wall. **D.** Arrived leukocyte subsets induce an inflammatory cascade which facilitates outward collateral artery growth, resulting in maturation of collateral arteries that restore distal blood flow. *(illustration adapted from: Heil et al, Coronary Artery Disease, 2004)*

During arteriogenesis, vascular wall remodeling of collateral arterioles is initiated by increased shear stress, which induces increased expression of genes encoding for chemo-attractants and activating cytokines or for adhesion molecules in the collateral endothelium [21]. This results in production of a wide variety of chemokines and adhesion molecules supporting attraction, adhesion and invasion of leukocytes in the

vessel wall, that in turn induce an inflammatory cascade facilitating an increase in collateral artery diameter.
To date, a great variety of leukocyte subsets have been identified to contribute to collateral artery growth. Leukocytes from the innate immune system, such as monocytes [18, 22] and NK cells [23], play an early role in collateral artery growth, whereas leukocytes from the adaptive immune system, for example CD4+ T (Th1) cells [23, 24], CD8+ T cells [25], and regulatory T-cells [26, 27], arrive at the site of vascular remodeling in a subsequent later phase [28].
Monocytes were initially demonstrated to play a contributory role in arteriogenesis [18, 19, 22]. Later on it was demonstrated that these cells are recruited by Monocyte Chemo-attractant Protein-1 (MCP-1), an important pro-arteriogenic chemo-attractant, produced by shear stress activated collateral endothelium [29, 30]. This pro-arteriogenic role was demonstrated by enhancement of arteriogenesis after local MCP-1 infusion in a rabbit hind limb ischemia model [31].
MCP-1 binds to the CCR2 receptor which is expressed on monocytes [32], but also on other inflammatory cells [28, 33, 34]. Monocytes migrate towards a high MCP-1 concentration gradient, roll, adhere and finally transmigrate into the vessel wall. This is all facilitated by different kinds of adhesion molecules such as vascular cellular adhesion molecules (VCAM-1) and intercellular adhesion molecules (ICAM-1 and 2) [3, 35] and by selectins and integrins [36], produced by the shear stress activated endothelium. Transmigrated monocytes can differentiate into macrophages or further initiate vessel wall remodeling by expression of cytokines, growth factors and proteases like matrix-metallo-proteinases and uPA [36]. Nowadays, evidence is accumulating that monocytes play a double role in neovascularization, since they are also involved in modulation of angiogenesis [7, 37]. A nice overview of the specific role of the monocytes is given by Schirmer et al [38].
The production of cytokines by monocytes induces an inflammatory reaction with subsequent invasion of different leukocytes such as Natural Killer (NK) cells [23], CD4+ [23, 24] and CD8+ T lymphocytes [25] around the collateral arteries. The invading cells migrate to the perivascular space and activate proteases in the peri-collateral space, which are important in digestion of interconnective tissue of arterial wall cells. The latter is a stimulus for smooth muscle cell proliferation and differentiation [39, 40]. It is necessary to allow enlargement of the collateral artery diameter [41]. In addition, these leukocytes can produce various factors leading to growth of pre-existing collateral arteries by proliferation and migration of smooth muscle cells and endothelial cells[42].

In **chapter 2** an extensive overview is provided about the role of different leukocyte subsets in arteriogenesis. In particular, the role of monocytes [22], Natural Killer (NK) cells [23] and T-cells [23-25], including various subpopulations of T-cells [26, 27], are

addressed.
In most of the studies described in this thesis we use a mouse model for hind limb ischemia, in which ischemia is induced by ligation of the femoral artery. In our studies we perform a single electro-coagulation of the left femoral artery, leaving all pre-existing collateral artery side branches intact, which allows us to study collateral artery growth. The type of surgery used in this animal model varies widely between different research groups. Several surgical options to obtain hind limb ischemia are described in **chapter 3**. Furthermore, the various end points of this mouse model, such as measurement of blood flow recovery, assessment of collateral artery growth and assessment of capillary sprouting are described. In **chapter 4**, we use this mouse model to investigate the role of different types of lymphocytes in arteriogenesis in two mouse strains. As also demonstrated by Stabile et al [24], we show a contributory role for CD4+ T lymphocytes and demonstrate for the first time that NK cells play a pivotal role in arteriogenesis. In this study we observed severely reduced arteriogenesis in BALB/c as compared to C57BL/6, as was also published by other groups [43-45].
For example, Chalothorn et al demonstrated that substantial variability exists in collateral density and collateral artery growth between C57BL/6 and BALB/c mice [43]. Another explanation for differences in collateral artery growth between C57BL/6 and BALB/c mice was documented by Dokun et al, who identified a quantitative trait locus on murine chromosome 7 (LSq-1) that is associated with the absence of tissue loss and may be useful in identifying genes involved in modulation of collateral artery growth [44].
In a cross-breeding experiment we also demonstrated that C57BL/6 genes have a dominant effect on arteriogenesis, since C57BL/6xBALB/c F1 mice had similar rapid arteriogenic response as compared to the C57BL/6 parent strain. Relevantly, C57BL/6 and BALB/c mice have different immune bias. Besides differences in T-helper response [46], they are genetically different in the content of the Natural Killer gene complex (NKC) [47]. The NKC encodes a great number of activating and inhibiting NK cell receptors and BALB/c mice lack a 200 Kb region in this complex [47], which is linked to a significant reduced level of resistance to murine Cytomegalovirus (mCMV) infections in these mice [48]. These NKC differences between the two strains and the observation of reduced arteriogenesis in NK cell depleted C57BL/6 mice, led us to the hypothesis that presence of the C57BL/6 NKC alleles in BALB/c might improve arteriogenesis. As described in **chapter 5** we study this in a BALB/c mouse strain congenic for the entire C57BL/6 NKC [49]. In these mice the arteriogenic response is similar to that of C57BL/6 mice and NK cell depletion in the congenic mice impaired arteriogenesis. Furthermore, we demonstrate that NK cell responsiveness in BALB/c mice is significantly reduced when compared to C57BL/6 and the congenic BALB/c strain. Altogether this suggests that the C57BL/6 NKC contains a NK cell related factor that contributes to arteriogenesis.

Vascular remodeling in arteriogenesis is a general concept that also applies to other types of vascular remodeling, such as vein graft thickening after bypass surgery or the formation of neo-intima after angioplasty and artery stenting [50]. After these vascular interventions blood vessels may react with a response that leads to extensive vascular remodeling. After bypass surgery, when a venous segment is positioned in an arterial location, this segment naturally displays a form of adaptive vascular remodeling, which should lead to arterialization of the vessel wall to meet requirements of the arterial circulation. This adaptive remodeling may overshoot resulting in extensive hyperplasia with a risk of insufficient luminal patency and graft failure. Similarly, after balloon angioplasty the injured vessel wall may display a repair process that is overbalanced and results in massive intimal hyperplasia [51]. Post interventional vascular remodeling is a common feature and the extent of remodeling is determining outcome of treatment. Variations in the genetic background, and variations in immune response between mouse strains have been shown to correlate with differences in (post-interventional) vascular remodeling [46, 52]. Therefore we hypothesized that the strain dependent differences in Natural Killer gene complex between C57BL/6 and BALB/c might induce differences in "post-interventional" vascular remodeling. We study this in **chapter 6** by using two additional mouse models, a mouse model for intimal hyperplasia to mimic post-angioplasty restenosis and a mouse model for vein graft surgery. In the previous model the femoral artery will be triggered for a response of intimal hyperplasia by perivascular cuff placement [53], and in the latter model a venous segment of a donor mouse will be placed as an interposition in the carotid artery of a recipient mouse [54, 55].

Angiogenesis

In the **second part** of this thesis several aspects of angiogenesis are covered. Angiogenesis is hypoxia induced sprouting of new capillary-like structures from an existing capillary network [37, 56]. Hypoxia induces production of hypoxia inducible factors such as vascular endothelial growth factor (VEGF) [57] through the increased availability of its transcription factor Hypoxia Inducible Factor-1 alpha (HIF-1α) [58, 59]. The total process of angiogenesis consists of a series of sequential events consisting of a VEGF induced increase of vascular permeability, extra-cellular matrix (ECM) degradation by proteases, and endothelial cell proliferation and migration [37, 56], as is schematically illustrated in figure 3. During this process endothelial cells from an existing capillary network start to infiltrate the ischemic tissue by protruding tip cells [57]. VEGF initiates the increase of vascular permeability and vasodilation in combination with endothelial nitric oxide (eNOS) to facilitate the protrusion of the tip cells [60]. Proteases, such as urokinase-Plasminogen Activator (u-PA) and Matrix Metalloproteinases (MMPs) are essential in degradation extracellular matrix molecules to facilitate endothelial cell migration, which

is required for capillary sprouting. During capillary sprouting, blood vessel endothelium specializes in tip and stalk cells to promote capillary network expansion in the extra-vascular space towards angiogenic stimuli. Stalk cell form a lumen through which blood is transported, whereas tip cells extend filopodia to detect chemotactic growth factor gradients, which are formed by a combination of VEGF isoforms with a differential affinity for the extracellular matrix. Fusion of tip cells with other capillary networks was recently demonstrated to be guided by a specific subset of monocytic cells that interact with the endothelial tip cells to promote vascular anastomosis [61]. After recruitment of perivascular pericytes, that provide mechanical support to the new formed vessel, the capillaries will mature. Ultimately, the new interconnected capillary networks should augment perfusion and subsequent oxygen diffusion in ischemic tissue.

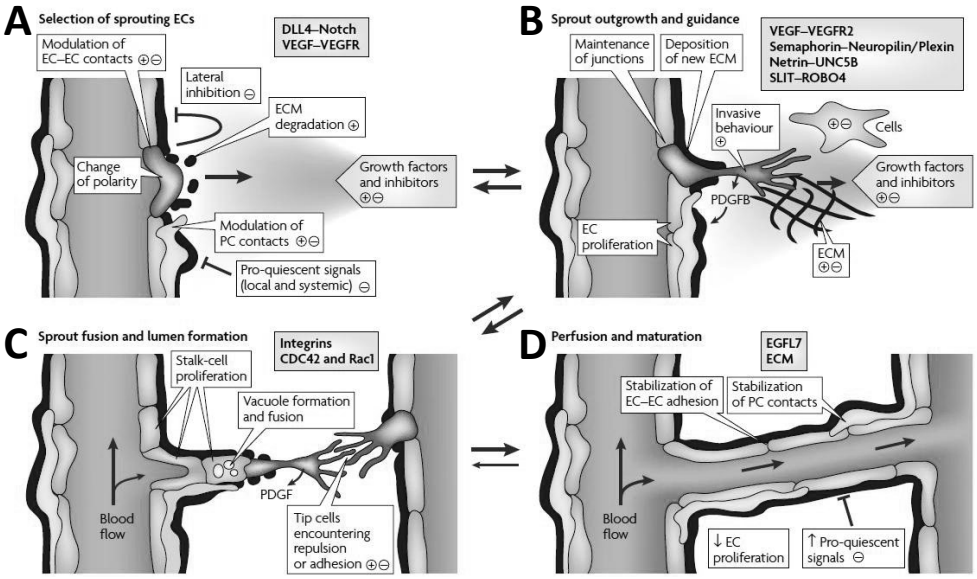

Figure 3 A. Angiogenesis is initiated by production of hypoxia inducible pro-angiogenic growth factors, such as VEGF, extra-cellular matrix degradation and sprouting of 'selected' endothelial cells. **B.** The 'selected' endothelial sprout extends filopodia to detect guiding signals of VEGF gradient and 'signals' from extra-cellular matrix products. **C.** Stalk cells proliferate and form a lumen through which blood is transported, whereas tip cells extend filopodia to detect chemotactic growth factor gradients, which are formed by a combination of VEGF isoforms with a differential affinity for the extracellular matrix. Fusion of tip cells with other capillary networks was recently demonstrated to be guided by a specific subset of monocytic cells that interact with the endothelial tip cells to promote fusion with other capillary sprouts and networks. **D.** Expansion of the capillary network is finalized when a continuous lumen is formed as a result of stabilized endothelial cell adhesion. Blood flow is improved and subsequent oxygen delivery reduces pro-angiogenic stimuli. As a consequence of improved perfusion cell junctions of endothelial cells and supporting pericytes are stabilized, which induces maturation of the new formed capillary network. *(illustration adapted from: Adams et al, Nature Reviews Molecular Cell Biology, 2007) See color figure on page 211.*

For the increase of the capillary network, it was thought that endothelial cells forming the new capillary structures were derived from the bone marrow, which functions as a reservoir for so called endothelial progenitor cells (EPC). Hypoxia induced VEGF production also induces production of the chemokines stromal derived factor-1 (SDF-1), that is necessary for recruitment of CXCR4 positive circulating progenitor cells [62, 63]. Initially these progenitor cells were thought to act as a source of new endothelial cells that could be directly incorporated in sprouting capillary vessel walls [64]. However, a recent study provides evidence that these CXCR4-positive progenitor cells play a paracrine role, since these cells accumulate perivascular and secrete a variety of chemokines that favor angiogenesis, such as SDF-1 [65].

The role of VEGF as a pro-angiogenic growth factor has been widely studied as a therapeutic application to augment vascularization in ischemic tissues [9, 10, 12]. However, these placebo controlled trials of therapeutic angiogenesis were inconsistent [66, 67]. This could be caused by differences in chronicity of ischemia and tissue condition, which may reflect on regulation of the expression of hypoxia inducible pro-angiogenic factors. To improve angiogenic strategies, more information is required on the regulation of vascular growth in ischemic tissues. In **chapter 7** we study expression of the hypoxia inducible factors VEGF, SDF-1 and the progenitor cell receptor CXCR4 in muscle from patients suffering from chronic and acute limb ischemia. It is demonstrated that hypoxia inducible factors are differentially expressed between acute-on-chronic and chronic ischemia. Furthermore, a role is provided for VEGF and SDF-1 in adult neovascularization via retention of CXCR4-positive cells in human ischemic skeletal muscle tissue.

During angiogenesis, several processes such as endothelial cell proliferation and migration are under constant control of growth factors. Recent studies have focused on the post-transcriptional regulation of angiogenesis. Cell specific profiles of micro interfering RNA (miR) molecules were identified [68], which is a class of highly conserved, non-coding small RNAs that regulate gene expression on the post-transcriptional level by inhibiting the translation of protein from messenger RNA (mRNA) or by promoting the degradation of mRNA [69]. miRs can be located in introns of coding and non-coding genes and miR transcription depends on the host genes [70]. Once miRs are expressed, two RNAse endonucleases Dicer and Drosha determine their maturation [71]. Mature miRs incorporate into a RNA-induced silencing complex (RISC), through which target mRNA is translational repressed or degraded [72]. Each miR can regulate hundreds of targets and evidence is increasing that specific miRs are involved in various biological processes such as cardiogenesis, skeletal muscle proliferation and haematopoetic lineage differentiation. One important characteristic is the tissue and cell specific expression pattern. To understand function of miRs in mammals, *in vivo* loss-of-function studies

are required. By using chemically engineered oligonucleotides, called antagomirs, endogenous miRs can be silenced efficiently and specifically. Intravenous administration of antagomirs results in marked reduction of corresponding miR levels *in vivo* [73, 74].
Recently, evidence supporting a role for endothelial miRs in the control of neovascularization has been provided [69]. A study on miR profiles of endothelial cells revealed that specific miRs, such as let-7b, miR-16, miR-21, miR-221 and miR-222 are enriched in endothelial cells. In addition, miR-126 is enriched in endothelium of heart and blood vessels of zebra fish. In these fish miR-126 regulated the response of endothelial cells to VEGF [69, 75], and targeted deletion of miR-126 resulted in abnormal vessel morphology [76]. We study the *in vivo* role of miR-126 on angiogenesis in **chapter 8**, where we use antagomirs to specifically silence miR-126 function in combination with our hind limb ischemia model. By using antagomir directed specifically against miR-126 it is possible to assess the regulatory function of miR-126. miR-126 silencing did not have an effect on collateral artery growth, whereas the effect on the angiogenic response was striking, as described in this chapter.
Another important growth factor and regulator of endothelial cell proliferation and migration is TGF-beta (TGF-β), a multifunctional cytokine involved in the control of cell division, differentiation, migration, adhesion and programmed cell death. TGF-β is known to mediate inflammation and contributes to vascular remodeling [77], for example after vessel wall injury and in stimulating blood flow recovery [78]. The latter was demonstrated in recent animal studies that have applied TGF-β as a successful therapeutic growth factor to enhance revascularization after arterial occlusion [78, 79]. TGF-β can signal through pathways that promote endothelial cell proliferation and migration, but also through pathways that inhibit this [80] (Figure 4). The TGF-β receptor ALK1 and its accessory receptor endoglin both promote endothelial cell proliferation and migration [81, 82], whereas ALK5 acts in an antagonistic fashion [83]. Furthermore, ALK1 and endoglin have been suggested to play a role in shear stress driven vascular adaptation [84, 85]. Defective signaling of one of these receptor pathways results in vascular malformations, as observed in patients with hereditary hemorrhagic telangiectasias [82]. Although the individual role of endoglin and ALK1 in vessel growth is well described, it is yet unknown how these receptors are involved in blood flow recovery after induction of hind limb ischemia. Therefore, the contributory role of these two receptor pathways in both shear induced collateral artery growth and ischemia induced capillary angiogenesis is studied in **chapter 9.** Mice that are heterozygous for ALK1 or endoglin exhibit disrupted TGF-β signaling through these pathways, enabling individual assessment of their contributory role in neovascularization. Finally, the studies described in this thesis are discussed and placed in a broader perspective in **chapter 10**.

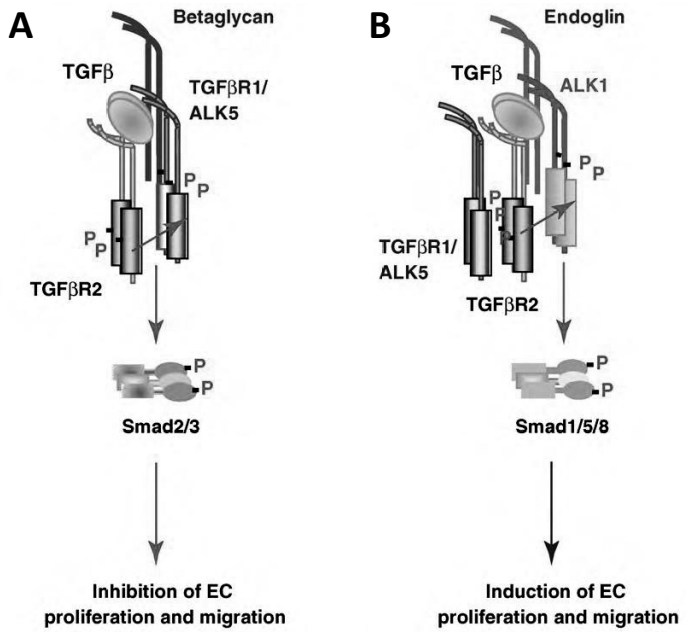

Figure 4. Promotion or inhibition of endothelial cell proliferation and migration by distinct sets of TGF-β signaling receptors. **A.** TGF-β binds to a membrane complex of TGF-β type I receptor ALK5 and TGF-β type II receptor. Binding with this receptor complex induces SMAD-2/3 phosphorylation that act as intracellular transcription factors. Phosphorylated SMAD-2/3 inhibit endothelial cell proliferation and migration. **B.** Binding of TGF-β to TGF-β type I receptor ALK1 results in phosphorylation of SMAD-1/5, which induces migration and proliferation of endothelial cells. The accessory TGF-β type III receptor endoglin is essential for efficient ALK1 signaling and ALK1 indirectly inhibits ALK5 signaling. *(illustration adapted from: Pardali et al, Trends in Cell Biology, 2010)* *See color figure on page 212.*

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