PETRA KORPISALO-PIRINEN

# Angiogenic Gene Therapy

## Vascular Endothelial Growth Factors and Platelet Derived Growth Factors in Vascular Growth and Stabilization

Doctoral dissertation

To be presented by permission of the Faculty of Medicine of the University of Kuopio
for public examination in Tietoteknia Auditorium, University of Kuopio,
on Friday 24th April 2009, at 12 noon

Department of Biotechnology and Molecular Medicine
A.I. Virtanen Institute for Molecular Sciences
University of Kuopio

KUOPION YLIOPISTO

KUOPIO 2009

Author's address:   Department of Biotechnology and Molecular Medicine
                    A.I. Virtanen Institute, University of Kuopio
                    P.O. Box 1627
                    FI-70211 KUOPIO
                    FINLAND
                    E-mail: Petra.Korpisalo@uku.fi

ABSTRACT

Cardiovascular diseases are, despite medical advances, the leading cause of death in Western countries. At the same time, patients with peripheral arterial disease (PAD) are increasingly older, have multiple diseases and are thus increasingly ineligible for conventional revascularization. The use of vascular growth factors to induce neovascularization in ischemic tissues - angiogenic gene therapy - could possibly offer a new treatment for these no-option patients and could also enhance the efficacy of current revascularization techniques.

The aim of this thesis project was to study the safety and the therapeutic potential of vascular endothelial growth factors (VEGFs) and platelet derived growth factor B (PDGF-B) separately or in combination on inducing functional neovascularization and vessel stabilization after adenovirally (Ad) mediated gene transfer (GT) in rabbit and mouse hind limbs. Also, imaging modalities suitable for detecting changes at capillary level vessels including ultrasound- and magnetic resonance imaging (MRI) and digital subtraction angiography were compared. Additionally, the functionality of the induced neovessels was studied using $^{31}$phosphorus magnetic resonance spectroscopy ($^{31}$P-MRS) and arterial acid-base analyses. Furthermore, factors mediating endogenous collateral growth in PAD patients were identified in a retrospective clinical study.

AdVEGF-A GT alone is shown to induce up to 20-fold increase in rabbit hind limb skeletal muscle perfusion, leading to the growth of the whole vascular tree, capillary arterialization and improved muscle aerobic energy metabolism. Using high-resolution contrast-enhanced ultrasound imaging the changes in capillary level vessels were detected non-invasively, in real time and with the accuracy approaching the level of histological examination. The benefits of the induced neovessels on muscle energy metabolism in ischemic rabbit hind limbs were objectively confirmed using use of $^{31}$P-MRS. In vivo titration of the dose of AdVEGF-A revealed a relationship between capillary enlargement and the metabolic benefits of angiogenic gene therapy, and also displayed the need for dose titration in order to avoid gene therapy related side-effects. With the use of an AdVEGF-A+AdPDGF-B combination GT the association of pericytes and neovessels is shown to be affected by the site of transgene expression. Finally, hemodynamics are shown to have an important role in all vascular growth and stabilization mediated by angiogenic gene transfers, and in the formation of endogenous collaterals in PAD.

In summary, the therapeutic potential of angiogenic gene therapy has been extensively verified as regards to the functionality and structure of induced vessels in animal models. Novel methods, suitable for both preclinical and clinical use, have also been tested for imaging microvascular growth and studying the functionality of vessels induced by angiogenic gene therapy. It is concluded that therapeutic angiogenesis is a promising new treatment option for patients with PAD.

"Life is like a box of chocolates.
You never know what you're gonna get"

*Forrest Gump*

## ACKNOWLEDGEMENTS

## ABREVIATIONS

| | |
|---|---|
| AAV | adeno associated virus |
| ABI | ankle brachial index |
| Ad | adenovirus |
| ALI | acute limb ischemia |
| Akt | serine-threonine kinase Akt (PKB) |
| Ang | angiopoietin |
| AP | alkaline phosphatase |
| α-sma | α-smooth muscle actin |
| CAR | coxsackie adenovirus receptor |
| CEU | contrast enhanced ultrasound |
| CLI | critical limb ischemia |
| CT | X-ray computed tomography |
| DCE-MRI | dynamic contrast enhanced-magnetic resonance imaging |
| DPP | di-peptityl-peptidase |
| EC | endothelial cell |
| ELISA | enzyme-linked immunosorbent assay |
| eNOS | endothelial nitric oxide synthase (NOS III) |
| F | French (a unit used to standardize catheter caliber 1 F = 0.33 mm) |
| FGF | fibroblast growth factor |
| FGFR | fibroblast growth factor receptor |
| Flk-1 | fetal liver kinase-1 (murine VEGFR-2) |
| Flt-1 | fms-like tyrosine kinase-1 (VEGFR-1) |
| Flt-4 | fms-like tyrosine kinase-4 (VEGFR-3) |
| G | Gauge (a unit used to measure the diameter of hypodermic needles) |
| GM-CSF | granulocyte macrophage-colony stimulating factor |
| GT | gene transfer |
| HIF | hypoxia inducible factor |
| IGF | insulin-like growth factor |
| iNOS | inducible nitric oxide synthase (NOS II) |
| i.a. | intra-arterial |
| i.m. | intra-muscular |
| i.v. | intra-venous |
| KDR | kinase domain region (human VEGFR-2) |
| LacZ | β-galactosidase (marker gene) |
| MRI | magnetic resonance imaging |
| MRS | magnetic resonance spectroscopy |
| NO | nitric oxide |
| NOS | nitric oxide synthase |
| NRP | neuropilin receptor |
| p | plasmid |
| PAD | peripheral arterial disease |
| PBS | phosphate buffered saline |
| PDGF | platelet derived growth factor |
| PDGFR | platelet derived growth factor receptor |
| PlGF | placental growth factor |
| r | recombinant protein |
| SPECT | single photon emission computed tomography |
| SMC | smooth muscle cell |
| VEGF | vascular endothelial growth factor |
| VEGFR | vascular endothelial growth factor receptor |
| vp | viral particles |
| VPF | vascular permeability factor |

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications which are referred to by their Roman numerals:

I       Rissanen TT, **Korpisalo P**, Markkanen JE, Liimatainen T, Orden MR, Kholova I, de Goede A, Heikura T, Gröhn OH, Ylä-Herttuala S. Blood flow remodels growing vasculature during vascular endothelial growth factor gene therapy and determines between capillary arterialization and sprouting angiogenesis. *Circulation*. 2005;20;112:3937-3946.

II      Rissanen TT\*, **Korpisalo P\***, Karvinen H, Liimatainen T, Laidinen S, Gröhn O, Ylä-Herttuala S. High-Resolution Ultrasound Perfusion Imaging of Therapeutic Angiogenesis. *Journal of American College of Cardiology: Cardiovascular Imaging*. 2008;1:83-91. (\*authors with equal contribution)

III     **Korpisalo P\***, Rissanen TT\*, Bengtsson T, Liimatainen T, Laidinen S, Karvinen H, Markkanen JE, Gröhn OH, Ylä-Herttuala S. Therapeutic angiogenesis with placental growth factor improves exercise tolerance of ischemic rabbit hind limbs. *Cardiovascular Research*. 2008;80(2): 263-270. (\*authors with equal contribution)

IV      **Korpisalo P**, Karvinen H, Rissanen TT, Kilpijoki J, Marjomäki V, Baluk P, McDonald DM, Cao Y, Eriksson U, Alitalo K, Ylä-Herttuala S. VEGF-A and PDGF-B combination gene therapy prolongs angiogenic effects via recruitment of interstitial mononuclear cells and paracrine effects rather than improved pericyte coverage of angiogenic vessels. *Circulation Research*. 2008;103: 1092-1099.

V       **Korpisalo P**, Laidinen S, Hytönen J, Karvinen H, Laitinen J, Parviainen H, Marjomäki V, Rissanen TT, Vajanto I, Ylä-Herttuala S. Optimal VEGF-A concentration enables the growth of physiological, non-leaky vessels. *Submitted 2009*.

VI      Vajanto I\*, **Korpisalo P\***, Karjalainen J, Hakala T, Mäkinen K, Ylä-Herttuala S. Antegrade flow and peripheral resistance determine the level of endogenous arteriogenesis in patients with superficial femoral artery occlusion. *Submitted 2009*. (\*authors with equal contribution)

CONTENTS

INTRODUCTION

Cardiovascular diseases are the main cause of death in Western countries causing on average almost half of the total mortality in Europe. In the forthcoming years the increase in again will inevitably further increase the burden of cardiovascular disease (Scholte op Reimer WJM et al., 2006). Peripheral arterial disease (PAD) leads to decreased blood flow and ischemia in the lower limbs resulting in pain and reduced function (Cotran et al., 1999; Beard, 2000; Norgren et al., 2007). Patients with PAD are increasingly older with several co-morbidities. Also, the characteristics of the disease have changed making patients with wide-spread changes a challenge to conventional revascularization (Kuukasjarvi et al., 1997). Despite medical development, 30 % of critical limb ischemia patients still end up with amputation within one year of diagnosis (Norgren et al., 2007). Angiogenic gene therapy aims to enhance nature's own regeneration capacity and may have potential in the revascularization of ischemic tissues (Yla-Herttuala and Alitalo, 2003). Angiogenesis is a process that includes the growth and branching of vascular structures to form new blood vessels and naturally occurs e.g. during growth and wound healing (Risau, 1997). Gene therapy means a transfer of nucleic acids into somatic cells of tissues where a beneficial effect can be achieved by replacing a missing or non-functioning protein by a functional one (Yla-Herttuala and Alitalo, 2003). In angiogenic gene therapy growth factors, such as vascular endothelial growth factors (VEGFs), are transferred into ischemic tissues where they stimulate the growth of new blood vessels and relieve ischemia (Yla-Herttuala and Alitalo, 2003). Currently, angiogenic gene therapy has been extensively tested in both preclinical and clinical trials. Although the initial clinical reports were promising, results from randomized, double-blind clinical trials have reported concerns about the efficacy of gene therapy (Rissanen and Yla-Herttuala, 2007; Yla-Herttuala et al., 2007) and the functionality of newly formed vessels (Richardson et al., 2001; Pajusola et al., 2005). The aim of this thesis was to study factors affecting the growth of functional vessels and to test the potential of angiogenic gene therapy in the revascularization of ischemic tissues.

REVIEW OF LITERATURE


**The blood vascular system and mechanisms of vascular growth**


Organization of the vascular system

The vascular system is the first organ to develop during fetal growth (Risau and Flamme, 1995). However, it remains dynamic, responding to changes such as inflammation, ischemia, growth and exercise throughout life. Blood flow and perfusion of each tissue are always precisely controlled in relation to the needs of the tissue (Guyton and Hall, 2000). Skeletal muscle for example has very low perfusion in resting conditions (about 4 ml/min/100g) but can increase perfusion up to 20-fold (as high as 80 ml/min/100g) during exercise to meet increased metabolic needs - the greatest adaptation of perfusion in the human body (Guyton and Hall, 2000). The regulation of flow and perfusion is maintained, besides the regulation of the cardiac function, by the structure of the vascular walls and the diameter and number of vessels (Guyton and Hall, 2000). According to Poiseuille's law, the rate of blood flow is directly proportional to the fourth power of the radius of a vessel, which demonstrates that the diameter of the vessel plays the greatest role in determining the rate of blood flow (Guyton and Hall, 2000).

Arteries carry oxygenated blood from the heart to the periphery with high pressure (fluctuates at rest between 80 mmHg and 120 mmHg) and velocity (average during rest: 33 cm/s) (Guyton and Hall, 2000). In humans arteries range from several centimeters to 100 μm in diameter and have strong muscular walls comprising of intimal, medial and adventitial layers separated by internal and external elastic laminas (Figure 1) (Cotran et al., 1999). A layer of smooth muscle cells (SMC) in the medial layer of the arterial wall generates active contractions that push blood forwards (Guyton and Hall, 2000). The SMCs also regulate the diameter of the arteries via constriction or relaxation mediated by nitric oxide (NO) released from the endothelium. To initiate a change in vascular diameter and blood flow, the endothelium can be stimulated by different factors such as growth factors, hormones and also by shear stress (Guyton and Hall, 2000). After branching to smaller arteries and further to arterioles (ranging from 100 μm to 5 μm in diameter), blood pressure and velocity in the arterial side are decreased to be more suitable for capillary circulation. A specific precapillary sphincter, situated between the end arterioles (metarterioles) and the capillary bed, controls the amount of blood going into the capillary level (Guyton and Hall, 2000).

The capillary network consists of fine tubes, 9 to 5 μm in diameter, comprising only of an endothelial layer and occasional pericytes (Cotran et al., 1999) covering 100 % capillary length but only 20 % of the surface area (Figure 1) (DM McDonald, unpublished). Capillaries exchange fluids, oxygen, nutrients, electrolytes and hormones with the surrounding tissues via diffusion. To manage that, the capillaries need to maintain blood pressure of 10 to 35 mmHg, blood velocity of approximately 0.3 mm/s and a tight physical connection with the surrounding cells (Guyton and Hall, 2000). Endothelial cells are joined together by tight junctions but small intercellular clefts (about 6 to 7 nm in width) between cells are needed for water and water-soluble substances to pass through (Guyton and Hall, 2000). In normal conditions, these junctions are just small enough to maintain homeostasis without plasma leakage. However, if the endothelial junctions are loosened during vascular growth for example or if capillary pressure is increased, albumin is able to leak out through these clefts, drive water to the interstitium and cause tissue edema. Also, cellular fenestration and increased vesicle transport (vesiculo-vacuolar organelle) have been identified as endothelium-based causes for the development of edema (Feng et al., 2000).

14

Post capillary venules (10 to 50 μm in diameter), muscular venules (50 to 100 μm) and veins (100 μm to several centimeters) conduct oxygen-deprived blood from the periphery back to the heart. Veins have thin walls comprising the endothelium, pericytes or SMCs and a loose adventitia (Figure 1) (Cotran et al., 1999). The SMCs in veins are less organized than in arteries but still able to induce small contractions to support blood flow due to a low, gradually decreasing blood pressure that nearly reaches 0 mmHg in the right atrium of the heart (Guyton and Hall, 2000). Other mechanisms that maintain the pressure in the venous circulation are: 1) the so called "venous or muscular pump" which means that tensing large muscles around the veins, during walking for example, pushes blood forwards, 2) venous valves that prevent backflow, and 3) a negative pressure caused in the right atrium of the heart during each heart beat. An important feature of the veins also is that they are capable of storing large amounts of blood, owing to their dilating potential, and to pump the blood back to the circulation if needed (Guyton and Hall, 2000).
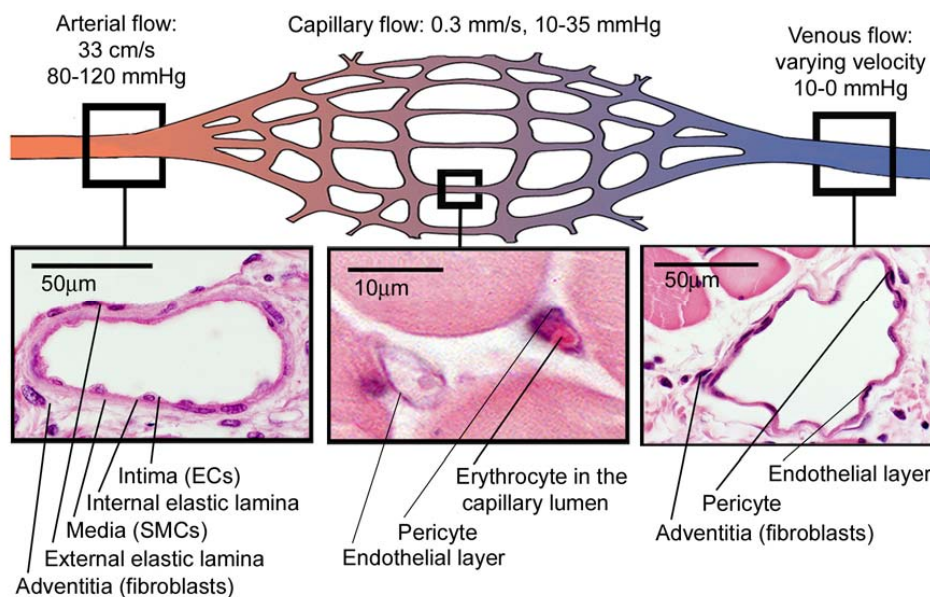


**Figure 1.** Functional and structural differences of arterial, capillary and venous vessels. The histological images have hematoxylin-eosin stainings.

Vasculogenesis

The *de novo* formation of vessels from endothelial precursors, angioblasts, during fetal development is called vasculogenesis (Risau and Flamme, 1995). During vasculogenesis fetal stem cells differentiate into hemangioblasts which serve as precursors to angioblasts and hematopoietic cells. Angioblasts then migrate and differentiate, guided by several growth factors, to form endothelial tubes (Figure 2) (Risau and Flamme, 1995). VEGFs have been identified as mediators of angioblast migration and differentiation (Risau and Flamme, 1995; Carmeliet et al., 1996). Vasculogenesis via bone marrow derived, circulating endothelial precursors has also been suggested to take place postnatally (Asahara et al., 1997; Rafii and Lyden, 2003). VEGF, granulocyte macrophage-colony stimulating factor (GM-CSF) and other cytokines have been proposed as the mediators of postnatal vasculogenesis and especially angioblast activation (Asahara et al., 1999). The current knowledge

of postnatal vasculogenesis, however, strongly suggests that the bone marrow derived cells do not actually incorporate into the endothelium of growing vessels in the adult as during fetal development but rather act as sources for growth factors that subsequently stimulate vascular growth (Rehman et al., 2003; Purhonen et al., 2008).

Angiogenesis

Angiogenesis is a process that includes the growth, branching and reorganization of the primitive vascular structures formed by vasculogenesis to form functional vascular networks (Conway et al., 2001). Types of angiogenesis include sprouting of new vascular branches, enlargement of pre-existing capillaries and bridging or intussusception of enlarged vessels to form smaller daughter vessels (Figure 2) (Risau, 1997; Conway et al., 2001). Loosening of the endothelial cell junctions is the first step in angiogenesis. This increases the permeability of the vessel leading to plasma extravasation and degradation of the extracellular matrix (Conway et al., 2001). These changes allow proliferation of endothelial cells in the vascular wall and their migration to the surrounding tissue to form expanding and elongating vascular branches that then form connections to the preexisting vascular network. At first, these structures are unstable and leaky but are stabilized by the recruitment of accessory cells such as pericytes or SMCs around the vessel. The accumulation of pericytes or SMCs inhibits additional endothelial proliferation and the endothelial junctions are once more re-established (Conway et al., 2001). The origin of pericytes surrounding the formed vessels is yet unclear but on site proliferation and differentiation from endothelial- or mesenchymal precursors, stem cells or macrophages have been suggested (Conway et al., 2001). Several cytokines mediating angiogenesis have been identified of which the most important are VEGFs and fibroblast growth factors (FGFs) (Conway et al., 2001). Angiogenesis is common postnatally for example during wound healing, the menstruation cycle in the uterus, and several disease processes such as diabetic retinopathy, cancer, ischemic diseases, arthritis and inflammation (Risau, 1997).

Breaking down of endothelial junctions and increasing vascular permeability are crucial for efficient angiogenesis (Conway et al., 2001). This process however, can lead to the formation of tissue edema via plasma protein extravasation and increased osmotic pressure. Plasma protein extravasation does also take place during normal conditions but only so modestly that the lymphatic system can easily compensate the changes (Guyton and Hall, 2000). During rapid vascular growth the surface area of vessels, perfusion and blood pressure of the vasculature increase so that plasma protein extravasation exceeds the capacity of the lymphatic system. Tissue edema has also been taken as a hallmark of efficient angiogenesis and in fact it has been shown that the changes in capillary size correlate with the increase in endothelial permeability in animal models (Rissanen et al., 2003b). However, formation of edema is also a side-effect of angiogenesis that can lead to a circulatory collapse or a tissue compartment syndrome (Baumgartner et al., 2000; Callum and Bradbury, 2000; Conway et al., 2001). Diuretics and cortisone have been used to treat edema (Nauck et al., 1998; Baumgartner et al., 2000) but also means of avoiding it remain an interesting field of study.

Arteriogenesis

Arteriogenesis is a mechanism by which arterial side vascular branches, arterioles, form larger conducting arteries (Conway et al., 2001; Schaper and Scholz, 2003). In the adult, arteriogenesis is prominently found in ischemic conditions where the formation of collateral arteries is a natural way of by-passing vascular blockages (Figure 2) (Schaper and Scholz, 2003). When a conducting artery

is blocked the routing of blood flow is changed. Collateral arteries, by-passing the blockage, are initially small arterial side branches of the conducting artery. Unlike in angiogenesis, where hypoxia is the main activator of vessel growth, in arteriogenesis hemodynamic factors drive vessel growth (Schaper and Scholz, 2003). By means of elevated blood pressure and shear stress, caused by the blood flow directing to the small arterial branches instead of the blocked main artery, gene expression profile in the endothelium of the collateral artery is changed (Schaper and Scholz, 2003). Monocyte chemotactic protein (MCP-1) recruits monocytes to the area that is believed to then loosen the basement membrane of the arteriole leading to the mobilization of smooth muscle cells in the vascular wall (Ito et al., 1997). The mobilization of SMCs is followed by proliferation and migration, and changes in the other vascular wall components to increase the diameter of the artery. The process continues until the pressure and shear stress are again normalized and the SMCs turn back into their constricting phenotype (Hoefer et al., 2001). Stimulation of arteriogenesis in the treatment of ischemia is interesting since large collateral vessels could conduct large amounts of blood across the blocked main artery (White et al., 1998; Hoefer et al., 2001).
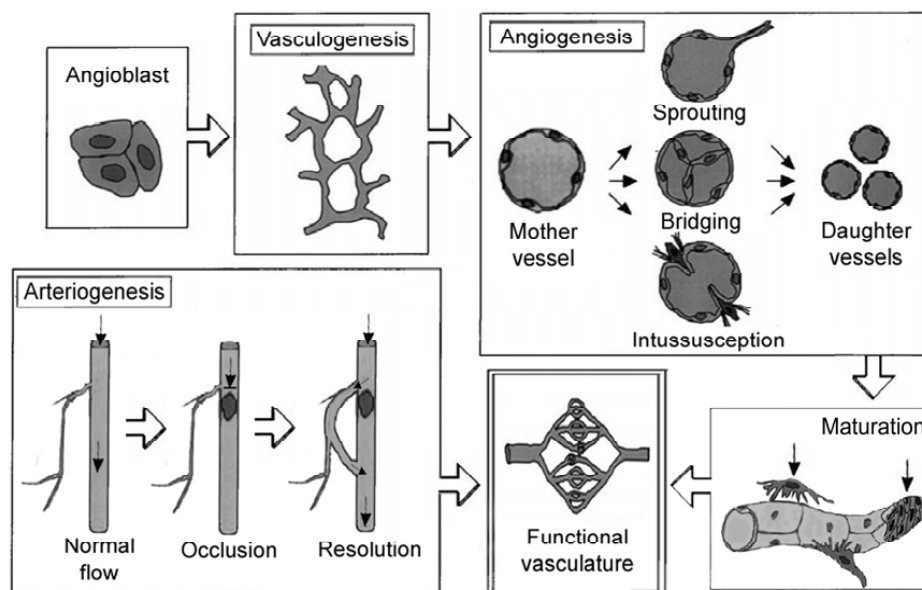


**Figure 2.** Mechanisms of vascular growth. Modified from Conway et al 2001.

**Peripheral ischemic diseases**

Atherosclerosis

Atherosclerosis is a disease process in which lipid, cholesterol-laden macrophages, SMCs, connective tissue and calcium-salt precipitates accumulate in the walls of arteries causing impairment of blood flow and tissue ischemia most often in the heart, peripheral muscles or in the brain (Cotran et al., 1999; Libby, 2002). It is widely accepted that the genetic background, high serum LDL and low serum HDL cholesterol levels, high blood pressure, diabetes, smoking, the male gender and the lack of physical activity are the main risk factors for atherosclerosis (Doevendans et al., 2001; Grech, 2003). Several hypotheses for the pathogenesis of atherosclerosis have been presented including the lipid theory, highlighting the role of oxidized LDL, and the

endothelial injury theory, considering atherosclerosis as a chronic inflammatory response (Ross, 1999). The development of an atherosclerotic lesion is a stepwise process (Figure 3) that involves monocyte adhesion, foam cell formation, SMC proliferation and fibrous cap formation.
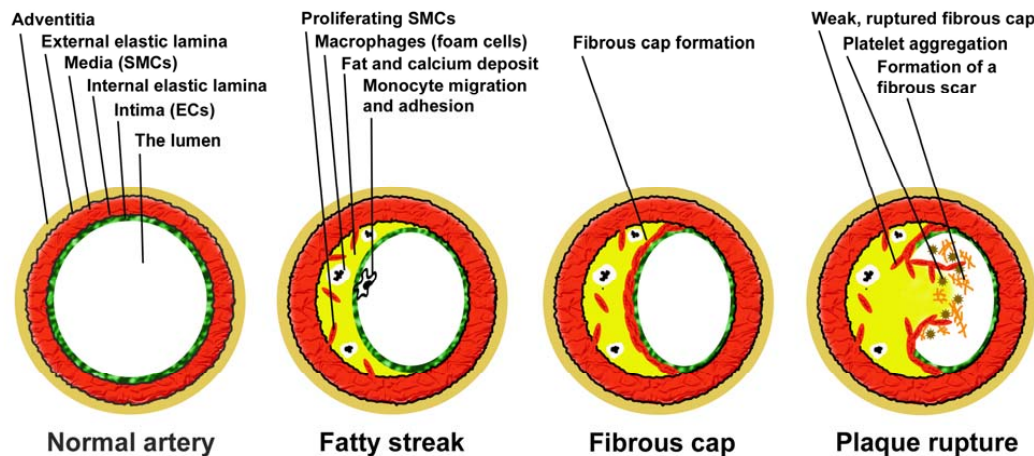


**Figure 3.** Development of an atherosclerotic plaque. Based on Cotran et al 1999.

Lower limb ischemia

In peripheral arterial disease (PAD) blood flow and subsequently the delivery of oxygen to the lower limbs is disrupted resulting in ischemia (Cotran et al., 1999). The prevalence of PAD is about 3-10 % in the total population (including asymptomatic patients) and is increased by age (15-20 % in over 70-year old persons) (Beard, 2000; Norgren et al., 2007). Atherosclerosis is the most common cause for PAD and chronic lower limb ischemia. Pain during walking that is relieved by a short rest (claudication) is usually the first symptom of lower limb ischemia. Claudication is caused by inadequate energy and blood supply to the peripheral muscles during exercise resulting in a metabolic acidosis and ischemic pain (Norgren et al., 2007). Diagnostic criteria used to evaluate limb ischemia are: maximal walking distance without stopping, loss of peripheral pulses, temperature differences, formation of ulcers, rest pain, decreased ankle-brachial systolic blood pressure index (ABI) and angiography (Beard, 2000). ABI under 0,5, rest pain or arterial ulcers are signs of critical chronic limb ischemia (CLI) that requires immediate medical attention (Norgren et al., 2007). Thrombus of an atherosclerotic lesion can lead to an acute worsening of a previous chronic disease to acute limb ischemia (ALI) that is presented by acute, severe pain during rest, cyanosis and the loss of motor- and sensory functions (Callum and Bradbury, 2000). ALI requires revascularization in a few hours to avoid limb loss (Norgren et al., 2007). The classification of PAD according to Fontaine, Rutherford and individual clinical signs in different stages of the disease are described in Figure 4.

Conventional treatment of chronic lower limb ischemia includes in its early stages lifestyle guidance encouraging physical activity, a low fat diet, smoking cessation and good foot care preventing formation of ulcers (Norgren et al., 2007). Also, a re-evaluation of pharmaceutical treatment of possible high serum LDL, high blood glucose and high blood pressure is needed since the risk of death from all cardiovascular causes is increased in patients with PAD. Active treatment of diabetes is highly important since the risk of diabetics to develop chronic ulcers is higher than that of non-diabetics (Norgren et al., 2007). Low dose, daily acetylsalicylic acid is a basic

preventive anti-platelet therapy recommended already at the early stages of PAD (Norgren et al., 2007). Embolectomy, intra-arterial thrombolytic treatment, percutaneous transluminal angioplasty (PTA) and by-pass surgery are the standard treatment options for patients with acutely or critically ischemic limbs or a chronic lower limb ischemia that affects the patient's ability to work. Furthermore, ulcers or gangrene need to be treated actively and sufficient pain relief taken care of. Also, the use of vasodilators, prostanoids or anticoagulants may benefit some patients (Norgren et al., 2007). The incidence of CLI is currently about 500-1000 new cases per year of one million people (Norgren et al., 2007). Despite active treatment 30 % of CLI patients will have amputation within one year of diagnosis (120-500 amputations/ million/ year) and the mortality during the first year after diagnosis among CLI patients is 25 %. Thus, there is a need for the development of improved treatment options for both the prevention and treatment of CLI.
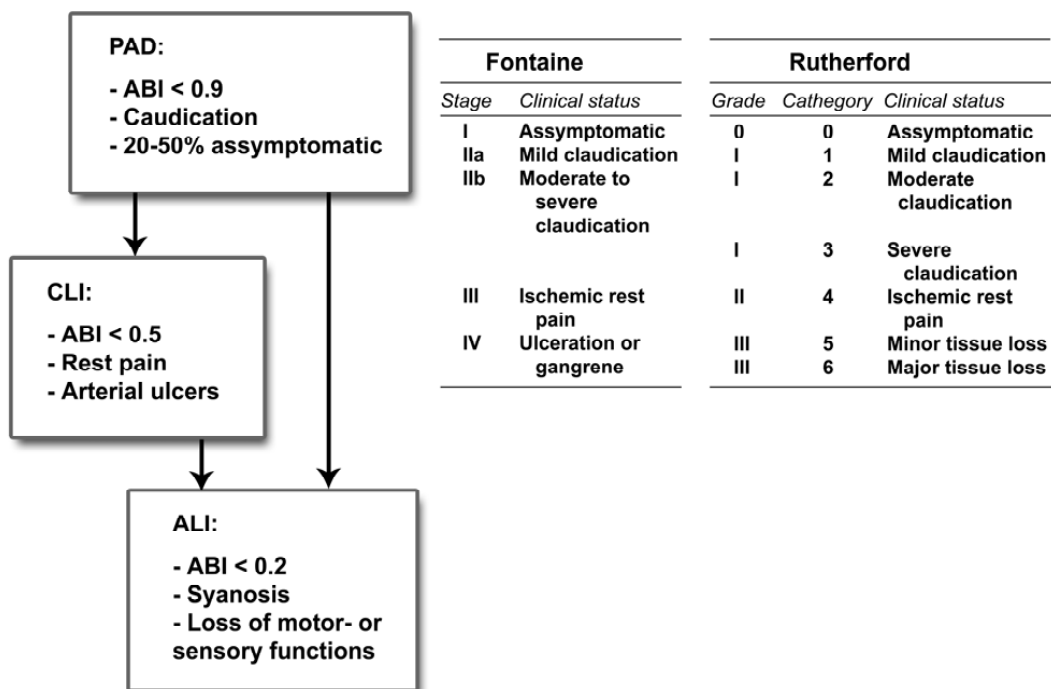


**Figure 4.** Classification of PAD by Fontaine and Rutherford, and clinical findings in different stages. PAD = peripheral arterial disease, CLI = critical chronic limb ischemia, ALI = acute limb ischemia. Based on Norgren et al 2007.

**Growth factors and receptors**

Vascular endothelial growth factors and their receptors

The first member of the VEGF family, VEGF-A, was discovered in 1989 by two independent research groups describing a factor that stimulated the growth of endothelial cells (Leung et al., 1989; Ferrara and Henzel, 1989). However, already in 1983 a cytokine stimulating endothelial migration and vascular growth, called vascular permeability factor (VPF), had been described

(Senger et al., 1983) and was in 1989 indentified the same growth factor than the newly purified VEGF-A (Keck et al., 1989). Human VEGF-A is currently known to have eight different splice forms consisting of 121, 145, 162, 165, 165b, 183, 189 or 206 amino acids (Tischer et al., 1991; Houck et al., 1991; Poltorak et al., 1997; Jingjing et al., 1999; Bates et al., 2002; Lange et al., 2003). VEGF-A[121] is acidic and thus freely soluble in the extracellular matrix. The other VEGF-A isoforms are increasingly basic, depending on their number of amino acids, insoluble and bound to the extracellular matrix and heparan sulphate on cell surfaces (Ferrara, 2004). The matrix-binding properties of VEGF-A have been shown critical for the formation of proper vascular networks (Carmeliet et al., 1999; Ruhrberg et al., 2002). VEGF-A[165] has intermediate matrix-binding properties and is the principal effector of VEGF-A actions (Ferrara, 2004). VEGF-A is a regulator of angiogenesis, arteriogenesis, vasodilatation and vascular permeability but is also associated with stem cell mobilization, monocyte chemoattraction, osteoblast-mediated bone formation and neuronal protection (Storkebaum et al., 2004; Ferrara, 2004). VEGF-A is indispensable for growth; the lack of only one VEGF-A allele leads to embryonic lethality due to defects in the formation of the vascular system (Carmeliet et al., 1996; Ferrara et al., 1996). In the adult VEGF-A is expressed widely in several organs and in many different cell types with low levels that for example in the vascular endothelium serve as survival factors for ECs (Gerber et al., 1998a; Gerber et al., 1998b). The expression of VEGF-A is tightly regulated and it is up-regulated by hypoxia but also by other growth factors and by vascular shear stress (Rissanen et al., 2002; Ferrara, 2004). VEGF-A levels lower than normal are associated with pre-eclampsia and postnatal respiratory distress syndrome, whereas overly high levels of VEGF-A have been associated with for example tumor growth, ascites formation, brain edema, polycystic ovary syndrome, macular generation and diabetic retinopathy (Ferrara, 2004).

VEGF-B, the second member of the VEGF family, was discovered in 1996 (Olofsson et al., 1996a). It is found in two isoforms, VEGF-B[167] and VEGF-B[186] (Olofsson et al., 1996b). VEGF-B is expressed in the heart, skeletal muscle and in the SMCs of large arteries together with VEGF-A (Olofsson et al., 1996a). The specific biological role of VEGF-B is currently under active research. Initially it was reported to regulate the proliferation of endothelial cells (Olofsson et al., 1996a). Later, knockout studies showed that VEGF-B^{-/-} mice, although viable, have developmental defects in their hearts and coronary vasculature, and they also recover less efficiently from myocardial ischemia than wild type animals (Bellomo et al., 2000). VEGF-B has also been suggested to have a role in pathological angiogenesis (Mould et al., 2003). VEGF-B has been found essential for the normal function of the heart but not for the development of the cardiovascular system or angiogenesis in the skeletal muscle of an adult (Aase et al., 2001). The most recent evidence indicates that VEGF-B would have a myocardial specific role in post natal angiogenesis (Li et al., 2008; Lahteenvuo et al., 2009).

VEGF-C was cloned in 1996 (Joukov et al., 1996) and shares high similarity in structure and binding with VEGF-D that was found in 1997 (Yamada et al., 1997). Both VEGF-C and -D are synthesized in long precursor forms that can then go through proteolytic processing to yield mature (ΔNΔC) forms (Joukov et al., 1997; Stacker et al., 1999). The unprocessed forms of VEGF-C and -D bind VEGF-receptor (VEGFR) 3 stimulating lymphatic growth, where as the processed forms also bind with high affinity to VEGFR-2 and stimulate angiogenesis as well as growth of lymphatic vessels (Joukov et al., 1996; Stacker et al., 1999; Rissanen et al., 2003b). During embryonic development VEGF-C is expressed in areas of lymphatic sprouts and is essential for lymphatic vessel development (Karkkainen et al., 2004). In contrast, VEGF-D is expressed more widely during development e.g. in limb buds, the heart and several other organs implicating a more diverse developmental role (Avantaggiato et al., 1998). In the adult, VEGF-C is expressed, besides the lungs, heart and kidneys, abundantly in lymph nodes (Lymboussaki et al., 1999) and also in

neuroendocrine cells of pancreas and thymus (Partanen et al., 2000). VEGF-D is expressed in the heart, skeletal muscle, lungs, colon, small intestine and in SMCs of normal and atherosclerotic arteries (Achen et al., 1998; Rutanen et al., 2003). Additionally, both VEGF-C and -D have reported to be up-regulated in several tumors and have been suggested to have a role in tumor invasion and metastasis (Karpanen et al., 2001; Skobe et al., 2001; Stacker et al., 2001; Saharinen et al., 2004). Neutralization of both VEGF-C and -D by a VEGFR-3 antibody has led to decreased lymphangiogenesis and lymph node metastasis in a mouse tumor model (He et al., 2002).

VEGF-E and -F are collective names for growth factors derived from Orf-viruses (Lyttle et al., 1994; Ogawa et al., 1998) and snake venom (Yamazaki et al., 2005), respectively. The VEGF-Es are only partly similar to VEGFs found in mammals and their receptor binding and biological effects differ (Wise et al., 1999). Overall, VEGF-Es are not bound to heparan sulphate and thus soluble in the matrix. One VEGF-E form has been reported to stimulate vascular growth without increasing vascular permeability (Kiba et al., 2003). VEGF-Fs consist of the VEGF -domain, bind VEGFR-2 and have been described to block the activity of VEGF-A[165] (Yamazaki et al., 2003; Yamazaki et al., 2005). The therapeutic potential of these growth factors is currently unknown.

Placental growth factor (PlGF) was first characterized in 1991 (Maglione et al., 1991). It is found in four isoforms, PlGF1-4 consisting of either 149 (Maglione et al., 1991), 170 (Maglione et al., 1993), 221 (Cao et al., 1997) or 252 amino acids (Yang et al., 2003), respectively. The isoforms differ in their solubility; PlGF-1 and PlGF-3 do not bind to heparin where as PlGF-2 and PlGF-4 are heparin-binding. PlGF is naturally expressed in the blood vessel endothelium in the human placenta during pregnancy, and a drop in placental PlGF levels is associated with pre-eclampsia (Yang et al., 2003). However, knockout studies have not revealed any pathological changes, infertility or premature death in PlGF^{-/-} animals, implicating that PlGF would not be necessary for normal development or for pregnancy (Carmeliet et al., 2001). These studies have shown however that in pathological conditions such as cancer, ischemia, inflammation or wound healing the lack of PlGF results in improper angiogenesis (Carmeliet et al., 2001). Over-expression of PlGF either in adulthood or during development has been shown to induce angiogenesis, vascular permeability and formation of vascular glomeruloid structures via up-regulation of VEGF-A (Odorisio et al., 2002; Oura et al., 2003; Lahteenvuo et al., 2009). Additionally PlGF stimulates migration and differentiation of bone marrow derived monocytes and hematopoietic cells (Hattori et al., 2002; Pipp et al., 2003).

The VEGF family growth factors have five receptors: VEGFR 1-3 (de Vries et al., 1992; Terman et al., 1992; Pajusola et al., 1992; Galland et al., 1992) and neuropilin receptors (NRP) 1-2 (Soker et al., 1998; Gluzman-Poltorak et al., 2000). The VEGFR-1-3 are tyrosine kinase receptors and are found in ECs and bone marrow derived cells (Ferrara, 2004). The NRPs are trans-membrane receptors and are present, besides the vascular wall, in neurons (Giger et al., 1998). The receptors also have soluble forms such as sVEGFR-1, sVEGFR-2, sNRP-1 and sNRP-2 that function as extracellular decoy receptors and regulate free growth factor concentration (Kendall and Thomas, 1993; Neufeld et al., 2002; Ebos et al., 2004). VEGFR-1 is a debated receptor. It binds VEGF-A, -B and PlGF and is reported to mediate endothelial-, hematopoietic cell- and monocyte chemotaxis (Luttun et al., 2002; Pipp et al., 2003). Some studies suggest that VEGFR-1 would not be able to induce signaling on its own but would rather act as an decoy receptor for VEGFR-2-activating ligands (Ferrara, 2004). Others report that VEGFR-1 enhances signaling via VEGFR-2 (Autiero et al., 2003). The soluble VEGFR-1 has been claimed to have a role, besides only being a decoy receptor, in the guidance and lodging of endothelial cells (Orecchia et al., 2003). VEGFR-2 mediates most of the angiogenesis and vascular permeability effects of VEGFs and is indispensable for growth (Shalaby et al., 1995; Rissanen et al., 2003b; Ferrara, 2004). It binds VEGF-A, VEGF-

Es, VEGF-Fs and both the mature and unprocessed forms of VEGF-C and -D (Yla-Herttuala et al., 2007). VEGFR-3 binds both the mature and unprocessed forms of VEGF-C and -D and mediates lymphangiogenesis and lymphatic vessel survival (Makinen et al., 2001). NRP-1 binds VEGF-A, -B, -E and PlGF. Traditionally, NRP-1 is believed to have a function only as an amplifier of the signaling via VEGFR-2 with respect to angiogenesis (Soker et al., 1998; Ferrara, 2004) but some reports have also indicated that it might have a role alone as an activator of EC migration and survival (Wang et al., 2003; Wang et al., 2007). NRP-2 binds VEGF-A, -C and -D and is currently thought to have a role in the formation of small lymphatics and capillaries (Yuan et al., 2002; Karpanen et al., 2006). The functions and binding properties of VEGFs and their receptors are summarized below in Figure 5.
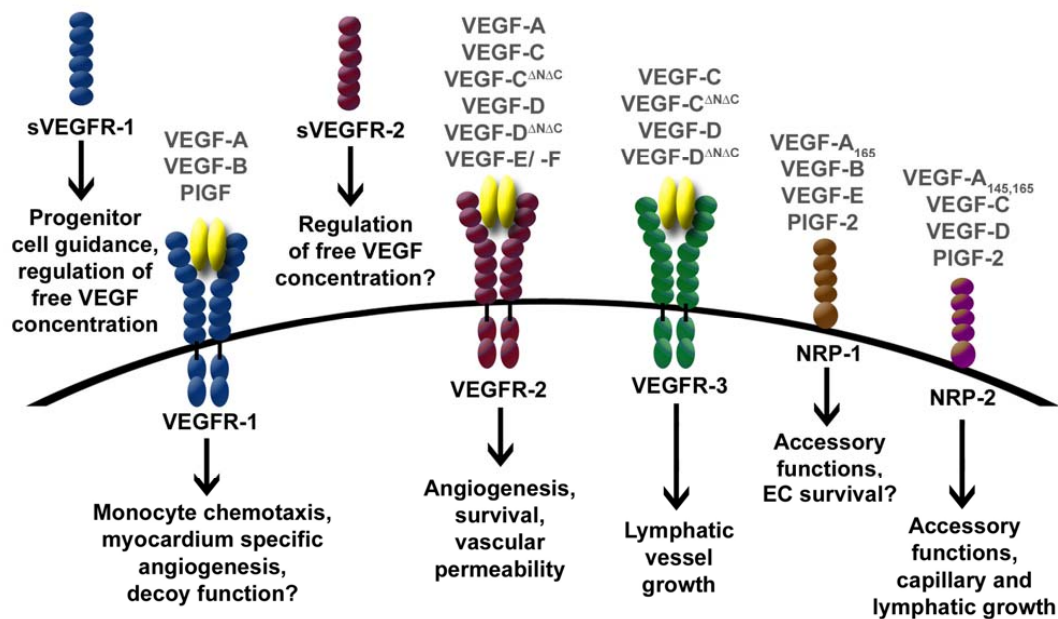


**Figure 5.** Binding of the VEGF family proteins to their receptors. Modified from Rissanen et al 2007.

Platelet derived growth factors and their receptors

Platelet derived growth factor (PDGF) was first identified in the 1970s (Ross et al., 1974; Heldin et al., 1979; Antoniades et al., 1979). Currently, the PDGF family of proteins consists of four members: PDGF-A (Heldin et al., 1979; Deuel et al., 1981; Bonthron et al., 1988), -B (Collins et al., 1985), -C (Li et al., 2000) and -D (Bergsten et al., 2001; LaRochelle et al., 2001), from which five dimeric forms: -AA, -AB, -BB, -CC, -DD have been described so far. PDGF-A is found in 196 and 211 amino acid containing splice forms, PDGF-B is 241 amino acids long, and PDGF-C and PDGF-D have 345 and 370 amino acids, respectively (Fredriksson et al., 2004). The PDGFs promote the proliferation of several types of cells with mesenchymal origin, such as fibroblasts and smooth muscle cells, and also promote cell migration and survival (Tallquist and Kazlauskas, 2004). In the adult, PDGFs are widely expressed in most human tissues. PDGF-A has the highest expression in the heart, pancreas and skeletal muscle, PDGF-B in the heart and placenta, PDGF-C in the heart, kidneys, pancreas and the liver, and PDGF-D in the heart, pancreas and the ovaries (Fredriksson et al., 2004). All PDGFs have been described to have angiogenic potential but due to

their specificity on SMCs and pericytes the PDGFs are likely to contribute mostly in the angiogenic process to vascular stabilization. The PDGFs signal via binding to PDGF receptors α and β (PDGFR-α and -β) (Heldin et al., 1988) that also form dimers (αα, αβ and ββ) (Fredriksson et al., 2004). Both PDGFRs are required during development (Tallquist and Kazlauskas, 2004). PDGFR-α is involved in the stimulation of several cell types including chondrocytes (Soriano, 1997), oligodentrocytes (Fruttiger et al., 1999), and kidney (Li et al., 2000) and dermal (Karlsson et al., 1999) mesenchyme. PDGFR-β mostly regulates the fate of vascular smooth muscle and pericytes (Soriano, 1994; Lindahl et al., 1997). The functions and binding properties of PDGFs and PDGFR are summarized in Figure 6.
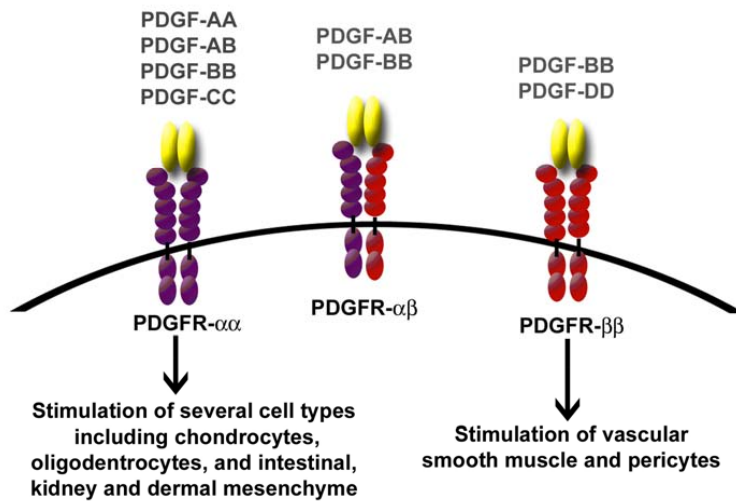


**Figure 6.** PDGFR ligands and functions. Modified from Fredriksson et al 2004.

Due to their binding ability to PDGFR-β and stimulation of vascular SMCs, PDGF-B and -D are interesting candidates for the stabilization of vessels induced by gene therapy. Endothelial-targeted PDGF-B and VEGF-A combination gene transfers have been reported to improve vascular wall maturation and duration of angiogenic effects (Richardson et al., 2001; Levanon et al., 2006). Endothelial PDGF-B retention has been demonstrated crucial for the formation of proper PDGF-B gradients and investment of pericytes on vessels (Lindblom et al., 2003; Bjarnegard et al., 2004). Accordingly, non-endothelial expression of PDGF-B in tumors has been reported to have caused pericyte detachment (Abramsson et al., 2003). Up-regulation of FGF-2 and the stimulation of FGF-receptor 1 have been shown to mediate PDGF-B induced SMC proliferation (Millette et al., 2005) and also FGF-2 and VEGF-A have been shown to induce PDGF-B expression and PDGFR-β activation (Kano et al., 2005).


Other angiogenic factors

Angiopoietins (Ang) are a group of growth factors implicated in remodeling and maturation of vasculature and are thus of interest to angiogenesis researchers (Davis et al., 1996; Thurston et al., 1999; Hawighorst et al., 2002). The Ang family consists of Ang-1 and -2 that bind Tie-1 and -2 receptors (Davis et al., 1996; Maisonpierre et al., 1997). However, current knowledge of the angiopoietins is largely controversial and thus the potential of the growth factors in angiogenic gene therapy is unclear. Binding of Ang-1 to Tie-2 is potentially interesting as it has been reported to mediate EC survival and the remodeling and stabilization of vasculature (Davis et al., 1996;

Thurston et al., 1999; Papapetropoulos et al., 2000; Hawighorst et al., 2002). However, Ang-1 has also been reported both to stimulate (Shyu et al., 1998; Suri et al., 1998; Chae et al., 2000) and suppress (Visconti et al., 2002) angiogenesis, and both to promote (Shim et al., 2002) and inhibit (Hawighorst et al., 2002) tumor growth. Ang-2 is most often described as a natural antagonist of Tie-2 leading to endothelial apoptosis and disruption of angiogenesis (Maisonpierre et al., 1997; Lobov et al., 2002). However, in some reports, Ang-2 has also been implicated as a cell type specific Tie-2 agonist (Gale et al., 2002) and in the presence of VEGF-A to stimulate sprouting angiogenesis (Lobov et al., 2002).

One of the most potent growth factors to induce angiogenesis besides the VEGFs is fibroblast growth factor (FGF) -4 (Rissanen et al., 2003a). The FGF family currently consists of 23 members (Yla-Herttuala and Alitalo, 2003) of which FGF-1, -2, -4 and -9 are described the most potent mitogens in vitro (Ornitz et al., 1996). Of these, at least FGF-2 has been reported to up-regulate VEGF-A expression and angiogenesis induced by FGF-2 can be inhibited by VEGFR-2 antagonism (Stavri et al., 1995; Tille et al., 2001). The FGFs bind five distinct receptors, namely FGFR-1-5, of which the fifth one is likely a decoy receptor due to a lack of a tyrosine kinase domain (Sleeman et al., 2001; Murakami and Simons, 2008). The stimulation of many cell types including both ECs and SMCs, has been described beneficial for the angiogenic properties of FGFs (Ornitz et al., 1996; Yablonka-Reuveni et al., 1999). The FGFs are involved in embryonic development, tissue regeneration, cell transformation, tumor growth and angiogenesis (Murakami and Simons, 2008).

Other growth factors that have been described to have angiogenic potential are for example hepatocyte growth factors (HGF) and granulocyte macrophage colony stimulating factor (GM-CSF) (Rissanen and Yla-Herttuala, 2007). HGF has been reported to induce angiogenesis at least partially mediated by VEGF-A up-regulation (Dong et al., 2001). Additionally, transcription factors such as hypoxia inducible factor (HIF) -1$\alpha$, and nitric oxide synthases (NOS) have been described to contribute to angiogenesis. HIF-1$\alpha$ is a factor, strongly up-regulated during acute ischemia, that can activate the transcription of several VEGF-A isoforms and has thus been suggested to stimulate more physiological angiogenesis than a gene transfer of a single VEGF-A isoforms (Pajusola et al., 2005). Adenoviral eNOS has been described to induce vasodilatation and to increase perfusion of ischemic tissues (Brevetti et al., 2003) but its therapeutic potential with respect to effects on endothelial proliferation and the stability of the vessels remains unclear (Cooney et al., 2006).

**Gene therapy for vascular growth**

Principles of gene therapy

The current treatments for cardiovascular diseases are based on prevention and revascularization of the blockages in the main conducting arteries (Norgren et al., 2007). It is, however, common that patients have wide-spread changes that propose a challenge for conventional revascularization approaches (Kuukasjarvi et al., 1997). Gene therapy is a promising tool for revascularization through the stimulation of growth of the whole vascular tree, including large conducting arteries, veins and capillary level vessels (Rissanen and Yla-Herttuala, 2007; Yla-Herttuala et al., 2007). The process by which new blood vessels are grown is called therapeutic angiogenesis. In gene therapy a gene construct, encoding a therapeutic protein, is transferred into somatic target cells by a process called transduction (Watson et al., 1992). In the target cells the gene construct causes the expression of the therapeutic protein in the target tissue. If the produced protein is secreted, it can then stimulate, depending on the protein, for example the growth of blood vessels in the surrounding

tissue. The efficacy of gene therapy is dependent on the success of all the following: 1) the delivery of the gene construct to the target tissues and target cells, 2) the efficacy of the gene construct to produce the therapeutic protein, 3) the duration of protein expression, 4) the solubility of the therapeutic protein and 5) the biological potency of the therapeutic protein as with respect to the receptors and intra-cellular signaling needed to mediate the therapeutic effect (Yla-Herttuala and Alitalo, 2003; Rissanen and Yla-Herttuala, 2007).

Gene transfer methods

In its ideal form, the gene therapy product could be administered systemically and the gene construct could find its way to the place where it is needed. Unfortunately, systemic administration usually has a high incidence of side-effects and thus the gene construct needed to be guided to the right destination (Hiltunen et al., 2000). In theory, this can be achieved by targeting the construct, by modifications of vector-receptor interaction or for example by a hypoxia-sensing element that then regulates the binding of the construct so that it can only bind to hypoxic areas (Wickham, 2000). In preliminary studies using targeted constructs, however, transduction efficacies have been decreased by the modifications to the construct (Rissanen and Yla-Herttuala, 2007). Other reasons for low efficacy of intra-vascular gene transfers are the dilution of the construct in the systemic circulation and the fact that the ischemic areas are by definition out of reach of the circulation and thus the construct can possibly only reach some of the hypoxic border areas but not the ischemic core (Rissanen and Yla-Herttuala, 2007). Thus, injections of gene constructs to target tissues are to date the most efficient gene transfer methods (Yla-Herttuala et al., 2007). Injections through the skin to skeletal muscle is the most convenient method used for the stimulation of vascular growth in lower limb ischemia (Yla-Herttuala and Alitalo, 2003; Rissanen and Yla-Herttuala, 2007). Catheter mediated injections to the myocardium can be used in myocardial ischemia (Sylven et al., 2002). Whereas intramuscular injections yield more widespread effects needed in the revascularization of ischemic tissues, targeted, local delivery to the vascular wall can be desirable, for example in the prevention of restenosis or in the stimulation of collateral artery growth. For that purpose special catheters and gene product containing, vessel surrounding capsules (collars) have also been developed to deliver the virus into the vascular wall without losing it to the systemic circulation (Laitinen et al., 1997). In "ex vivo" gene transfers cells from the target tissue are collected, cultivated in cell culture and transduced in the laboratory. The transduced cells are then returned to the target tissue and only they will contain the gene product and secrete the therapeutic protein (Cavazzana-Calvo et al., 2000). However, the "ex vivo" approach is highly laborious and thus the method is likely not suitable for clinical applications requiring immediate action.

Gene transfer vectors

To achieve an efficient delivery of the gene product to the target tissue, a gene delivery vehicle, a vector, is needed (Yla-Herttuala and Alitalo, 2003). Vectors can be divided into viral vectors and non-viral vectors based on their biological background, and the viral vectors can be further divided into integrating and non-integrating vectors depending on their ability to integrate into to the host cell genome. The type of vector used partly determines the efficacy of the gene transfer and how long the gene product will be expressed in the target cells. An ideal vector efficiently delivers the gene product into the target cells and also assists in nuclear transport. Long-term expression is achieved by the integration of the gene product into the host cell genome, where as a short-term expression is a result of an extra-chromosomal placement. Some modified viral vectors can, however, also yield relatively long expression by division in the extra-chromosomal position using

their own reproduction genes (Grieger and Samulski, 2005). Long gene expression can be useful in cases where the depletion of a protein is treated with a gene product producing a replacement protein such as in X-scid (Cavazzana-Calvo et al., 2000). However, a long-term expression can also be harmful, for example in the case of stimulation of vascular growth that could possibly contribute to tumor growth or macular degeneration (Yla-Herttuala and Alitalo, 2003; Ferrara, 2004). Integration of the gene product into the host genome can also result in insertional mutagenesis and activation of malignancies (Hacein-Bey-Abina et al., 2003).

**Table 1.** Characteristics of the most common gene transfer vectors

| Vector | Advantages | Disadvantages |
|---|---|---|
| **Non-viral vectors** (plasmids, oligonucleotides and their modified complexes) | Safe<br>Easy to produce | Poor efficacy<br>Very short expression |
| **Adenoviruses** | High transduction efficacy<br>Easy to produce<br>High titers<br>Transduces both proliferative and quiescent cells<br>Does not integrate to genome | Transient expression (~two weeks)<br>Inflammatory reactions<br>Immunity towards the virus (repeated administration requires another serotype) |
| **Adeno-associated viruses** (AAV) | Long-term expression<br>Tropism towards specific tissues (skeletal muscle, myocardium) | Difficult to produce in high titers<br>Expression duration currently unknown<br>Safety data conflicting |
| **Retroviruses** | Stable gene expression | Non-specific integration to the genome - possibility for induction of malignancies<br>Transduces only dividing cells<br>Low efficacy<br>Difficult to produce in high titers |
| **Lentiviruses** | Stable gene expression<br>Transduces both proliferative and quiescent cells<br>High DNA-capacity | Non-specific integration to the genome<br>Difficult to produce in high titers<br>Poor efficacy in other than hematopoietic cells |

Modified from Rissanen et Ylä-Herttuala 2007.

Modified adenoviruses are the most frequently used and most effective vectors to date (http://www.wiley.co.uk/genetherapy/clinical/). They have a high transduction efficacy and are relatively easy to produce even in high concentrations (Kovesdi et al., 1997; Raty et al., 2008). Adenoviruses are DNA-viruses that can carry up to 30 kb of DNA (gutless vectors) and can transfect both dividing and non-dividing cells (Kovesdi et al., 1997). Gene expression length after adenoviral transduction is about 1-2 weeks (Muruve, 2004; Korpisalo P et al., 2007). The gene construct remains non-dividing in an extra-chromosomal position and is destroyed among the cell

cycle and by the immune system. Accordingly, the greatest weakness of adenoviruses as vectors is related to the immune response they elicit (Muruve, 2004). In nature they cause respiratory infections and thus many people might already have antibodies against them. Formation of antibodies destroys the virus immediately and that is why a repeated administration of the virus using a same serotype does not lead to sufficient transduction (Muruve, 2004; Korpisalo P et al., 2007). Adenoviruses enter the cells using coxsackie-adenovirus receptors (CAR) and integrin receptors whose expressions partly determine the transduction efficacy of the virus (Wickham et al., 1993; Bergelson et al., 1997). CARs are highly expressed in the respiratory epithelial cells, cardiomyocytes and hepatocytes, whereas in skeletal muscle the amount of CAR is limited in other than regenerating myofibers (Bergelson et al., 1997). Modification of the capsid proteins of adenoviruses might improve its transduction efficacy in skeletal muscle but the results remain to be seen (Wickham, 2000). Adjusting the transcription efficacy by special tissue specific promoters has also been suggested (Fadel et al., 1998; Hiltunen et al., 2000; Wickham, 2000; Laurema et al., 2003). The characteristics of adenoviruses in comparison to other viral and non-viral vectors are described in Table 1.


Preclinical experiments

The purpose of preclinical experiments is to find out the safest and most efficient gene products for clinical testing. Both *in vitro* and *in vivo* tests have an important role in preclinical testing of gene therapy. *In vitro* cell culture tests allow the investigation of mechanism of action and verification of gene product functionality without confounding effects of e.g. the immune system or injection trauma. However, the cell culture experiments cannot at least yet mimic the complexity of organ systems and thus the *in vivo* animal experiments are crucial.

The existence of factors inducing vascular growth (tumor angiogenesis factor) was identified already in 1939 when the relationship of vascular growth and tumors was studied (Ide et al., 1939). In the 1970s it was discovered that factors inducing endothelial proliferation could also be used to grow new blood vessels in a therapeutic way (Folkman, 1971; Svet-Moldavsky and Chimishkyan, 1977). For limb ischemia gene transfers (GTs) were first tested in 1992 when bFGF recombinant protein was injected into a rabbit hind limb after an ischemia operation (Baffour et al., 1992). Ischemia operations have since been an important part of preclinical tests and models have been developed for all small rodents. The use of small rodents is more cost effective than the use of rabbits for example, but the results are compromised by the small muscle mass and the difficulty of the operations due to extremely small proportions (Rissanen and Yla-Herttuala, 2007). Importantly, also the well-being of the animals has been considered and the models have been developed into less traumatic (Rissanen et al., 2003a). Unfortunately, findings from only ischemic tissues may have some over-interpretation as acutely ischemic tissues yield very high levels of endogenously up-regulated growth factors (Rissanen et al., 2002). Thus, the studies should be performed also in normoxic muscles to be able to separate the effects of endo- and exogenous factors (Rissanen and Yla-Herttuala, 2007). Many other models besides the hind limb ischemia model have also been used to study angiogenesis, to mention few: cardiac models (Rutanen et al., 2004), a mouse ear model (Frank et al., 1994), a tracheal model (Baluk et al., 2005), zebra fish and tadpole models (Ny et al., 2006) and a normoxic hind limb model (Rissanen et al., 2003b). These models do not necessarily give information needed for the treatment of an ischemic leg of a patient but they do provide important information on the mechanisms of vascular growth and the structure of the formed vascular systems with fewer confounding factors and less harm to the animals as compared to the ischemia models. In addition to the acute ischemia models, the induction of chronic ischemia has been pursued in order to better resemble human chronic ischemia with less endogenous growth

factor stimulation (Baffour et al., 2000). However, these models suffer from high variation. The use of genetic models is also an important achievement to make animal models better mimic the human disease. Diabetes and hypercholesterolemia, factors often associated with human lower limb ischemia, have for example been induced in animals (Roy et al., 2006; Heinonen et al., 2007).

To date several genes have been tested for their therapeutic potential for vascular growth in animal models. The most investigated and also the most potent angiogenic growth factors have shown to be VEGFs and FGFs. They have also been the only ones reported to have induced efficient capillary enlargement (Pettersson et al., 2000; Rissanen et al., 2003a; Rissanen et al., 2003b; Ozawa et al., 2004). In contrast, sprouting angiogenesis has been described more often and in relation to several different growth factors such as PDGFs (Cao et al., 2002; Li et al., 2003), Ang-1 (Shyu et al., 1998), HGFs (Toyoda et al., 2001) and insulin-like growth factors (Su et al., 2003). Interestingly, HGF, IGF-1, PDGF-B, PlGF and FGFs have all been shown to at least partially mediate their angiogenic effects via VEGF-A up-regulation (Ferrara, 2004; Yla-Herttuala et al., 2007). Tissue edema, a side-effect of efficient angiogenic gene therapy and also a marker of efficient vascular growth, has been described after VEGFs and FGFs (Pettersson et al., 2000; Rissanen et al., 2003a; Rissanen et al., 2003b; Rutanen et al., 2004). There are also reports of presumably efficient angiogenesis without edema formation (Kiba et al., 2003; Pajusola et al., 2005; Zheng et al., 2006) but these results might be compromised by the fact that in small rodents the detection of moderate edema is very difficult. Recently, combination GTs with VEGFs or FGFs and PDGFs or angiopoietins have become an interesting target to reduce edema associated with angiogenic gene therapy and to improve the maturity and stability of induced vessels (Richardson et al., 2001; Hao et al., 2007). The unsolved issues regarding angiogenic gene therapy in the preclinical experiments still are: 1) Stability of vessels - chronic ischemia models have been difficult to develop and thus the results from long-term time points are often inconclusive. 2) Is there a need for long-term expressing vectors or is a short up-regulation of growth factors enough to induce the growth of functional vessels? 3) Are the induced vessels metabolically active? 4) How to avoid the formation of edema? 5) Are gene combinations more potent and more physiological than an administration of a single growth factor?

Clinical trials

The first clinical attempt for the treatment of lower limb ischemia with gene therapy was performed in 1996 when an intravascular plasmid (p)VEGF-A[165] GT was reported to induce an improved blood flow to an ischemic area (Isner et al., 1996). Edema was then detected as a side-effect of the therapy (Isner et al., 1996). Promising angiographic results, improved ABI, improved blood flow and healing of ischemic wounds were also found in phase I studies in 1998 after pVEGF-A[165] GT (Baumgartner et al., 1998; Isner et al., 1998; Baumgartner et al., 2000). However, none of these studies had placebo controls and thus the results remained inconclusive. Trials with adenoviruses also yielded initially promising results with the therapy being well tolerated and patients only showing slight fever (Makinen et al., 2002; Rajagopalan et al., 2002). Overall, angiogenic gene therapy has been well tolerated and safe in clinical trials with no evidence of malignancies related to the gene product (Yla-Herttuala and Alitalo, 2003). However, looking beyond lower limb ischemia trials one death has been associated with a high dose of adenovirus in an immunoincompetent patient (Lehrman, 1999). In the past few years data from the randomized, controlled, phase II/III trials have emerged. Most limb ischemia trials with naked plasmid failed to reach their goals (Rissanen and Yla-Herttuala, 2007), except the TRAFFIC-study that reported an increase in peak walking time in patients that received intra-arterially administered FGF-2 as compared to placebo controls (Lederman et al., 2002). Most studies with adenoviruses have also been negative (Rissanen

and Yla-Herttuala, 2007) and only VEGF-A[165] has been reported efficient (Makinen et al., 2002). In the treatment of myocardial ischemia, phase II/III studies have yielded slightly more positive results but only with AdVEGF[165] (Hedman et al., 2003), pVEGF-A[165] (Kastrup et al., 2005), AdVEGF[121] (Stewart et al., 2006) and AdFGF-4 (Grines et al., 2003). Low efficacy of the gene constructs for angiogenesis, too short expression of the gene product to result in efficient vascular growth, low tissue concentrations of the gene products, inefficient gene delivery routes, poorly designed time points with respect to the duration of gene expression and highly subjective end-points such as treadmill tests and the evaluation of pain relief have been suggested among the reasons for the vast failures in the randomized controlled trials (Rissanen and Yla-Herttuala, 2007).


**Imaging vascular growth**

As the effects of angiogenic gene therapy take place mostly at the capillary level vessels, the imaging of capillary level circulation is crucial to demonstrate the efficacy of the treatment. None of the current noninvasive perfusion measurement techniques, such as dynamic contrast enhanced steady-state magnetic resonance imaging (DCE-MRI), x-ray computed tomography (CT), single photon emission computed tomography (SPECT), laser Doppler, and power Doppler based ultrasound techniques, can provide simultaneous capillary level imaging at a high frame-rate, good penetration, efficient contrast agent separation from background tissue, and a contrast agent that is retained in leaky angiogenic vessels. DCE-MRI offers relatively good sensitivity and spatial resolution in vascular imaging, the possibility to perform whole organ scans, as well as functional imaging (Miller et al., 2005). However, in DCE-MRI perfusion imaging, much of the spatial resolution must be sacrificed to get a sufficient frame rate for bolus tracking. Moreover, MRI devices are not widely accessible to experimental researchers. Although highly sensitive, SPECT suffers from poor spatial resolution (Miller et al., 2005). Micro-CT is suitable for very accurate (up to 5 μm) postmortem anatomical vascular imaging but the protocols for dynamic studies in vivo are less well established, in addition to well known drawbacks of ionizing radiation and the low sensitivity of CT to contrast agents. Laser Doppler and optical imaging methods suffer from low resolution and poor tissue penetration (Miller et al., 2005).

Contrast enhanced ultrasound (CEU) is increasingly used for perfusion measurement in various tissues including human skeletal muscle (Krix et al., 2005). One of the most interesting, novel aspects of ultrasound imaging is the use of microbubble contrast agents (large, >1 μm microbubbles) that not only remain in circulation despite vessel leakiness (Orden et al., 2003; Miller et al., 2005) but can also have antibodies attached to their surface, allowing the possibility of imaging at molecular level (Ellegala et al., 2003). Previously it has been shown that the backscatter signal from microbubbles correlates in a linear manner with the concentration of bubbles *in silico* and *in vitro* as well with blood flow *in vivo* according to the classical tracer-dilution theory (Cosgrove et al., 2001; Galbraith et al., 2002; Thijssen and de Korte, 2005). Contrast enhanced ultrasound (CEU) with Power Doppler has been validated in skeletal muscle perfusion measurement both in animals and humans (Arsic et al., 2003; Krix et al., 2005). However, power Doppler based ultrasound techniques, although they have good penetration and high frame rates, lack in their ability to separate contrast agent and tissue signals from each other and are also highly affected by movement artifacts. Contrast pulse sequencing (CPS) is a recently introduced, highly promising contrast-enhanced ultrasound imaging technology that uses nonlinear fundamental frequencies, resulting in improved spatial resolution and higher sensitivity to microbubble contrast media (Phillips and Gardner, 2004). Furthermore, CPS provides better tissue penetration, less attenuation, and more improved tissue subtraction than previous ultrasound perfusion imaging techniques (Phillips and Gardner, 2004). Importantly, CPS is technically less demanding, and the imaging

protocol is much quicker than, for example, that of DCE-MRI. The properties of the most common imaging techniques are summarized in Table 2.

**Table 2.** Comparison of the most typical imaging methods for imaging of the vasculature

|  | Spatial resolution | Advantages | Disadvantages |
|---|---|---|---|
| **DCE-MRI** | ~100 μm | Contrast agent sensitivity, whole body scans possible, functional imaging possible. | Expensive, poor availability, post processing needed and technically demanding. |
| **Micro-CT** | 10-100 μm | Good resolution, whole body scans possible. | Low contrast sensitivity, radiation exposure, post mortem imaging, highly time consuming, poor availability and expensive. |
| **SPECT** | 5-6 mm | Whole body imaging and molecular imaging possible. | Expensive, radionuclides needed, laborious, poor availability, poor resolution, post processing needed. |
| **Laser Doppler** | ~1 cm | Inexpensive, quick and easily available. | Poor penetration (only ~1 mm) and resolution. |
| **Power Doppler ultrasound** | 0.5 mm | Inexpensive, quick and easily available, real-time imaging with high frame rates. | Susceptible to movement artifacts, penetration limited to a few centimeters, limited sensitivity. |
| **CPS ultrasound** | 20-100 μm | Quick, real-time imaging with high frame rates, good tissue-contrast agent separation, high sensitivity. | Contrast agent needed, still quite poorly available, penetration limited to a few centimeters. |

Modified from Miller et al 2005. DCE-MRI = dynamic contrast enhanced-magnetic resonance imaging, CT = computed tomography, SPECT = single photon emission computed tomography, CPS = contrast pulse sequence.

AIMS OF THE STUDY

The aim of this thesis was to study angiogenic gene therapy as a potential treatment for lower limb ischemia in animal models, as well as to elucidate factors involved in the growth of functional vessels and to compare methods suitable for imaging angiogenic vessels.

The specific aims of this work were (according to each sub-study):

I       To evaluate the arteriogenic potential of AdVEGF-A after an intra-muscular GT.

II      To test the feasibility of CPS ultrasound imaging in the assessment of angiogenesis.

III     To verify the functionality of vessels grown using gene therapy by studying the energetic reserve and exercise tolerance of ischemic muscles before and after AdPlGF GT.

IV      To study the potential of AdPDGF-B GT in the stabilization of vessels induced by a GT of AdVEGF-A.

V       To examine the effects of different AdVEGF-A doses on the induction of sprouting angiogenesis, capillary enlargement and formation of tissue edema, and to study the safety of efficient angiogenic gene therapy.

VI      To identify factors involved in the formation of endogenous collateral vessels in patients with peripheral arterial disease and chronic limb ischemia.

MATERIALS AND METHODS

**Ischemia operations**

Two models of acute ischemia induced by a surgical operation have been used in this thesis work (Figure 7). Despite their limitations, surgically induced ischemia models are feasible approaches to study therapeutic angiogenesis as means for revascularization of ischemic tissues (Rissanen et al., 2003a). Ligation of arteria femoralis profunda (PFA) was used in studies I and IV, and the ligation of arteria femoralis superficialis (SFA) (Rissanen et al., 2003a) in study III. The ligation of SFA was carried out according to previous description (Rissanen et al., 2003a) in the right hind limbs of New Zealand White rabbits a day before GT. Briefly, SFA and the re-entry branches for the collaterals growing from the lateral circumflex and deep femoral arteries were ligated under ketamine (Ketalar, Pfizer 0.3 ml/kg) and medetomidine (Domitor, Orion, 0.3 ml/kg) anesthesia. In this model the calf region is ischemic while the thigh remains normoperfused. The ligation of PFA was carried out under similar conditions but no re-entry branches for collaterals were closed. The GT was also performed immediately after the operation. In the PFA ligation model, the ischemia is much milder than that induced by the ligation of SFA, and is restricted to the adductor muscles making the model less painful for the animals. All animal experiments were approved by the Experimental Animal Committee at the University of Kuopio.
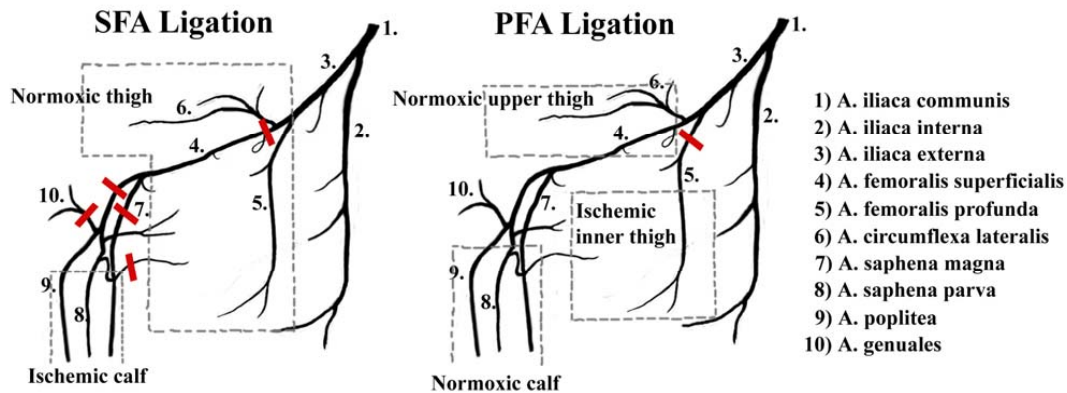


**Figure 7.** Ischemia models used in the studies.

**Gene Transfer**

GT was performed to New Zealand White rabbits (studies I-V) or 57Bl/6Ja mice (study II) either intra-muscularly (i.m., studies I-V) or intra-arterially (i.a., study V) under ketamine-medetomidine anesthesia in rabbits and ketamine (1,0mg/kg) -medetomidine (75mg/kg) anesthesia in mice. Human clinical grade, first generation, serotype 5, replication-deficient adenoviruses analyzed to be free from contaminants were used with viral doses ranging from $1x10^9$ to $5x10^{11}$ vp/ml. Adenoviral human VEGF-A[165], mouse PlGF-2 and human PDGF-B under the cytomegalovirus (CMV) promoter were used in different studies as described in Table 3. Adenoviral β-galactosidase marker gene (LacZ) with the same promoter was used as a control in all studies. In rabbits 0.1 ml intra-muscular injections were performed using a 1 ml syringe and a 25 G needle (studies I-V), and in mice 50 μl injections with a 50 μl Hamilton syringe and a 30 G needle (study II), respectively. In

rabbits, semimembranosus (10 injections), abductor cruris cranialis (8 inj.), quadriceps femoris (5 inj.), tibialis anterior (3 inj.) or gastrocnemius (6-8 inj.) was injected as described in Table 3. In mice, one injection was performed in the inner thigh and one in the posterior calf (study II). In the intra-arterial GTs 1.0 ml of $5x10^{11}$ vp/ml AdLacZ or AdVEGF-A was injected through a butterfly needle selectively into the profound femoral artery while all arterial circulation into and from the transduced limb was closed. The virus was left to incubate in the circulation for 15 min after which the artery was flushed with 20 ml of NaCl and 15 ml of the lower limb venous return was drained via a 22 G cannula. After the operation, all vascular access points were sutured with 10.0 sutures restoring normal circulation. The effects of the GTs were studied six, 14 or 28 days after the GT as described in Table 3.

**Table 3.** Summary of animal studies.

| Study no | Animal species | Ischemia operation | Gene transfers | Total doses | Transduced muscles | Time points |
|---|---|---|---|---|---|---|
| I | Rabbit | PFA ligation | AdLacZ AdVEGF-A | $10^{11}$ vp | Semimembranosus | d6, d14 |
| II | Rabbit, mouse | - | AdLacZ AdVEGF-A | $10^{11}$ vp | Semimembranosus | d6 |
| III | Rabbit | SFA ligation | AdLacZ AdPlGF | $3x10^{11}$ vp | Semimembranosus, cruris cranialis, quadriceps femoris, tibialis anterior, gastrocnemius | d6, d14, d28 |
| IV | Rabbit | PFA ligation | AdLacZ AdVEGF-A AdPDGF-B AdV-A+ AdL-Z AdV-A+ AdP-B | $10^{11}$ vp, $2x10^{11}$ vp for combi-nations | Semimembranosus | d6, d14, d28 |
| V | Rabbit | - | AdLacZ AdVEGF-A | $1.6x10^{11}$ vp | Semimembranosus, gastrocnemius | d6, d14 |

**Assessment of skeletal muscle perfusion and blood volume**

Ultrasound imaging

Three different ultrasound approaches have been used to assess skeletal muscle perfusion in the studies: Native Doppler (Studies I-V), Contrast enhanced Doppler (CEU, studies I and IV) and Contrast Pulse Sequence ultrasound (CPS, studies II, III and V). All ultrasound imaging was performed with Acuson Sequoia 512 system and 15L8 transducer (Siemens). For CEU and CPS applications a second generation contrast agent (sulfur hexafluoride in a phospholipid shell, approx. $2x10^8$ bubbles/ml, mean diameter 2.5 μm, SonoVue, Bracco) was used. Native Doppler imaging was performed without contrast administration in both rabbits and in mice with the following parameters: 12.5 frames/s, power Doppler at 14 MHz, dynamic range 20 dB, power -5 dB, gain 50 and depth 20 mm. For CEU in rabbits transversal plane video clips of 20 sec were captured using the power Doppler (mechanical index=0.6) mode at 8.5 MHz (dynamic range 10 dB, power -18 dB, mechanical index 0.60, gain 40 and depth 20 mm) with the administration of a 0.3 ml bolus of

SonoVue via the ear vein. CPS imaging was performed in rabbits after an i.v. bolus injection of 0.5 ml SonoVue with the following parameters at 8.0 MHz: power -16 dB, mechanical index 0.31, CPS gain -10 and depth 20 mm. In mice, 50 µl of SonoVue was injected as a bolus with a Hamilton syringe and a 25 G needle via a silastic tube (diameter 1 mm) placed in the right external jugular vein under anesthesia. The following parameters were used for CPS imaging in mice: frequency 14 MHz, power -8 dB, mechanical index 0.25, CPS gain -15 and depth 20 mm. Signal intensities (dB) of the ultrasound video clips were quantified with Datapro (v2.13, Noesis) and signal intensity-time curves were generated. Results are reported as ratios to intact values.

Magnetic resonance imaging

Magnetic resonance imaging (MRI) of rabbit thighs (studies I and II) was carried out in a 4.7 T magnet (Magnex, Abington, United Kingdom) interfaced to a Varian $^{UNITY}$INOVA (Varian, Palo Alto, California) console with actively shielded gradients and with an in-house-built surface coil (diameter 38 mm). Dynamic contrast-enhanced (DCE)-MRI of perfusion with a FLASH pulse sequence (repetition time 9 ms, echo time 5 ms, field of view 6 x 6 cm$^2$, resolution 64 x 64, slice thickness 5 mm, number of averages 1, acquisition time 0.58 s/image) was done after an i.v. 0.7 ml bolus injection of superparamagnetic iron oxide particles (Resovist, Schering; mean size 62 nm). The signal intensity–time curves were derived from the DCE-MRI data, and perfusion ratios were calculated. To obtain high-quality images of blood volume, T2*- weighted gradient echo images (repetition time 2 s, echo time 18 ms, field of view 6 x 6 cm$^2$, resolution 256 x 128, slice thickness 2.5 mm, and number of averages 2) were acquired before and 6 min after the bolus contrast agent injection to determine the steady-state distribution of the i.v. contrast agent. Finally, blood volume-MRI maps ($\Delta$R2*) were reconstructed.

Microspheres

Microsphere perfusion index between transduced semimembranosus muscles was measured at rest (study I) with red fluorescent microspheres (2x10$^6$, 15µm in diameter, FluoSpheres, Molecular Probes) injected into the left ventricle just before sacrifice (Pettersson et al., 2000; Rissanen et al., 2003a; Krix et al., 2005). After sacrifice, microspheres were extracted from muscle samples with a sedimentation method according to the manufacturer's instructions. The microsphere perfusion index was calculated as the ratio of red fluorescence between the transduced and contralateral intact semimembranosus muscles. Yellow-green microspheres were used as internal controls for pipeting errors.

**Assessment of skeletal muscle vascularity and structure of vessels**

Digital subtraction angiography

To demonstrate collateral artery growth in ischemia operated rabbit hind limbs and the extravasation of X-ray contrast medium from growing VEGF-induced vessels (study I), selective digital subtraction angiography (DSA) was performed with a 4 F right coronary artery catheter (Cordis) introduced into the common carotid artery using a power-injection of 6 ml contrast medium (320 mg iodine/ml, Omnipaque, Amersham). Serial DSA images were recorded at the rate

of two images/s for 5 s (Siremobil 2000, Siemens), and the image representing the best arterial filling was chosen for analysis.

Histological analysis

Mean capillary area ($\mu m^2$) was measured, at 200x magnification, from CD31 immunostained sections obtained from areas covered entirely by skeletal muscle tissue (studies I, IV and V) (Rissanen et al., 2003b). All measurements were performed in a blinded manner from 10 fields representing maximal angiogenic effects of each muscle section using AnalySIS software (Soft Imaging System). To avoid ambiguous data caused from trauma effects of the needle injection, the analysis was made outside the needle track area. Means of the measurements were reported. Total area of arteries and veins (% of the total muscle area) was quantified from α-sma stained sections at 40x magnification covering the entire muscle (studies I, IV, V).

The percentage of α-sma positive pericytes, SMCs and myofibroblasts (% of the skeletal muscle area) were measured by immunofluorescence (FITC) of α-sma-stained sections at 200x magnification (study IV). All measurements were performed, using AnalySIS software (Soft Imaging System), in a blinded manner from 5 fields that represented maximal α-sma immunofluorescence of each muscle section. Measurements were taken from areas that did not contain large arteries or veins since their SMC layer could affect the results.

**Analysis of muscle energy metabolism**

$^{31}$phosphorus -magnetic resonance spectroscopy (study III)

The degree of ischemia and the level of aerobic metabolism (aerobic capacity) of rabbit calf muscles before the ischemia operation, and 6 and 28 days after GT were evaluated using $^{31}$phosphorus -magnetic resonance spectroscopy ($^{31}$P-MRS). $^{31}$P-MRS was performed with a $^{UNITY}$INOVA imaging console (Varian) interfaced to a 4.7 T horizontal magnet (Magnex Scientific) with actively shielded gradients (Magnex Scientific). In-house built linear surface RF-coil consisting of two separate loops tuned to $^1$H and $^{31}$P frequencies with diameters of 38mm and 25mm, respectively, were used for reception. Region of interest under the surface coil was shimmed using $^1$H signal and $^{31}$P data were acquired after ~70 degree hard pulse excitation using a repetition time of 1.5 s, spectral bandwidth of 10 kHz covered by 4096 data points and a number of averages of 16. Small needle electrodes (27 G) were placed on both sides of the sciatic nerve on the lateral side of the rabbit thigh. During the whole duration of the experiment (20 min) $^{31}$P-MRS spectra were collected each taking 30 s. First, resting spectra were collected for 2 min. Thereafter, the sciatic nerve was electrically stimulated for 6 min at 3 Hz using a voltage of 75-125 V (14A11 Electromyograph, Disa) to induce maximal contractions in the calf muscles. Then, the stimulation was ceased and muscle recovery was followed for 12 min. JMRui 2.1 was used for line shape fitting analyses of the spectra after pre-processing the data by discarding the two first data points, using a DC correction of 500 and 15 Hz line broadening. The ratio between the peak areas of phosphocreatine (PCr) and the sum of PCr and inorganic phosphate (Pi) i.e. PCr/(PCr+Pi) was calculated and used as a measure of aerobic capacity and fatigue. Additionally, pH was derived from the chemical shift (placement on the peak on the x-axis) difference between PCr and Pi peaks.

Arterial acid-base analysis (study V)

For systemic acid-base balance and blood gas analysis 1 ml of arterial blood was collected into heparinized 1 ml syringes before and immediately after electrically stimulated exercise before, 6 and 14 days after GT. The samples were immediately analyzed using an ABL625 analyzer (Radiometer Copenhagen) and blood gas concentrations, electrolyte values and metabolite values were recorded.

**Tissue edema and vascular permeability**

The Modified Miles assay was used in rabbits for the evaluation of tissue edema at sacrifice (studies I, III-V). Evans Blue dye (30 mg/kg, Sigma) was injected i.v. 30 min before sacrifice. After sacrifice, the animals were perfusion-fixed with 1 l of 1 % paraformaldehyde (PFA) in 0.05 M citrate buffer (pH 3.5) via the left ventricle. Extravasated Evans Blue dye bound to plasma was extracted from transduced and contralateral intact muscle samples by incubation in formamide at 60 °C for 48 h. The amount of extravasated Evans Blue dye was determined on the basis of absorbance at 610 nm (Rissanen et al., 2003b). The results are represented as absorbance ratios between the transduced and contralateral intact muscles. The absorbances were normalized to the weight of the muscle sample.

**Immunohistological and histological stainings**

After sacrifice, the animals were perfusion-fixed with 1 % paraformaldehyde in 0.05 M citrate buffer (pH 3.5) via the left ventricle. Sample tissues were then immersion-fixed in 4 % paraformaldehyde/ 15 % sucrose (pH 7.4) for 4 h, immersed in 15 % sucrose (pH 7.4) overnight (Rissanen et al., 2003b). Confocal microscopy samples (studies IV and V) were then frozen on dry side embedded in Tissuetech embedding medium. Samples for light microscopy (studies I-V) were embedded in paraffin. Samples for histochemical alkaline phosphatase (AP) and dipeptidyl peptidase IV (DPP) stainings (study I) were collected from non-fixed animals and the samples were immediately frozen in isopentane cooled down with liquid nitrogen.

Avidin-biotin-HRP and alkaline-phosphatase systems (Vector Laboratories) with 3'-5'-diaminobenzidine (DAB, Zymed) and Vector Blue (Vector) color substrates were used, respectively, for immunohistochemistry on 7 μm thick light microscopy sections. The range of antibodies used in immunostainings is described in Table 4. To visualize vessels that are perfused, Rhodamine-labeled Ricinus Communis lectin (1 mg in 10 ml of saline, Cat.# RL-1082, Vector) was injected into the common femoral artery using a 4 F catheter just before sacrifice in a subset of animals (study I, IV and V). Proliferation marker BrdU (20 mg/kg, Sigma) was given 3 h before sacrifice to detect proliferation (study I). Intra-arterially injected Rhodamine-labeled Ricinus Communis lectin and FITC-conjugates were used for immunocytochemistry on 50 μm thick confocal sections. Controls for immunostainings included incubations with irrelevant class- and species-matched immunoglobulins and incubations in which the primary antibody was omitted (studies I-V).

Alkaline phosphatase (AP) and di-peptityl-peptidase (DPP) activity on blood vessels was demonstrated by the azo-coupling method on frozen sections (study I) (Langille and O'Donnell, 1986; Hansen-Smith et al., 1992; Saltin et al., 1998; Giatromanolaki et al., 2001; Giatromanolaki et al., 2002). Briefly, for the AP staining the sections were incubated in medium containing 10 mg of

Naphthol AS-BI phosphate (disodium salt, Sigma) dissolved in 0.5 ml of N,N-dimethylformamide (Aldrich) and 10 mg of Fast Blue BB Salt (Fluka) dissolved in 10 ml of veronal-acetate buffer (pH 9.2) for 90 min. For the DPP staining the sections were incubated in Gly-Pro-4-β- naphthylamide (Cat no G-9262, Sigma) at room temperature under microscopic control for 90 minutes.

Photographs of the 7 μm thick histological sections were taken with an Olympus AX70 microscope (Olympus Optical) and analySIS software (Soft Imaging System) (studies I-V). Fluorescent images were taken using an Olympus U-RFL-T burner (study I). Confocal images of the 50 μm thick sections were taken with an Olympus IX81 microscope and a Fluoview-1000 confocal setup (studies IV and V). Reconstructions of the confocal images were done with an open source software package, BioImageXD. Images were further processed for publication with Adobe Photoshop 7.0 (Adobe) (studies I-V).

**Table 4.** Antibodies used in immunohistology.

| Antibody | Specificity | Clone/ Code | Manufacturer | Dilution used | Pretreatment | Used in studies |
|---|---|---|---|---|---|---|
| CD31 | Endothelium | JC/70A | Dako | 1:50 | - | I - V |
| Griffonia simplifolia lectin I | Endothelium | B-1105 | Vector | 1:100 | - | II |
| α-sma | Pericytes, smooth muscle | 1A4 | Sigma | 1:250 | - | I, IV |
| VEGF | VEGF$_{121-206}$ | c-1/ Sc-7269 | Santa Cruz | 1:500 | citrate boiling | I, IV, V |
| PDGF-B | PDGF-B | AF-220-NA | R&D | 1:500 | citrate boiling | IV |
| VEGFR-1 | VEGFR-1 | Sc-316 | Santa Cruz | 1:250 | - | IV |
| VEGFR-2 | VEGFR-2 | MFLK1 | RDI | 1:250 | citrate boiling | IV |
| PDGFR-β | PDGFR-β | Sc-6252 | Santa Cruz | 1:200 | - | IV |
| β-Gal | β-galactosidase | Z3783 | Promega | 1:500 | - | I, V |
| BrdU | Proliferating cells | Bu20a | Dako | 1:100 | BrdU injected | I |
| Ki67 | Proliferating cells | MIB-1 | Dako | 1:100 | citrate boiling | IV |
| RAM11 | Macrophages | M0633 | Dako | 1:200 | - | IV |
| RAM11 | Macrophages | M0633 | Dako | 1:200 | trypsin | V |
| HAM56 | Macrophages, monocytes | M0632 | Dako | 1:500 | trypsin | IV |
| Desmin | | D33 | Sigma | 1:100 | - | IV |
| Vimentin | | V9 | Sigma | 1:50 | - | IV |

**Transgene expression**

mRNA expression analysis (study V)

The localization of VEGF-A mRNA was shown by non-radioactive in situ hybridization (Roche Diagnostics Co., Mannheim, Germany) from paraffin sections using a digoxigenin- (DIG) labeled antisense and sense probes for VEGF-A according to previous description (Ylä-Herttuala et al., 1991; Karvinen et al., 2009). Paraffin sections were pretreated with Proteinase K (Roche Diagnostics Co., Mannheim, Germany) (10 µg/ml). Hybridization of slides was carried out in a hybridization buffer (40 % deionized formamide, 10 % dextran sulfate, 1 × Denhardt's solution, 4 × saline sodium citrate buffer (SSC), 10 mM ditiothreitol, 1 mg/ml yeast tRNA, 1 mg/ml denatured ssDNA) containing antisense or sense probes with the concentration of 0.33 ng/µl overnight at 58 °C. After hybridization the slides were washed in a shaking water bath at 58 °C with decreasing SCC concentration. Immunological detection of DIG-labeled probes was done according to the manufacturer's protocol (Roche Diagnostics Co., Mannheim, Germany) using anti-DIG-alkaline phosphatase Fab fragments (Roche Diagnostics Co., Mannheim, Germany). A color reaction was detected with Nitro blue tetrazolium chloride/ 5-Bromo-4-chloro-3-indolyl phosphate, toluidine salt (NTB/BCIP) solution (Roche Diagnostics Co., Mannheim, Germany).

Protein expression analysis

Muscle samples taken at sacrifice were frozen in liquid nitrogen and stored at -70 °C. T-Per buffer (Thermo Scientific) with 1x Halt protease inhibitor (Thermo Scientific) was used for protein extraction from homogenized muscle samples. The amount of protein in each sample was quantified with hVEGF-A (studies IV-V) and hPDGF-B (study IV) enzyme-linked-immuno-sorbent-assay kits (R&D Systems) and further normalized to the amount of total protein in each protein extract. The amount of total protein in each sample was quantified with BCA protein assay kit (Thermo Scientific).

**Assessment of gene therapy related side-effects**

Inflammatory cells

Inflammatory responses induced by different adenoviral doses (study V) were measured from trypsin treated and RAM11 immunostained sections of semimembranosus muscles at 100x magnification. All measurements were performed in a blinded manner from 5 fields representing maximal staining of each muscle section using analySIS software (Soft Imaging System). To avoid confounding effect of the needle injection trauma the analyses were made outside the needle track area. Means of the measurements are reported.

Clinical chemistry

Arterial blood samples were collected from a subgroup of animals (study V) to 9 ml Z Serum Clot Activator Vacuette tubes (Greiner Bio-One) before, 6 and 14 days after GT. Serum was extracted from the samples and frozen to -70 °C until analysis. Clinical chemistry including analysis of

kreatinin, inorganic phosphate, alanine aminotransferase, aspartate aminotransferase, amylase, C-reactive protein, haptoglobin and alkaline phosphatase was performed in the Eastern-Finland laboratory centre (ISLab) in Kuopio University Hospital.

Electrocardiography

Electrocardiograms were registered using a 12 -lead electrocardiograph (Sicard 460, Siemens) in a subgroup of rabbits (study V) before, and 6 and 14 days after GT. Each electrocardiogram was analyzed systematically for heart rate, conduction, configuration, duration and amplitudes of different components.

**Analysis of collateral growth in CLI patients (study VI)**

Patient selection

Preoperative angiograms and medical records for all patients (N=96) scheduled for femoropopliteal or femorodistal revascularization at the department of Vascular Surgery, Kuopio University Hospital, between January 2004 and December 2005 were retrospectively reviewed. Patients were included in the study when indication for surgery was claudication or chronic critical leg ischemia, due to occlusion of the superficial femoral artery (SFA) with open popliteal artery and at least one patent calf artery. Patients with a significant aortic stenosis or those who had previously been treated with ipsilateral surgical bypass reconstruction were excluded. Finally, 70 patients formed the study population. Demographic data, risk factors and co-morbidities were studied and recorded. The information on the disease onset, stage, duration of symptoms, functional capacity, claudication distance and ankle/brachial indexes (ABI) were registered.

Angiographic findings

A systematic quantification and qualification of collateral vessels bypassing the occluded SFA was performed. A re-elaboration of the angiograms was performed using digital subtraction angiography (DSA) workstation software (Sectra AB, Sweden), adjusting zoom, contrast and brightness parameters to visualize also the smallest collaterals. Image readings were done independently by two vascular surgeons blinded for patients' medical history. In the assessment all arterial vessels bypassing a horizontal plane at the proximal, medial and inferior third of the obstructed SFA segment were registered. The quality and stenosis of the inflow vessels (the iliac - common femoral region), outflow vessels (the popliteal region) and the number of patent outflow arteries below the knee were recorded. In addition, the length of the SFA occlusion was measured. The mean difference and standard deviation in regard of collateral counts between the two readers were small (< 10 %). The collateral count was the highest and the difference between the counters was the smallest in the counting line crossing the inferior third of the obstructed femoral artery segment. The mean value of the readings on this line was used for analysis of each patient (Sorlie et al., 1978).

**Statistical analysis**

Results are expressed as mean $\pm$ standard error of mean (SEM). Statistical significance was evaluated using Kruskal-Wallis test followed by Mann-Whitney U-test where appropriate (studies I-V) with non-parametric data. Differences in continuous and categorized variables were tested by unpaired t-test and one-way Anova, respectively (study VI). Correlation analyses were performed using Pearson's test for continuously distributed variables (Studies I, II and VI) and by Spearman's Rho correlation (studies I-V) test with categorized variables. $P < 0.05$ was considered statistically significant. If a risk factor predicted the number of collateral vessels in the univariate analysis (study VI) ($P < 0.10$) it was entered into regression analysis in order to assess the independent factors affecting collateral vessel formation. All statistical procedures were performed using SPSS software (SPSS Inc., Chicago, IL, USA).

RESULTS

## Imaging angiogenesis

We compared five different imaging modalities in the assessment of vascular growth associated with gene therapy (studies I and II). To calculate a perfusion index in normal muscles transduced with AdVEGF-A or AdLacZ in all methods, the peak tracer signal intensities between the transduced and contralateral limbs were used. The perfusion indices calculated with the use of native power Doppler ultrasound, CEU, CPS or DSC-MRI were very similar after AdVEGF GT (20- to 27-fold). Importantly, the microsphere method likely underestimated perfusion (15-fold increase after AdVEGF-A, as potent angiogenic growth factors such as VEGF-A significantly enlarged capillary size (diameter >15 μm) so that microspheres (15 μm) could not be retained in them. With respect to the feasibility of the methods, DSC-MRI produced high-resolution images of both capillary level vessels and large arteries but it was hard and time-consuming to perform and required extensive post-processing of the images unlike the real-time ultrasound techniques. The ultrasound analyses also provided information on the structure and function of the vascular bed (Figure 8).
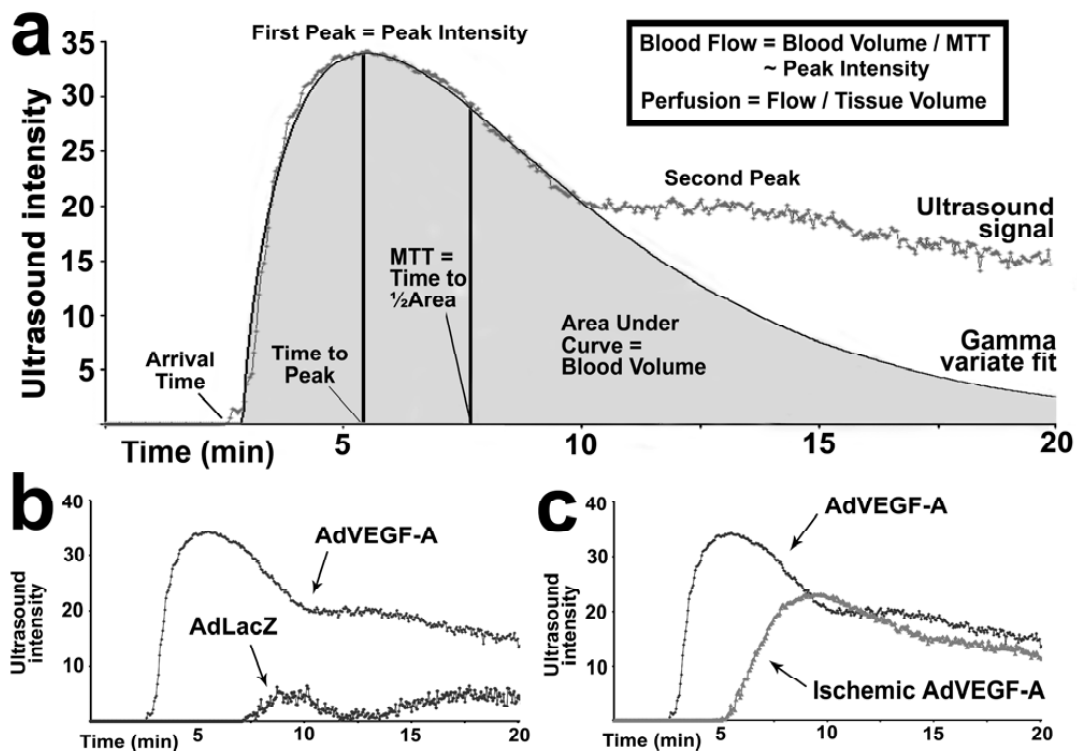


**Figure 8.** Information obtained from the ultrasound intensity vs. time graph. **a)** Blood volume and flow can be derived from the ultrasound signal using a Gamma variate function. The time to arrival of the i.v. contrast agent describes peripheral vascular resistance. **b)** The shape of the curve reflects different vessel types in the vascular bed; the presence of large arteries and shunting produced a steep curve with a high peak intensity (such as with AdVEGF-A), whereas a normal vascular network with normal-sized capillaries yielded a flat curve (AdLacZ). **c)** Furthermore, after AdVEGF GT in limbs with ligated PFA, the peak intensity was lower and the shape of the curve was flatter, reflecting the absence of the large conducting artery.

The Power Doppler CEU imaging was capable of detecting only the large (~100 μm) vessels, whereas microcirculation was invisible (study I). On the contrary, CPS ultrasound had a high spatial resolution allowing the detection of perfusion throughout the vascular bed (study II). CPS was able to detect moving microbubbles in enlarged capillaries that were only 10 to 20 μm in diameter. Blood flow in normal-sized capillaries colocalized with very low CPS signal, whereas blood flow in enlarged capillaries colocalized with high CPS signal. Due to high spatial resolution of CPS ultrasound we were also able to show that intramuscularly injected AdVEGF-A induced angiogenesis mainly in the surrounding adipose tissue rather than in the targeted skeletal muscle itself in mouse hind limbs. This finding was confirmed by histological staining of capillaries (Figure 9).
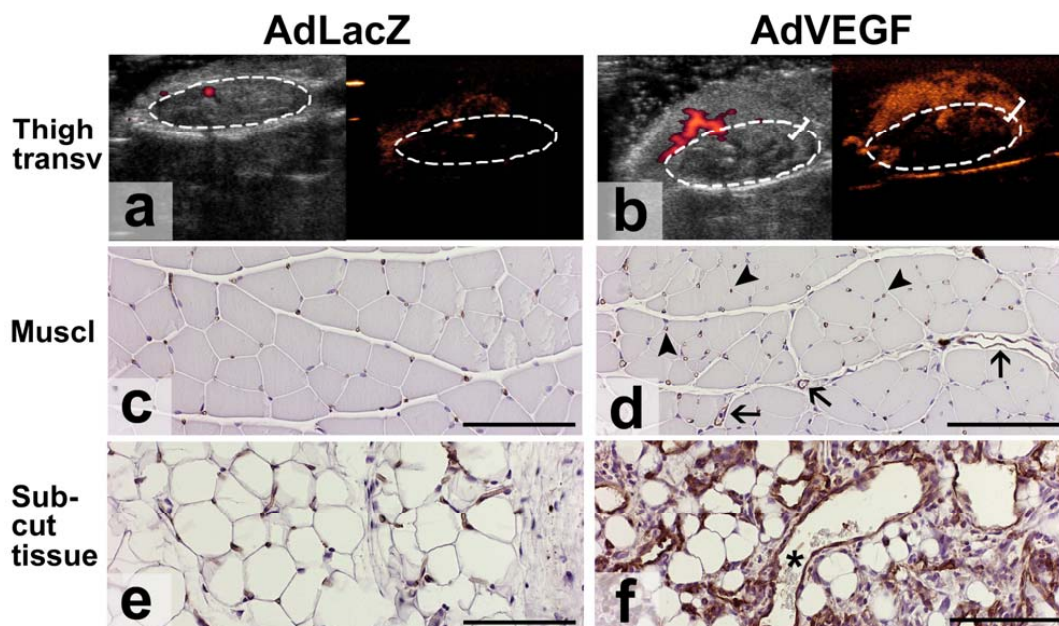


**Figure 9.** Angiogenesis is mainly induced in the subcutaneous tissue of mice after intra-muscular AdVEGF-A injection. **a)** Normal perfusion in AdLacZ transduced thigh muscles (muscle borders marked with white dotted line). **b)** Increased perfusion in AdVEGF-A transduced limbs (white parenthesis) but outside the muscle borders (white dotted line). **c)** Normal sized capillaries in AdLacZ transduced control muscles (brown). **d)** A few enlarged arterioles /venules in AdVEGF-A transduced hind limb muscles (arrows) and normal sized capillaries (arrowheads). **e)** Normal sized capillaries in AdLacZ transduced subcutaneous tissue (brown). **f)** Highly enlarged vascular structures in AdVEGF-A transduced subcutaneous tissue (brown). Erythrocytes are also visible in some of the vessels indicating functionality (asterisks). Scale bars 100 μm.

**Stimulation of vascular growth by gene therapy**

Intra-muscular adenoviral injection was found more effective than selective intra-arterial injection in stimulation of skeletal muscle angiogenesis in rabbits (study V) (Figure 10/1). Both target muscle transgene expression analysis and CPS perfusion analysis showed no effect with the intra-arterial approach while intra-muscular injections with much lower dose resulted in significant effects.

Intra-muscular AdVEGF-A GT into rabbit hind limbs induced dose dependent growth of the whole vascular tree (studies V) (Figure 10/2), increased perfusion (Figure 10/3) (studies I-V) and reduced peripheral resistance (study I) at six days in both intact and ischemic muscles. Enhanced formation

42

of collateral arteries was detected in angiograms of AdVEGF-A transduced limbs as compared with AdLacZ transduced limbs (study I). Capillary size measured from CD31 immunostained muscle sections was increased dose-dependently up to 12- and 17-fold after AdVEGF-A in intact and ischemic muscles, respectively, as compared to AdLacZ controls (Study V).
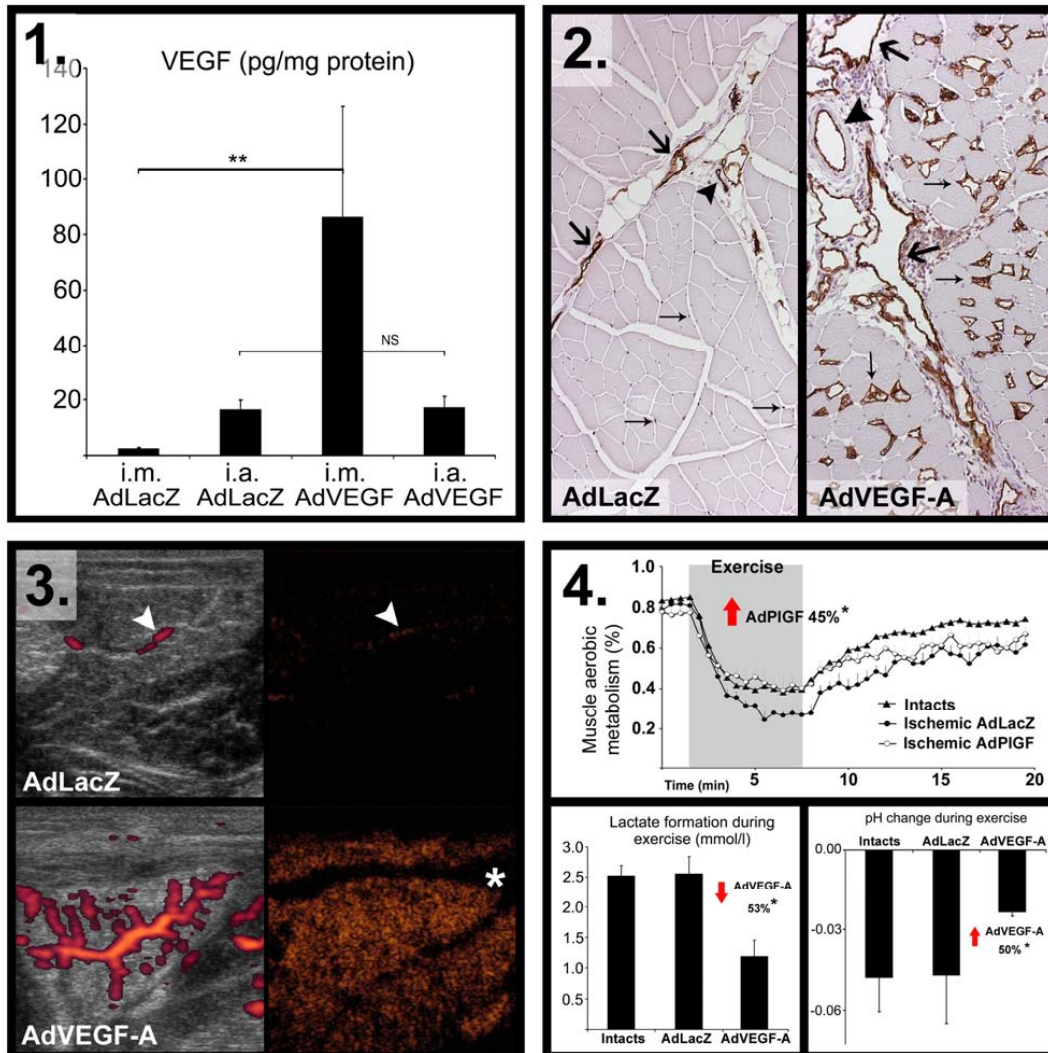


**Figure 10.** Four steps from gene transfer efficacy to vascular growth and metabolic benefits. **1)** Sufficient transgene expression needs to be achieved in the transduced muscles to reach therapeutic effects. Intramuscular GT (i.m.) was more efficient than intra-arterial GT (i.a.). **2)** Growth of the whole vascular tree needs to be stimulated. Arteries (arrowheads), veins (thick arrows) and capillaries (thin arrows) are enlarged after AdVEGF-A GT as compared to the AdLacZ control. **3)** Functional vessels are needed to increase the perfusion of transduced muscles. Perfusion is very low in the resting AdLacZ transduced muscle (arrowhead). Perfusion is highly increased after AdVEGF-A GT. Also tissue edema and free fluid (asterisk) are visible. **4)** Only if efficient vascular growth and perfusion are stimulated can metabolic benefits be expected. Efficient angiogenic gene therapy can improve muscle aerobic energy production, decrease lactate formation and formation of metabolic acidosis. *P<0.05, **P<0.01.

With arterial and venous vasculature, there was a dose-dependent increase until $5 \times 10^{10}$ vp/ml AdVEGF-A, but after that a plateau was detected in histological analysis. At maximum 11- and 5.5-fold increases were detected after AdVEGF-A in arterial area in intact and ischemic muscles, respectively. In venous vascular area 8- and 14-fold increases were observed at maximum in intact and ischemic muscles, respectively. As a result of vascular growth, maximum of 29- and 14.8-fold increases in skeletal muscle perfusion in intact and ischemic muscles, respectively, were found with AdVEGF-A as compared to AdLacZ controls (study IV). Also, AdPlGF was found to significantly increase perfusion, 15.8- and 7.8-fold in intact and ischemic muscles, respectively (study III). Additionally, as the result of vascular enlargement the ultrasound contrast agent arrived ~2-fold faster in AdVEGF-A transduced muscles than AdLacZ transduced muscles, reflecting a significant reduction in peripheral vascular resistance (study I).

$^{31}$P-MRS was used to objectively assess muscle energy metabolism in rabbit hind limbs (study III). Decreased aerobic capacity (36%) was observed during electrically stimulated exercise in calf muscles of ischemic limbs compared to intact limbs one day after the ischemia operation. AdPlGF improved aerobic capacity of ischemic limbs 45% compared to AdLacZ controls six days after GT (Figure 10). Also, the formation of metabolic acidosis during exercise and histologically detected signs of ischemia, such as necrosis and inflammation, were reduced in AdPlGF transduced ischemic limbs as compared to AdLacZ control limbs. 28 days after GT, aerobic capacity had recovered completely also in the control AdLacZ animals, due to the formation of endogenous collateral arteries and thus no difference could be detected between AdPlGF, AdLacZ and intact limbs. No effect on phosphorus metabolism was detected with $^{31}$P-MRS after AdPlGF GT in normoxic limbs. However, the formation of metabolic acidosis was reduced also in normoxic AdPlGF transduced limbs compared to normoxic controls (Figure 10/4). Analysis of blood pH and lactate levels after electrically stimulated exercise of AdVEGF-A transduced limbs also displayed dose-dependent decreases in the formation of lactate and metabolic acidosis during exercise as compared to AdLacZ controls (Figure 10/4) (study V).

**Stimulation of physiological angiogenesis**

Local concentration of VEGF-A (Figure 11, a-b) was found to determine the type of angiogenesis in normoxic rabbit hind limbs six days after GT (study V). Whereas sprouting angiogenesis could be stimulated already with small AdVEGF-A doses, capillary enlargement was dose-dependently increased even with a 100-fold greater dose (Figure 12). Furthermore, in ischemic muscles blood flow (Figure 11, c-d) affected the type of angiogenesis and stimulated capillary arterialization (study I). In PFA operated limbs the angiogenic response was more variable than in intact limbs, resulting in sprouting angiogenesis and formation of blood lacunae depending on low and high flow, respectively. Inflammation and paracrine growth factors secreted by inflammatory cells accumulating in transduced muscles may also have an effect on angiogenesis. The main response to AdPDGF-B transduction was, instead of angiogenesis, proliferation of cells in the muscle interstitium (study IV). Some of these cells were α-sma positive pericytes and fibroblasts but a remarkable fraction consisted CD31-, HAM56- and RAM11-positive cells that strongly expressed VEGF-A in immunostainings. In the very close proximity of these accumulating cells, occasional capillary enlargement could be detected after AdPDGF-B GT. Also, the distribution of growth factors within the muscle strongly affected the type of angiogenesis (Figure 11, e-f). After an intra-muscular adenoviral transduction many cell types including satellite cells, vascular endothelium, muscle fibers and fibroblasts in the muscle interstitium and in muscle fascias were found expressing transgene mRNA with in situ hybridization (study V). However, immunostainings demonstrated that most of the produced protein was localized in the muscle interstitium and at the borders of the

muscles, near the muscle fascias (studies IV and V). As a result, physiological, dose-dependent vessel growth was induced in the middle parts of the muscle where VEGF-A concentration was more uniform but large torturous hemangioma-like lacunae were formed near the muscle fascias having strongly immunostained VEGF-A deposits (study V). Also, after AdVEGF-A+AdPDGF-B combination GT the expression of PDGF-B in the muscle interstitium instead of vascular endothelium caused the detachment of pericytes from vascular structures and their migration towards the higher PDGF-B gradient in the muscle interstitium.
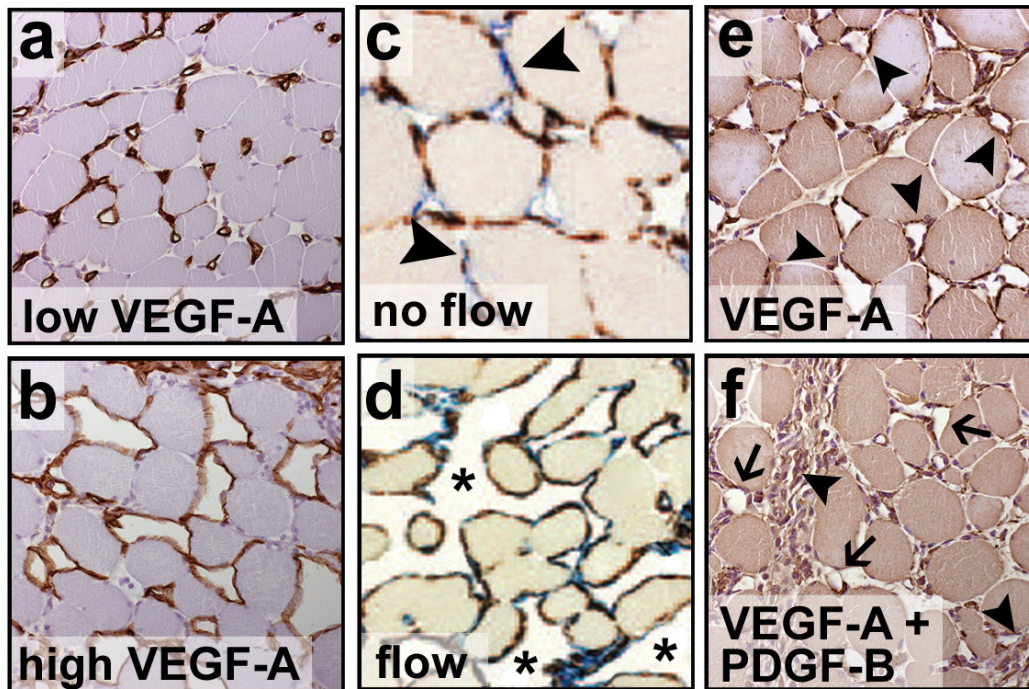


**Figure 11.** Factors affecting the type of angiogenesis. Blood flow, local VEGF-A concentration and the placement of the transgene in the tissue were found to affect the type of angiogenesis induced at six days. **a)** With low VEGF-A concentrations sprouting angiogenesis was induced but enlargement was minimal (endothelium brown). **b)** With high VEGF-A concentrations sprouting was similar to that with lower concentrations but enlargement was dose-dependently increased. **c)** In the absence of flow, sprouting angiogenesis was induced (endothelium blue, pericytes brown, sprouts indicated by arrowheads) after AdVEGF-A GT. **d)** In the presence of flow aberrant lacunae like vascular structures were formed (asterisks indicate vascular lumina). **e)** AdVEGF-A alone could induce efficient pericyte (brown) recruitment (arrowheads) likely via shear stress mediated mechanisms. **f)** Over-expression of PDGF-B besides VEGF-A outside endothelial cells created improper PDGF-B gradients that led to detachment of pericytes from capillaries (arrows). Pericytes were abundantly detected in the muscle interstitium (arrowheads).

AdVEGF-A transduction resulted in arterialization of capillary structures including the formation of pericyte coverage already 6 days after GT (studies I, III and IV). Also, AP (arterial capillary marker) and DPP (venous capillary marker) activity was decreased on endothelium of enlarged capillaries after AdVEGF-A GT, suggesting transformation toward arterioles (study I). However, this did not imply improved stability of the newly formed vessels as most of the enlarged capillaries in AdVEGF-A transduced muscles regressed by 14 days after GT (studies I, IV and V). A similar trend was observed also with perfusion in AdVEGF-A transduced muscles; after reaching the maximal increase at six days, perfusion decreased rapidly, reaching control levels at 2 weeks.

Perfusion increases induced by AdPDGF-B and AdVEGF-A+AdPDGF-B were still visible 14 days after GT. In addition to the ultrasound findings, the analysis of CD31 stained muscle sections 14 days after GT revealed that in AdPDGF-B and AdVEGF-A+AdPDGF-B transduced muscles large arteries and veins, and, to some extent capillaries, were still enlarged. Also, cell density was still increased in the muscle interstitium and often colocalized with persistent angiogenic vessels indicating a role of the inflammatory cells in the increased duration of changes. Importantly, also in AdVEGF-A treated animals, collateral arteries and the effects on arterioles and venules persisted for at least two weeks (studies I and V).
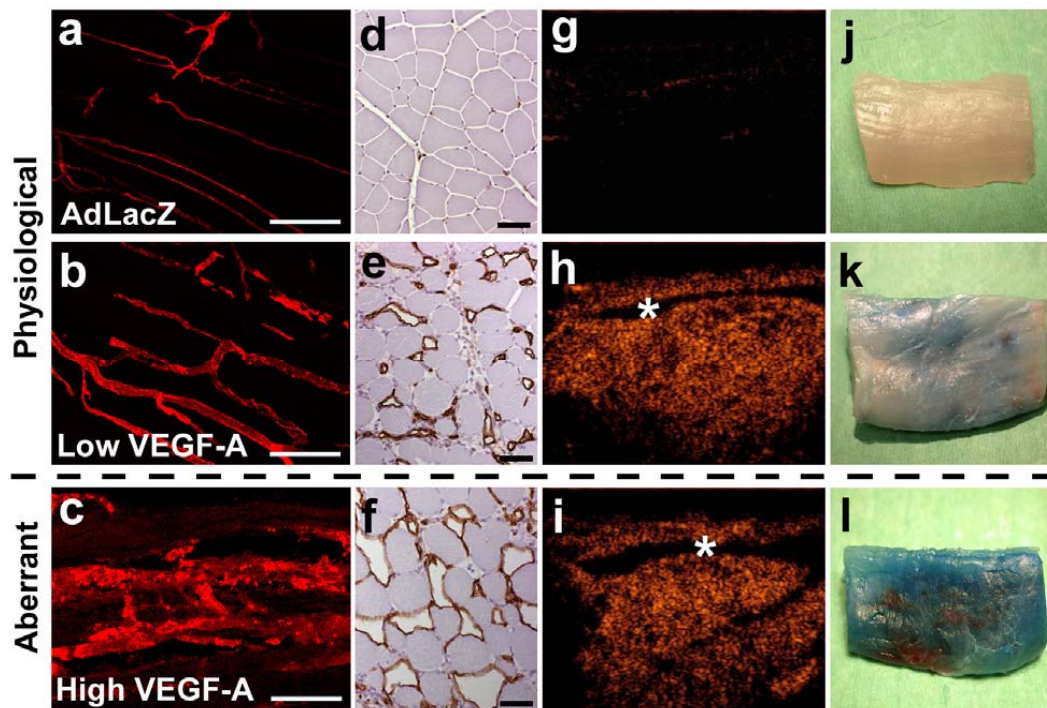


**Figure 12.** Physiological vessel growth is desired after angiogenic gene therapy. Whereas high VEGF-A concentrations can induce aberrant vascular structures and severe plasma protein extravasation leading to tissue edema, lower VEGF-A concentrations yield physiological, less leaky vessels still having as high perfusion as the higher concentration six days after GT. **a-c)** Longitudinal confocal images of control (a) and angiogenic capillary vessels (b-c). **d-f)** Transverse light microscopy images of control (d) and angiogenic vessels (e-f). **g-i)** CPS ultrasound images of control (g) and AdVEGF-A transduced muscles (h-i). Free edematous fluid is visible between the AdVEGF-A transduced muscles (asterisks). **j-l)** Plasma protein extravasation (blue) as a mark of tissue edema after control (j) and AdVEGF-A GTs (k-l). Scale bars 50 μm.

## Side-effects of angiogenic gene therapy

Formation of tissue edema during angiogenesis was found to correlate best with the increase in capillary area, less with the increases in perfusion and arterial or venous vasculature, and not to correlate with capillary density (study V). Tissue edema could not be inhibited by a combination GT with AdVEGF-A and AdPDGF-B (study IV). Instead, even an increase in edema formation was observed in ischemic limbs transduced with AdVEGF-A+AdPDGF-B as compared to AdVEGF-A alone. Instead, by titration of the overall viral dose physiological vessel growth could be stimulated and tissue edema avoided by a GT of a single growth factor (Figure 12) (study V). The

heterogeneity of the angiogenic response due to uneven distribution of VEGF-A within the transduced muscle seemed not to have a large effect on edema formation.

Dose-dependent increase in blood VEGF-A levels was observed six days after intra-muscular AdVEGF-A GT in rabbit hind limbs (Figure 13). As high as a 83-fold increase in blood VEGF-A was observed with the $10^{11}$ vp/ml AdVEGF-A dose as compared to the AdLacZ control with the same dose. Systemic side-effects including transient increases in liver, kidney and pancreatic enzymes, decrease in arterial blood pH, increase in blood lactate levels, and severe electrolyte changes, such as hyponatremia, hyperkalemia and hypocalsemia were detected six days after the $10^{11}$ vp/ml AdVEGF-A GT. $10^{11}$ vp/ml AdLacZ control or AdVEGF-A with lower doses ($10^9$ or $10^{10}$ vp/ml) had none of these effects. Analysis of electrocardiograms from animals that had received AdVEGF-A GTs revealed no dose dependent effects due to a high variation within the dose groups. However, when all the VEGF-A animals were pooled and compared to intacts and AdLacZ controls, significant effects on maximal R-wave amplitude and P-wave area could be detected, possibly indicating cardiac hypertrophy. In addition to the spread of the gene product through the systemic circulation, immunohistochemical analysis of lymph nodes six days after AdLacZ GT revealed that the transgene was expressed and widely distributed along the lymph node track after an intra-muscular GT (Figure 13).
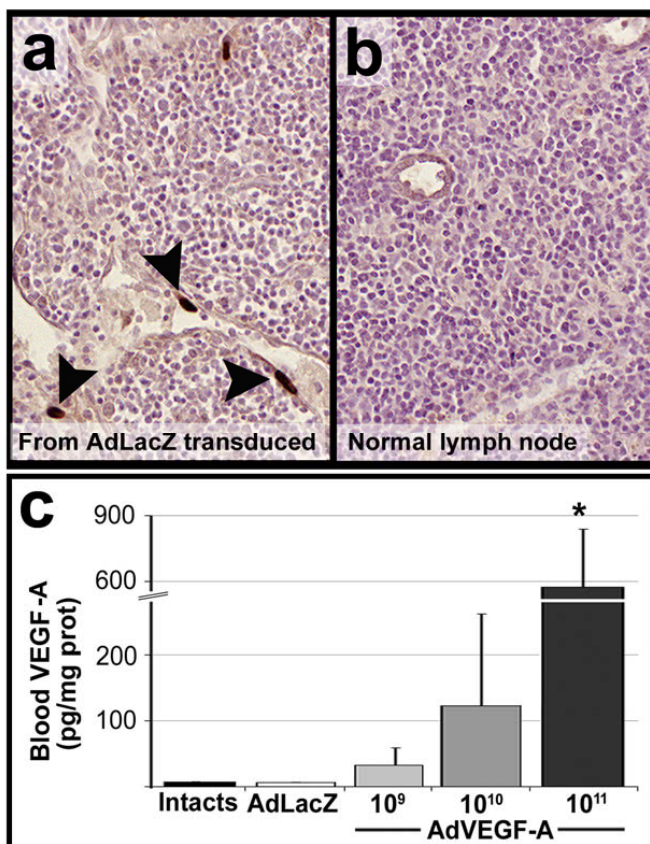


**Figure 13.** Both blood and lymphatic circulation serve as distributors of the transgene after an intra-muscular GT. **a)** Cells expressing β-galactosidase (arrowheads) were found in the lymph nodes of AdLacZ transduced animals. **b)** Lymph nodes from normal animals showed no positivity. **c)** Blood VEGF-A levels were found to increase dose-dependently after intra-muscular AdVEGF-A GT. *P< 0.05.

**Factors affecting collateral growth (study VI)**

Hemodynamics were identified critical for collateral artery formation in patients with lower limb ischemia (Figure 14). In univariate analysis, the number of patent calf arteries was found to correlate with the collateral count (Pearson's correlation 0.603, $p<0.0001$).  A correlation was also found between the collateral count and non-stenosed inflow arteries (Spearman's Rho correlation 0.304, $p<0.05$). Additionally, the presence of hypertension displayed an association with collateral count in variance analysis. Finally, in multivariate analysis only the quality of inflow and the number of patent calf vessels demonstrated an independent association ($p<0.05$) with the number of collaterals. Interestingly, the analysis revealed no relationship between low number of collaterals and a history of diabetes, chronic obstructive pulmonary disease, coronary artery disease, rheumatoid disease, renal disease, hypercholesterolemia, malignant disease or smoking. Age or the length of occlusion had also no effect of collateral formation. Supporting the role of hemodynamics, the number of collateral arteries was dramatically reduced after the studied patients had had surgical revascularization.
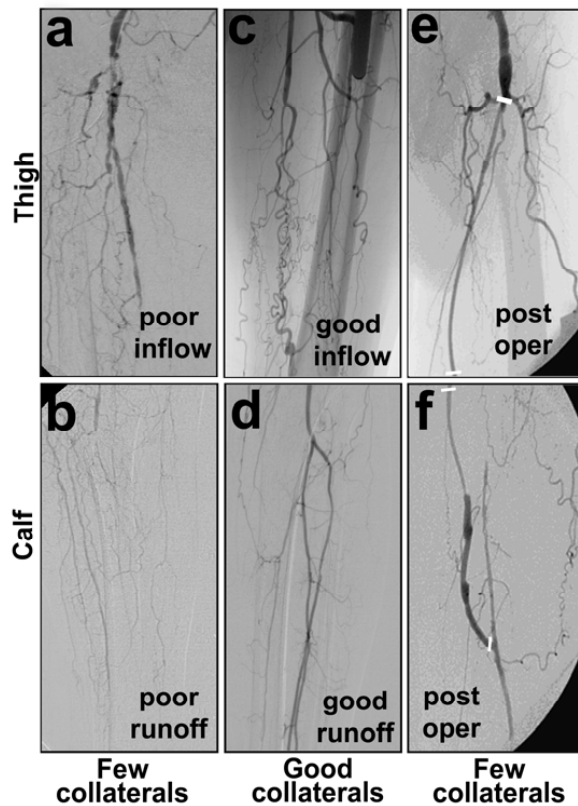


**Figure 14.** Antegrade flow and peripheral runoff affect collateral formation. **a-b)** In the presence of poor antegrade flow and poor distal runoff very few functional collaterals are formed. **c-d)** In the presence of good antegrade flow and good distal runoff functional collaterals are formed. **e-f)** After a femoro-popliteal bypass operation (white transversal lines marking the bypass craft) formed collaterals regress.

DISCUSSION

**Tools for studying vascular growth**

As the effects of angiogenic gene therapy take place mostly at the capillary level vessels, imaging changes occurring in capillaries is crucial to demonstrate the efficacy of the treatment also in clinical trials (Rissanen and Yla-Herttuala, 2007). The assessment of changes taking place at the capillary level has been so far out of reach due to the lack of proper non-invasive imaging modalities for the capillary level vessels and the difficulty of obtaining histological biopsies. Digital subtraction angiography, CT-angiography or MRI approaches have been used to show changes in collateral vessels in clinical trials (Makinen et al., 2002) but the results from these trials are compromised by the lack of evidence of angiogenesis actually taking place. Additionally, strong vascular growth by AdVEGF-A involving manifold capillary enlargement and increased vascular permeability interferes with many standard vascular imaging and perfusion measurement techniques such as x-ray angiography and the microsphere method as shown in this study and, as shown previously, contrast-enhanced MRI with the use of low-molecular-weight extracellular contrast agents such as gadolinium (Rissanen et al., 2003a; Rissanen et al., 2003b). In these circumstances, the contrast agent must not extravasate and the method must not assume physiological capillary size. Contrast enhanced ultrasound imaging is very attractive for quantitative perfusion measurement after angiogenic therapies because it meets these requirements, is noninvasive, is approved for humans, provides quantitative data on blood flow kinetics, and enables 3D reconstruction of vasculature without nefrotoxic contrast agents or ionizing radiation (Krix et al., 2005; Miller et al., 2005).

Here it has been shown that by contrast enhanced ultrasound imaging changes at capillary level vasculature can be followed non-invasively with high correlation to histological evaluation of angiogenesis. Additionally, the ultrasound data can be used to obtain information about the function of the vascular bed e.g. peripheral resistance, types of vessels and shunting. Ultrasound imaging and especially CPS can also be utilized in animal models of angiogenesis. With CPS, a higher frequency (14 MHz) can be used in mice than in rabbits (8.5 MHz), owing to a smaller region of interest and a higher amount of contrast agent used in proportion to the body size, and thus the spatial resolution is even better in mice than in rabbits. Indeed, the accuracy of CPS imaging is very closely correlated to histological findings of angiogenesis reaching 10-20 µm in spatial resolution with 14 MHz. In contrast to imaging techniques such as Laser Doppler which penetrates only ~1 mm in the skin (Miller et al., 2005), ultrasound imaging also provides superior tissue penetration. For example, Laser Doppler could not have been able to differentiate between subcutaneous and skeletal muscle perfusion increases in our mouse hind limb model. CPS ultrasound is expected to be a valuable tool in the future trials of therapeutic angiogenesis.

Besides imaging angiogenesis and perfusion, it is very important to actually measure the functionality of the induced vessels, as the measurement of perfusion alone does not always reflect the actual metabolic effects. In this study two methods have been used to evaluate the metabolic activity of the induced neovessels: 1) $^{31}$P-MRS in ischemic animals and 2) the measurement of acid-base balance and lactate formation in unoperated animals. The measurement of lactate formation from arterial blood samples is a simple method suitable for experimental research in relatively large animals. However, it is affected by the distribution of the metabolites in the systemic circulation and thus may not be used reliably in ischemic tissues. In contrast, $^{31}$P-MRS, although more laborious, can be used to reliably study the energy metabolism of ischemic muscles (Isbell et al., 2006). $^{31}$P-MRS is also feasible in clinical settings and can be used to objectively assess exercise

tolerance of ischemic muscles unlike many of the current techniques, such as the treadmill test that is strongly dependent on the co-operation of the tested (Isbell et al., 2006). With these tools it was here demonstrated in a rabbit model that the neovessels induced by angiogenic gene therapy could improve the aerobic energy metabolism of ischemic muscles and decrease the formation of metabolic acidosis also in normoxic muscles. In the future, a similar demonstration of a functional benefit from ischemic human limbs would be needed in order to actually assess the benefits of angiogenic gene therapy in humans.

**Growing functional vasculature**

Vascular growth is a complicated process and regardless of whether it takes place in ischemic muscle or during embryonic development it is orchestrated by several different growth factors and their isoforms (Conway et al., 2001). The role of VEGFs in vascular growth, although essential, has mainly been considered to be the stimulation of endothelial cell proliferation, migration and the relaxation of the vascular wall allowing enlargement and sprouting of vessels (Ferrara, 2004). PDGFs on the other hand are regarded as the stimulators of smooth muscle and pericytes needed to build a stable vessel (Lindahl et al., 1997; Tallquist and Kazlauskas, 2004). Thus, the functionality of vessels grown by gene therapy with a single growth factor or growth factor isoform has been questioned (Whitlock et al., 2004; Pajusola et al., 2005) and several gene combination cocktails have been tested in order to create more physiological vessels (Richardson et al., 2001; Hao et al., 2007). Here the combination of adenoviral VEGF-A and PDGF-B was tested with unexpected results. It was demonstrated that it is simply not enough to inject the growth factors into the target tissue but the growth factors need to be expressed in the right cell types. After an intra-muscular GT growth factor expression was shown to take place in several cell types. The secreted growth factor was also often bound to the extra cellular matrix with high quantities inducing high growth factor gradients towards the interstitium. After an intra-muscular injection AdVEGF-A alone could induce relatively efficient vascular growth and pericyte recruitment in the presence of efficient blood flow even if the transgene was not expressed in the vessel wall, likely due to blood flow and shear-stress mediated mechanisms. In contrast, the non-targeted expression of PDGF-B after an intra-muscular adenoviral injection appeared to cause, besides pericyte proliferation, detachment of pericytes from vessels, likely due to improper endothelial PDGF-B retention (Lindblom et al., 2003). When blood vessels dilate, blood flow and pressure are increased inside the vessels leading to increased shear stress on the vessel walls (Schaper and Scholz, 2003). Shear stress induces stimulation of pressure receptors that in response stimulate the production of several growth factors, including PDGFs, that induce changes in the vessel wall, such as attracting pericytes, to make it more resistant to the increased blood pressure (Schaper and Scholz, 2003). Thus, the expression of AdVEGF-A or another angiogenic growth factor alone can induce the growth of vessels having functional pericyte coverage (Dor et al., 2002) as it not only stimulates vascular growth but also starts a cascade of growth factor expression leading to the stabilization of the induced vessels.

The functionality of newly formed vessels has often been linked to the structure of vessels. Sprouting angiogenesis has often been considered the more physiological type of angiogenesis after angiogenic GTs in comparison to capillary enlargement that has been associated to aberrant angiogenesis taking place in tumors (Dor et al., 2002; Ozawa et al., 2004). Especially angiopoietins have been implicated to have a role in the normalization of aberrant vessels induced by a VEGF-A GT (Davis et al., 1996; Thurston et al., 1999; Papapetropoulos et al., 2000; Hawighorst et al., 2002). However, the potential of sprouting angiogenesis without enlargement in revascularization of ischemic tissues is remarkably less than that with enlargement, as blood flow is greatly dependent on vascular diameter according to Poiseuille's law (Guyton and Hall, 2000). Here in a AdVEGF-A

dose titration study it has been demonstrated that whereas high VEGF-A doses can induced the growth of torturous, hemangioma-like vessels, uniformly enlarged vessels can be induced by simply lowering the amount of AdVEGF-A. It was found that all the tested AdVEGF-A doses could stimulate sprouting angiogenesis whereas capillary enlargement and finally the formation aberrant lacunae required increasingly higher VEGF-A levels. Interestingly, the functionality of the vessels formed after AdVEGF-A GT, measured by improved aerobic energy production, was found to increase dose-dependently along with the increase in capillary area, even with the highest doses. With the lowest AdVEGF-A dose tested ($10^9$ vp/ml) sprouting angiogenesis was induced with the same magnitude as with the higher doses but capillary enlargement was very minimal. With this dose no effect on muscle energy metabolism was detected indicating that there is a threshold in angiogenesis, namely on capillary enlargement, before effects on muscle energy metabolism can be detected.

Capillary enlargement as a form of angiogenesis after angiogenic gene therapy is still often not recognized. As shown here, capillary sprouting can be stimulated already with very low VEGF-A doses, where as capillary enlargement requires higher VEGF-A doses and sufficient blood flow. A large portion of gene therapy studies, to date, have still been conducted with plasmids and recombinant proteins leading to relatively low levels of growth factors in target tissues. Also, growth factors and viral vectors vary in their efficacy of promoting angiogenesis depending on their biological activity and modifications. Furthermore, the most used animal model in angiogenic gene therapy is mouse and as shown here, the efficacy of angiogenic gene therapy with adenoviral vectors is much lower in mouse skeletal muscle than that with rabbit skeletal muscle or mouse subcutaneous tissue, possibly due to differences in vector or growth factor related receptors. Thus, the significance of capillary enlargement may still be unclear to many researchers due to the fact that they have not seen it in their studies. These differences in models and GT tools create a great variability in determining what is efficient angiogenesis and may also have led to the use of ineffective gene products in clinical trials, explaining the lack of positive results (Rissanen and Yla-Herttuala, 2007). Based on the results presented here, the efficacy of angiogenic gene therapy should not only be based on the level of sprouting angiogenesis induced but the level of capillary enlargement should also be evaluated - especially as the beneficial effects on muscle energy metabolism seemed to be highly correlated to the level of capillary enlargement and not on sprouting angiogenesis.
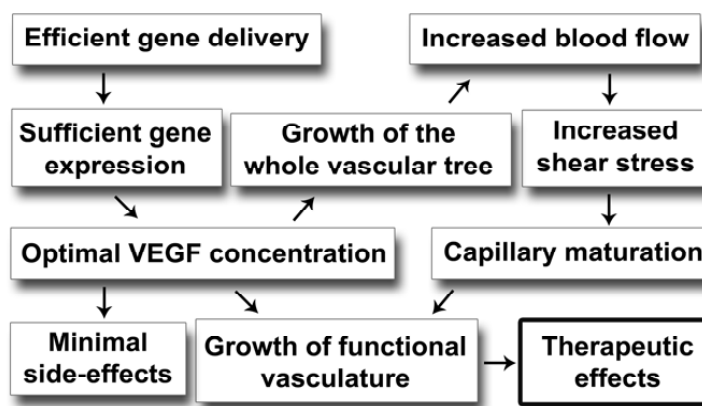


**Figure 15.** A summary of steps essential for efficient angiogenic gene therapy.

The efficacy of gene therapy is also highly related to the method of gene delivery, especially in regards to the injection site (Yla-Herttuala and Alitalo, 2003). An intra-vascular GT has appeal as for its ability to spread the virus to large areas. However, it obviously then can also easily transduce

unwanted tissues and could, in theory, result in acceleration of tumor growth for example (Hiltunen et al., 2000). Additionally, in regard of ischemic tissues the gene product would not actually reach the ischemic areas as by definition the ischemic areas are void of circulation (Rissanen and Yla-Herttuala, 2007). Gene transfer efficacy after an intra-vascular administration is also hampered by endonucleases and efficient immune responses neutralizing circulating vectors quickly, as well as constant blood flow that would make it hard for the vector to attach to vascular endothelium and enter endothelial cells (Guyton and Hall, 2000). Here it was demonstrated that although much higher adenoviral doses would be used in selective intra-arterial administration than in intra-muscular administration, the efficacy of the intra-vascular approach is questionable. A summary of proposed steps needed for efficient angiogenic gene therapy are presented in Figure 15.

**Duration of angiogenic changes**

To gain a true clinically relevant angiogenic response, the vessels induced by gene therapy need to be stable and functional for long periods of time. Adenoviral vectors induce transient but very high expression of the encoded growth factor but due to the nature of the virus the expression is quickly shut down after about two weeks (Muruve, 2004; Korpisalo P et al., 2007). With AdVEGF-A this means that a highly efficient angiogenic stimulus is first achieved but most of the angiogenic changes do not persist beyond the cessation of the growth factor expression at two weeks. The maturity of the vessel wall structure has been suggested to determine the stability of vessels after gene therapy (Benjamin et al., 1998; Conway et al., 2001). A four week expression of VEGF-A has been described to produce vessel structures stabile enough to resist regression after VEGF-A withdrawal (Dor et al., 2002). Thus, long expression vectors such as adeno-associated viruses (AAV) have been suggested more suitable for angiogenic gene therapy than adenoviruses (Dor et al., 2002; Yla-Herttuala et al., 2007). Here, an increase in the duration of adenovirally induced angiogenesis was desired and a combination GT of AdPDGF-B and AdVEGF-A stimulating both endothelial cells and pericytes was used in order to gain more mature vessels. As a result, an increase in the duration of angiogenesis was obtained after AdPDGF-B GT alone or in combination with AdVEGF-A. However, this increase was most likely not related to improved structure of vessels but rather mediated by paracrine secretion of growth factors from cells that had accumulated into the transduced muscles. The beneficial role of these, possibly bone marrow derived precursors or inflammatory cells and paracrine growth factors on vascular growth and stabilization after AdVEGF-A GT could have therapeutic implications but requires further research and development (Rehman et al., 2003; Kastrup et al., 2006).

The level of perfusion in resting skeletal muscle is very low (Guyton and Hall, 2000). It is only natural that a balance in the amount of blood vessels is maintained at a level responding to the need of the tissue (Risau, 1997; Guyton and Hall, 2000). By gene therapy an overload of vasculature can be stimulated even in ischemic muscle. Here it has been shown that collateral vessels induced by AdVEGF-A GT as well as changes in arterial and venous vascular area remained elevated after the expression of adenovirus ended and most of the changes in capillary level vessels returned to baseline, in line with previously published results (Vajanto et al., 2002; Rissanen et al., 2003a). This might imply that vessels that are truly needed and are continuously perfused will survive even after just a short initial boost of growth factors, possibly due to blood flow and shear stress mediated up-regulation of growth factors in the blood vessel endothelium (Vajanto et al., 2002). This theory is hard to test in practice as chronic ischemia, lasting over two weeks has been difficult to achieve in animal models so far. However, promising results have been published concerning gene transfers done before the ischemia operation that report improved recovery from acute ischemia after the expression boost of growth factor has passed (Gowdak et al., 2000).

**Side-effects of angiogenic gene therapy**

In general, only mild side-effects have been reported to have occurred in clinical angiogenic gene therapy trials (Makinen et al., 2002; Rajagopalan et al., 2002; Yla-Herttuala et al., 2007). Besides mild immune reactions such as fever, one death associated with an overdose of adenovirus has been reported in an immunoincompetent patient (Lehrman, 1999). However, side-effects related especially to growth factors and efficient angiogenesis, besides moderate edema, have been more rarely described likely due to low efficacy of the clinical trials so far (Baumgartner et al., 2000; Rajagopalan et al., 2002). A common feature of the clinical trial protocols that might also have reduce the observation of side-effects is that the patients are not monitored at early time points (< one week) after the GT (Rajagopalan et al., 2002). As shown here the highest prevalence of side-effects when using adenoviral vectors is around one week after the GT and most of the side-effects are diminished by two weeks. High circulating levels of VEGF-A were shown to induce liver, kidney and pancreatic effects. Additionally, cardiac changes were observed and could be in theory related to both the circulating VEGF-A or hemodynamic changes. Tissue edema was shown to develop dose-dependently after an angiogenic GT and to correlate with the level of capillary enlargement. While tissue edema can be largely avoided by the titration of the overall viral dose, as shown here, at the same time a compromise has to be made in the level of angiogenesis. It is also possible that increased perfusion and vascular permeability associated with efficient angiogenesis in the transduced muscles facilitates the release of the growth factor into the systemic circulation. Also, the lymphatic system was here shown to be a route for growth factor biodistribution. Thus, as the trials are constantly aiming towards better efficiency and using more powerful vectors than before, it can be assumed that the prevalence of angiogenesis-related side-effects will be increasing in future trials before optimal growth factor levels have been established. The safety data here demonstrates that subjects receiving gene therapy with adenoviral vectors, inducing high transient doses of growth factors and efficient angiogenesis, need to be observed carefully for the first two weeks after GT so that the side-effects can be recognized, and with optimization of the viral dose avoided in future trials.

**The potential of gene therapy in revascularization of ischemic tissues**

By natural repair, blood flow through collateral vessels compensates reduced blood flow through stenotic and occluded arteries (Schaper and Scholz, 2003). The degree of collateralization after an arterial occlusion has been suggested to be related to several factors, such as duration and level of ischemia, diabetes and pulmonary obstructive disease (Wahlberg, 2003; De Vivo et al., 2005; Topsakal et al., 2005). Our results from CLI patients indicate that while several factors, such as hypertension, may partially affect collateral vessel formation, only efficient antegrade flow and the functionality of vessels distal to the occlusion have an independent association on collateral vessel formation. Similarly to collateral formation and patency the success of radiological and surgical revascularization is dependent on these hemodynamic factors. As the hemodynamic factors mediating collateral formation upstream to an occlusion are often sufficient, the functionality of the runoff vessels remains a significant clinical problem. Angiogenic gene therapy could provide a precise treatment for opening up the distal vascular network and to improve the distal runoff in ischemic tissues. Here it has been shown in animal models that with angiogenic gene therapy it is possible to stimulate the growth of the whole vascular tree, increase skeletal muscle perfusion manifold, increase the level of aerobic metabolism in ischemic muscle and to reduce ischemic changes. Angiogenic gene therapy could be used not only as a single treatment but as an adjuvant therapy to surgical bypass and percutaneous revascularization operations to improve the runoff from crafts, maintain higher blood flow and pressure in stents or crafts and thus help to avoid restenosis.

SUMMARY AND CONCLUSIONS

In summary, the main findings of this work were (according to each sub-study):

I       AdVEGF-A induces arteriogenesis, capillary arterialization and a decrease in peripheral resistance after an intra-muscular GT to rabbit hind limbs. Furthermore, blood flow modulates vascular growth in ischemic tissues.

II      CPS ultrasound imaging is feasible in the assessment of angiogenesis and could be used to evaluate capillary level vascular growth non-invasively with almost histological precision in both animal models and clinical trials.

III     Vessels grown with AdPlGF GT are metabolically functional as verified by studying the energetic reserves of ischemic rabbit muscles with $^{31}$P-MRS. AdPlGF GT results in a decrease in metabolic acidosis during exercise and decreases the amount of ischemic changes such as necrosis or inflammation in transduced muscles. $^{31}$P-MRS is feasible in the objective assessment of muscle energy metabolism and could also be used in clinical trials.

IV      Non-targeted intra-muscular AdPDGF-B induces detachment of pericytes from vessels when delivered in combination with AdVEGF-A likely due to formation of improper PDGF-B gradients. An increase in the duration of angiogenic changes observed after the AdPDGF-B GT is due to paracrine secretion of VEGF-A from recruited mononuclear cells rather than improved vascular maturity.

V       The dose of AdVEGF-A determines between sprouting angiogenesis and capillary enlargement. There seems to be a threshold with capillary enlargement after which metabolic benefits are induced. Additionally, by titration of the viral dose the formation of physiological vessels without major side-effects can be induced. The side-effects of angiogenic gene therapy are the most prominent one week after adenoviral transduction and are mediated by the spread of the growth factor via both blood and lymphatic routes.

VI      Hemodynamics, mainly the functionality of inflow and outflow vessels, determine the level of endogenous collateral vessel formation in patients with limb ischemia.

This thesis project describes factors mediating endogenous collateral vessel formation in humans and presents means for induction of functional neovascular growth via angiogenic gene therapy in animal models. The potential of angiogenic gene therapy in supporting endogenous collateral formation has been extensively verified as regards to the functionality and structure of induced vessels. Additionally, novel methods, suitable for both preclinical and clinical use, have also been tested for imaging microvascular growth and studying the functionality of vessels induced by angiogenic gene therapy. It is concluded that therapeutic angiogenesis is a promising new treatment option for patients with severe peripheral vascular disease.

REFERENCES

Aase K, von Euler G, Li X, Ponten A, Thoren P, Cao R, Cao Y, Olofsson B, Gebre-Medhin S, Pekny M, Alitalo K, Betsholtz C, Eriksson U. Vascular endothelial growth factor-B-deficient mice display an atrial conduction defect. Circulation 2001; (104): 358-364.

Abramsson A, Lindblom P, Betsholtz C. Endothelial and nonendothelial sources of PDGF-B regulate pericyte recruitment and influence vascular pattern formation in tumors. J Clin Invest 2003; (112): 1142-1151.

Achen M G, Jeltsch M, Kukk E, Makinen T, Vitali A, Wilks A F, Alitalo K, Stacker S A. Vascular endothelial growth factor D (VEGF-D) is a ligand for the tyrosine kinases VEGF receptor 2 (Flk1) and VEGF receptor 3 (Flt4). Proc Natl Acad Sci U S A 1998; (95): 548-553.

Antoniades H N, Scher C D, Stiles C D. Purification of human platelet-derived growth factor. Proc Natl Acad Sci U S A 1979; (76): 1809-1813.

Arsic N, Zentilin L, Zacchigna S, Santoro D, Stanta G, Salvi A, Sinagra G, Giacca M. Induction of functional neovascularization by combined VEGF and angiopoietin-1 gene transfer using AAV vectors. Mol Ther 2003; (7): 450-459.

Asahara T, Murohara T, Sullivan A, Silver M, van der Zee R, Li T, Witzenbichler B, Schatteman G, Isner J M. Isolation of putative progenitor endothelial cells for angiogenesis. Science 1997; (275): 964-967.

Asahara T, Takahashi T, Masuda H, Kalka C, Chen D, Iwaguro H, Inai Y, Silver M, Isner J M. VEGF contributes to postnatal neovascularization by mobilizing bone marrow-derived endothelial progenitor cells. EMBO J 1999; (18): 3964-3972.

Autiero M, Waltenberger J, Communi D, Kranz A, Moons L, Lambrechts D, Kroll J, Plaisance S, De Mol M, Bono F, Kliche S, Fellbrich G, Ballmer-Hofer K, Maglione D, Mayr-Beyrle U, Dewerchin M, Dombrowski S, Stanimirovic D, Van Hummelen P, Dehio C, Hicklin D J, Persico G, Herbert J M, Communi D, Shibuya M, Collen D, Conway E M, Carmeliet P. Role of PlGF in the intra- and intermolecular cross talk between the VEGF receptors Flt1 and Flk1. Nat Med 2003; (9): 936-943.

Avantaggiato V, Orlandini M, Acampora D, Oliviero S, Simeone A. Embryonic expression pattern of the murine figf gene, a growth factor belonging to platelet-derived growth factor/vascular endothelial growth factor family. Mech Dev 1998; (73): 221-224.

Baffour R, Berman J, Garb J L, Rhee S W, Kaufman J, Friedmann P. Enhanced angiogenesis and growth of collaterals by in vivo administration of recombinant basic fibroblast growth factor in a rabbit model of acute lower limb ischemia: dose-response effect of basic fibroblast growth factor. J Vasc Surg 1992; (16): 181-191.

Baffour R, Garb J L, Kaufman J, Berman J, Rhee S W, Norris M A, Friedmann P. Angiogenic therapy for the chronically ischemic lower limb in a rabbit model. J Surg Res 2000; (93): 219-229.

Baluk P, Tammela T, Ator E, Lyubynska N, Achen M G, Hicklin D J, Jeltsch M, Petrova T V, Pytowski B, Stacker S A, Yla-Herttuala S, Jackson D G, Alitalo K, McDonald D M. Pathogenesis of persistent lymphatic vessel hyperplasia in chronic airway inflammation. J Clin Invest 2005; (115): 247-257.

Bates D O, Cui T G, Doughty J M, Winkler M, Sugiono M, Shields J D, Peat D, Gillatt D, Harper S J. VEGF165b, an inhibitory splice variant of vascular endothelial growth factor, is down-regulated in renal cell carcinoma. Cancer Res 2002; (62): 4123-4131.

Baumgartner I, Pieczek A, Manor O, Blair R, Kearney M, Walsh K, Isner J M. Constitutive expression of phVEGF165 after intramuscular gene transfer promotes collateral vessel development in patients with critical limb ischemia. Circulation 1998; (97): 1114-1123.

Baumgartner I, Rauh G, Pieczek A, Wuensch D, Magner M, Kearney M, Schainfeld R, Isner J M. Lower-extremity edema associated with gene transfer of naked DNA encoding vascular endothelial growth factor. Ann Intern Med 2000; (132): 880-884.

Beard J D. ABC of arterial and venous disease: Chronic lower limb ischaemia. BMJ 2000; (320): 854-857.

Bellomo D, Headrick J P, Silins G U, Paterson C A, Thomas P S, Gartside M, Mould A, Cahill M M, Tonks I D, Grimmond S M, Townson S, Wells C, Little M, Cummings M C, Hayward N K, Kay G F. Mice lacking the vascular endothelial growth factor-B gene (Vegfb) have smaller hearts, dysfunctional coronary vasculature, and impaired recovery from cardiac ischemia. Circ Res 2000; (86): E29-E35.

Benjamin L E, Hemo I, Keshet E. A plasticity window for blood vessel remodelling is defined by pericyte coverage of the preformed endothelial network and is regulated by PDGF-B and VEGF. Development 1998; (125): 1591-1598.

Bergelson J M, Cunningham J A, Droguett G, Kurt-Jones E A, Krithivas A, Hong J S, Horwitz M S, Crowell R L, Finberg R W. Isolation of a common receptor for Coxsackie B viruses and adenoviruses 2 and 5. Science 1997; (275): 1320-1323.

Bergsten E, Uutela M, Li X, Pietras K, Ostman A, Heldin C H, Alitalo K, Eriksson U. PDGF-D is a specific, protease-activated ligand for the PDGF beta-receptor. Nat Cell Biol 2001; (3): 512-516.

Bjarnegard M, Enge M, Norlin J, Gustafsdottir S, Fredriksson S, Abramsson A, Takemoto M, Gustafsson E, Fassler R, Betsholtz C. Endothelium-specific ablation of PDGFB leads to pericyte loss and glomerular, cardiac and placental abnormalities. Development 2004; (131): 1847-1857.

Bonthron D T, Morton C C, Orkin S H, Collins T. Platelet-derived growth factor A chain: gene structure, chromosomal location, and basis for alternative mRNA splicing. Proc Natl Acad Sci U S A 1988; (85): 1492-1496.

Brevetti L S, Chang D S, Tang G L, Sarkar R, Messina L M. Overexpression of endothelial nitric oxide synthase increases skeletal muscle blood flow and oxygenation in severe rat hind limb ischemia. J Vasc Surg 2003; (38): 820-826.

Callum K, Bradbury A. Acute limb ischaemia. BMJ 2000; (320): 764-767.

Cao R, Brakenhielm E, Li X, Pietras K, Widenfalk J, Ostman A, Eriksson U, Cao Y. Angiogenesis stimulated by PDGF-CC, a novel member in the PDGF family, involves activation of PDGFR-alphaalpha and -alphabeta receptors. FASEB J 2002; (16): 1575-1583.

56

Cao Y, Ji W R, Qi P, Rosin A, Cao Y. Placenta growth factor: identification and characterization of a novel isoform generated by RNA alternative splicing. Biochem Biophys Res Commun 1997; (235): 493-498.

Carmeliet P, Ferreira V, Breier G, Pollefeyt S, Kieckens L, Gertsenstein M, Fahrig M, Vandenhoeck A, Harpal K, Eberhardt C, Declercq C, Pawling J, Moons L, Collen D, Risau W, Nagy A. Abnormal blood vessel development and lethality in embryos lacking a single VEGF allele. Nature 1996; (380): 435-439.

Carmeliet P, Moons L, Luttun A, Vincenti V, Compernolle V, De Mol M, Wu Y, Bono F, Devy L, Beck H, Scholz D, Acker T, DiPalma T, Dewerchin M, Noel A, Stalmans I, Barra A, Blacher S, Vandendriessche T, Ponten A, Eriksson U, Plate K H, Foidart J M, Schaper W, Charnock-Jones D S, Hicklin D J, Herbert J M, Collen D, Persico M G. Synergism between vascular endothelial growth factor and placental growth factor contributes to angiogenesis and plasma extravasation in pathological conditions. Nat Med 2001; (7): 575-583.

Carmeliet P, Ng Y S, Nuyens D, Theilmeier G, Brusselmans K, Cornelissen I, Ehler E, Kakkar V V, Stalmans I, Mattot V, Perriard J C, Dewerchin M, Flameng W, Nagy A, Lupu F, Moons L, Collen D, D'Amore P A, Shima D T. Impaired myocardial angiogenesis and ischemic cardiomyopathy in mice lacking the vascular endothelial growth factor isoforms VEGF164 and VEGF188. Nat Med 1999; (5): 495-502.

Cavazzana-Calvo M, Hacein-Bey S, de Saint B, Gross F, Yvon E, Nusbaum P, Selz F, Hue C, Certain S, Casanova J L, Bousso P, Deist F L, Fischer A. Gene therapy of human severe combined immunodeficiency (SCID)-X1 disease. Science 2000; (288): 669-672.

Chae J K, Kim I, Lim S T, Chung M J, Kim W H, Kim H G, Ko J K, Koh G Y. Coadministration of angiopoietin-1 and vascular endothelial growth factor enhances collateral vascularization. Arterioscler Thromb Vasc Biol 2000; (20): 2573-2578.

Collins T, Ginsburg D, Boss J M, Orkin S H, Pober J S. Cultured human endothelial cells express platelet-derived growth factor B chain: cDNA cloning and structural analysis. Nature 1985; (316): 748-750.

Conway E M, Collen D, Carmeliet P. Molecular mechanisms of blood vessel growth. Cardiovasc Res 2001; (49): 507-521.

Cooney R, Hynes S O, Duffy A M, Sharif F, O'Brien T. Adenoviral-mediated gene transfer of nitric oxide synthase isoforms and vascular cell proliferation. J Vasc Res 2006; (43): 462-472.

Cosgrove D, Eckersley R, Blomley M, Harvey C. Quantification of blood flow. Eur Radiol 2001; (11): 1338-1344.

Cotran R S, Kumar V, Collins T. Robbins pathologic basis of disease. W.B. Saunders Company, Philadelphia 1999.

Davis S, Aldrich T H, Jones P F, Acheson A, Compton D L, Jain V, Ryan T E, Bruno J, Radziejewski C, Maisonpierre P C, Yancopoulos G D. Isolation of angiopoietin-1, a ligand for the TIE2 receptor, by secretion-trap expression cloning. Cell 1996; (87): 1161-1169.

De Vivo S, Palmer-Kazen U, Kalin B, Wahlberg E. Risk factors for poor collateral development in claudication. Vasc Endovascular Surg 2005; (39): 519-524.

de Vries C, Escobedo J A, Ueno H, Houck K, Ferrara N, Williams L T. The fms-like tyrosine kinase, a receptor for vascular endothelial growth factor. Science 1992; (255): 989-991.

Deuel T F, Huang J S, Proffitt R T, Baenziger J U, Chang D, Kennedy B B. Human platelet-derived growth factor. Purification and resolution into two active protein fractions. J Biol Chem 1981; (256): 8896-8899.

Doevendans P A, Jukema W, Spiering W, Defesche J C, Kastelein J J. Molecular genetics and gene expression in atherosclerosis. Int J Cardiol 2001; (80): 161-172.

Dong G, Chen Z, Li Z Y, Yeh N T, Bancroft C C, Van Waes C. Hepatocyte growth factor/scatter factor-induced activation of MEK and PI3K signal pathways contributes to expression of proangiogenic cytokines interleukin-8 and vascular endothelial growth factor in head and neck squamous cell carcinoma. Cancer Res 2001; (61): 5911-5918.

Dor Y, Djonov V, Abramovitch R, Itin A, Fishman G I, Carmeliet P, Goelman G, Keshet E. Conditional switching of VEGF provides new insights into adult neovascularization and pro-angiogenic therapy. EMBO J 2002; (21): 1939-1947.

Ebos J M, Bocci G, Man S, Thorpe P E, Hicklin D J, Zhou D, Jia X, Kerbel R S. A naturally occurring soluble form of vascular endothelial growth factor receptor 2 detected in mouse and human plasma. Mol Cancer Res 2004; (2): 315-326.

Ellegala D B, Leong-Poi H, Carpenter J E, Klibanov A L, Kaul S, Shaffrey M E, Sklenar J, Lindner J R. Imaging tumor angiogenesis with contrast ultrasound and microbubbles targeted to alpha(v)beta3. Circulation 2003; (108): 336-341.

Fadel B M, Boutet S C, Quertermous T. Functional analysis of the endothelial cell-specific Tie2/Tek promoter identifies unique protein-binding elements. Biochem J 1998; (330): 335-343.

Feng D, Nagy J A, Dvorak A M, Dvorak H F. Different pathways of macromolecule extravasation from hyperpermeable tumor vessels. Microvasc Res 2000; (59): 24-37.

Ferrara N. Vascular endothelial growth factor: basic science and clinical progress. Endocr Rev 2004; (25): 581-611.

Ferrara N, Carver-Moore K, Chen H, Dowd M, Lu L, O'Shea K S, Powell-Braxton L, Hillan K J, Moore M W. Heterozygous embryonic lethality induced by targeted inactivation of the VEGF gene. Nature 1996; (380): 439-442.

Ferrara N, Henzel W J. Pituitary follicular cells secrete a novel heparin-binding growth factor specific for vascular endothelial cells. Biochem Biophys Res Commun 1989; (161): 851-858.

Folkman J. Tumor angiogenesis: therapeutic implications. N Engl J Med 1971; (285): 1182-1186.

Frank J M, Kaneko S, Joels C, Tobin G R, Banis J C, Jr., Barker J H. Microcirculation research, angiogenesis, and microsurgery. Microsurgery 1994; (15): 399-404.

Fredriksson L, Li H, Eriksson U. The PDGF family: four gene products form five dimeric isoforms. Cytokine Growth Factor Rev 2004; (15): 197-204.

Fruttiger M, Karlsson L, Hall A C, Abramsson A, Calver A R, Bostrom H, Willetts K, Bertold C H, Heath J K, Betsholtz C, Richardson W D. Defective oligodendrocyte development and severe hypomyelination in PDGF-A knockout mice. Development 1999; (126): 457-467.

Galbraith S M, Lodge M A, Taylor N J, Rustin G J, Bentzen S, Stirling J J, Padhani A R. Reproducibility of dynamic contrast-enhanced MRI in human muscle and tumours: comparison of quantitative and semi-quantitative analysis. NMR Biomed 2002; (15): 132-142.

Gale N W, Thurston G, Hackett S F, Renard R, Wang Q, McClain J, Martin C, Witte C, Witte M H, Jackson D, Suri C, Campochiaro P A, Wiegand S J, Yancopoulos G D. Angiopoietin-2 is required for postnatal angiogenesis and lymphatic patterning, and only the latter role is rescued by Angiopoietin-1. Dev Cell 2002; (3): 411-423.

Galland F, Karamysheva A, Mattei M G, Rosnet O, Marchetto S, Birnbaum D. Chromosomal localization of FLT4, a novel receptor-type tyrosine kinase gene. Genomics 1992; (13): 475-478.

Gerber H P, Dixit V, Ferrara N. Vascular endothelial growth factor induces expression of the antiapoptotic proteins Bcl-2 and A1 in vascular endothelial cells. J Biol Chem 1998a; (273): 13313-13316.

Gerber H P, McMurtrey A, Kowalski J, Yan M, Keyt B A, Dixit V, Ferrara N. Vascular endothelial growth factor regulates endothelial cell survival through the phosphatidylinositol 3'-kinase/Akt signal transduction pathway. Requirement for Flk-1/KDR activation. J Biol Chem 1998b; (273): 30336-30343.

Giatromanolaki A, Sivridis E, Athanassou N, Zois E, Thorpe P E, Brekken R A, Gatter K C, Harris A L, Koukourakis I M, Koukourakis M I. The angiogenic pathway "vascular endothelial growth factor/flk-1(KDR)-receptor" in rheumatoid arthritis and osteoarthritis. J Pathol 2001; (194): 101-108.

Giatromanolaki A, Sivridis E, Maltezos E, Koukourakis M I. Down-regulation of intestinal-type alkaline phosphatase in the tumor vasculature and stroma provides a strong basis for explaining amifostine selectivity. Semin Oncol 2002; (29): 14-21.

Giger R J, Urquhart E R, Gillespie S K, Levengood D V, Ginty D D, Kolodkin A L. Neuropilin-2 is a receptor for semaphorin IV: insight into the structural basis of receptor function and specificity. Neuron 1998; (21): 1079-1092.

Gluzman-Poltorak Z, Cohen T, Herzog Y, Neufeld G. Neuropilin-2 is a receptor for the vascular endothelial growth factor (VEGF) forms VEGF-145 and VEGF-165 [corrected]. J Biol Chem 2000; (275): 18040-18045.

Gowdak L H, Poliakova L, Wang X, Kovesdi I, Fishbein K W, Zacheo A, Palumbo R, Straino S, Emanueli C, Marrocco-Trischitta M, Lakatta E G, Anversa P, Spencer R G, Talan M, Capogrossi M C. Adenovirus-mediated VEGF(121) gene transfer stimulates angiogenesis in normoperfused skeletal muscle and preserves tissue perfusion after induction of ischemia. Circulation 2000; (102): 565-571.

Grech E D. Pathophysiology and investigation of coronary artery disease. BMJ 2003; (326): 1027-1030.

Grieger J C, Samulski R J. Adeno-associated virus as a gene therapy vector: vector development, production and clinical applications. Adv Biochem Eng Biotechnol 2005; (99:119-45.): 119-145.

Grines C L, Watkins M W, Mahmarian J J, Iskandrian A E, Rade J J, Marrott P, Pratt C, Kleiman N. A randomized, double-blind, placebo-controlled trial of Ad5FGF-4 gene therapy and its effect on myocardial perfusion in patients with stable angina. J Am Coll Cardiol 2003; (42): 1339-1347.

Guyton A C, Hall J E. Textbook of Medical Physiology. W.B. Saunders, Philadelphia 2000.

Hacein-Bey-Abina S, von Kalle C, Schmidt M, Le Deist F, Wulffraat N, McIntyre E, Radford I, Villeval J L, Fraser C C, Cavazzana-Calvo M, Fischer A. A serious adverse event after successful gene therapy for X-linked severe combined immunodeficiency. N Engl J Med 2003; (348): 255-256.

Hansen-Smith F M, Blackwell L H, Joswiak G R. Expression of muscle capillary alkaline phosphatase is affected by hypoxia. J Appl Physiol 1992; (73): 776-780.

Hao X, Silva E A, Mansson-Broberg A, Grinnemo K H, Siddiqui A J, Dellgren G, Wardell E, Brodin L A, Mooney D J, Sylven C. Angiogenic effects of sequential release of VEGF-A165 and PDGF-BB with alginate hydrogels after myocardial infarction. Cardiovasc Res 2007; (75): 178-185.

Hattori K, Heissig B, Wu Y, Dias S, Tejada R, Ferris B, Hicklin D J, Zhu Z, Bohlen P, Witte L, Hendrikx J, Hackett N R, Crystal R G, Moore M A, Werb Z, Lyden D, Rafii S. Placental growth factor reconstitutes hematopoiesis by recruiting VEGFR1(+) stem cells from bone-marrow microenvironment. Nat Med 2002; (8): 841-849.

Hawighorst T, Skobe M, Streit M, Hong Y K, Velasco P, Brown L F, Riccardi L, Lange-Asschenfeldt B, Detmar M. Activation of the tie2 receptor by angiopoietin-1 enhances tumor vessel maturation and impairs squamous cell carcinoma growth. Am J Pathol 2002; (160): 1381-1392.

He Y, Kozaki K, Karpanen T, Koshikawa K, Yla-Herttuala S, Takahashi T, Alitalo K. Suppression of tumor lymphangiogenesis and lymph node metastasis by blocking vascular endothelial growth factor receptor 3 signaling. J Natl Cancer Inst 2002; (94): 819-825.

Hedman M, Hartikainen J, Syvanne M, Stjernvall J, Hedman A, Kivela A, Vanninen E, Mussalo H, Kauppila E, Simula S, Narvanen O, Rantala A, Peuhkurinen K, Nieminen M S, Laakso M, Yla-Herttuala S. Safety and feasibility of catheter-based local intracoronary vascular endothelial growth factor gene transfer in the prevention of postangioplasty and in-stent restenosis and in the treatment of chronic myocardial ischemia: phase II results of the Kuopio Angiogenesis Trial (KAT). Circulation 2003; (107): 2677-2683.

Heinonen S E, Leppanen P, Kholova I, Lumivuori H, Hakkinen S K, Bosch F, Laakso M, Yla-Herttuala S. Increased atherosclerotic lesion calcification in a novel mouse model combining insulin resistance, hyperglycemia, and hypercholesterolemia. Circ Res 2007; (101): 1058-1067.

Heldin C H, Backstrom G, Ostman A, Hammacher A, Ronnstrand L, Rubin K, Nister M, Westermark B. Binding of different dimeric forms of PDGF to human fibroblasts: evidence for two separate receptor types. EMBO J 1988; (7): 1387-1393.

Heldin C H, Westermark B, Wasteson A. Platelet-derived growth factor: purification and partial characterization. Proc Natl Acad Sci U S A 1979; (76): 3722-3726.

Hiltunen M O, Turunen M P, Turunen A M, Rissanen T T, Laitinen M, Kosma V M, Yla-Herttuala S. Biodistribution of adenoviral vector to nontarget tissues after local in vivo gene transfer to arterial wall using intravascular and periadventitial gene delivery methods. FASEB J 2000; (14): 2230-2236.

Hoefer I E, van Royen N, Buschmann I R, Piek J J, Schaper W. Time course of arteriogenesis following femoral artery occlusion in the rabbit. Cardiovasc Res 2001; (49): 609-617.

Houck K A, Ferrara N, Winer J, Cachianes G, Li B, Leung D W. The vascular endothelial growth factor family: identification of a fourth molecular species and characterization of alternative splicing of RNA. Mol Endocrinol 1991; (5): 1806-1814.

Ide A, Baker N, Warren S. Vascularization of the Brown Pearce rabbit epithelioma transplant as seen in the transparent ear chamber. Am J Roentgenol 1939; (42): 891-899.

Isbell D C, Berr S S, Toledano A Y, Epstein F H, Meyer C H, Rogers W J, Harthun N L, Hagspiel K D, Weltman A, Kramer C M. Delayed calf muscle phosphocreatine recovery after exercise identifies peripheral arterial disease. J Am Coll Cardiol 2006; (47): 2289-2295.

Isner J M, Baumgartner I, Rauh G, Schainfeld R, Blair R, Manor O, Razvi S, Symes J F. Treatment of thromboangitis obliterans (Buerger's disease) by intramuscular gene transfer of vascular endothelial growth factor: preliminary clinical results. J Vasc Surg 1998; (28): 964-973.

Isner J M, Pieczek A, Schainfeld R, Blair R, Haley L, Asahara T, Rosenfield K, Razvi S, Walsh K, Symes J F. Clinical evidence of angiogenesis after arterial gene transfer of phVEGF165 in patient with ischaemic limb. Lancet 1996; (348): 370-374.

Ito W D, Arras M, Winkler B, Scholz D, Schaper J, Schaper W. Monocyte chemotactic protein-1 increases collateral and peripheral conductance after femoral artery occlusion. Circ Res 1997; (80): 829-837.

Jingjing L, Xue Y, Agarwal N, Roque R S. Human Muller cells express VEGF183, a novel spliced variant of vascular endothelial growth factor. Invest Ophthalmol Vis Sci 1999; (40): 752-759.

Joukov V, Pajusola K, Kaipainen A, Chilov D, Lahtinen I, Kukk E, Saksela O, Kalkkinen N, Alitalo K. A novel vascular endothelial growth factor, VEGF-C, is a ligand for the Flt4 (VEGFR-3) and KDR (VEGFR-2) receptor tyrosine kinases. EMBO J 1996; (15): 290-298.

Joukov V, Sorsa T, Kumar V, Jeltsch M, Claesson-Welsh L, Cao Y, Saksela O, Kalkkinen N, Alitalo K. Proteolytic processing regulates receptor specificity and activity of VEGF-C. EMBO J 1997; (16): 3898-3911.

Kano M R, Morishita Y, Iwata C, Iwasaka S, Watabe T, Ouchi Y, Miyazono K, Miyazawa K. VEGF-A and FGF-2 synergistically promote neoangiogenesis through enhancement of endogenous PDGF-B-PDGFRbeta signaling. J Cell Sci 2005; (118): 3759-3768.

Karkkainen M J, Haiko P, Sainio K, Partanen J, Taipale J, Petrova T V, Jeltsch M, Jackson D G, Talikka M, Rauvala H, Betsholtz C, Alitalo K. Vascular endothelial growth factor C is required for sprouting of the first lymphatic vessels from embryonic veins. Nat Immunol 2004; (5): 74-80.

Karlsson L, Bondjers C, Betsholtz C. Roles for PDGF-A and sonic hedgehog in development of mesenchymal components of the hair follicle. Development 1999; (126): 2611-2621.

Karpanen T, Egeblad M, Karkkainen M J, Kubo H, Yla-Herttuala S, Jaattela M, Alitalo K. Vascular endothelial growth factor C promotes tumor lymphangiogenesis and intralymphatic tumor growth. Cancer Res 2001; (61): 1786-1790.

Karpanen T, Heckman C A, Keskitalo S, Jeltsch M, Ollila H, Neufeld G, Tamagnone L, Alitalo K. Functional interaction of VEGF-C and VEGF-D with neuropilin receptors. FASEB J 2006; (20): 1462-1472.

Karvinen H, Rutanen J, Leppänen O, Lach R, Levonen A-L, Eriksson U, Ylä-Herttuala S. PDGF-C and -D and their receptors PDGFR-a and PDGFR-b in atherosclerotic human arteries. Eur J Clin Invest 2009.

Kastrup J, Jorgensen E, Ruck A, Tagil K, Glogar D, Ruzyllo W, Botker H E, Dudek D, Drvota V, Hesse B, Thuesen L, Blomberg P, Gyongyosi M, Sylven C. Direct intramyocardial plasmid vascular endothelial growth factor-A165 gene therapy in patients with stable severe angina pectoris A randomized double-blind placebo-controlled study: the Euroinject One trial. J Am Coll Cardiol 2005; (45): 982-988.

Kastrup J, Ripa R S, Wang Y, Jorgensen E. Myocardial regeneration induced by granulocyte-colony-stimulating factor mobilization of stem cells in patients with acute or chronic ischaemic heart disease: a non-invasive alternative for clinical stem cell therapy? Eur Heart J 2006; (27): 2748-2754.

Keck P J, Hauser S D, Krivi G, Sanzo K, Warren T, Feder J, Connolly D T. Vascular permeability factor, an endothelial cell mitogen related to PDGF. Science 1989; (246): 1309-1312.

Kendall R L, Thomas K A. Inhibition of vascular endothelial cell growth factor activity by an endogenously encoded soluble receptor. Proc Natl Acad Sci U S A 1993; (90): 10705-10709.

Kiba A, Sagara H, Hara T, Shibuya M. VEGFR-2-specific ligand VEGF-E induces non-edematous hyper-vascularization in mice. Biochem Biophys Res Commun 2003; (301): 371-377.

Korpisalo P, Rissanen TT, Ylä-Herttuala S. The strengths and weaknesses of VEGF adenovirus-driven angiogenesis. In: Therapeutic Neovascularization - Quo Vadis? (Eds.Deindl E, Kupatt C). Springer, 2007; 23-32.

Kovesdi I, Brough D E, Bruder J T, Wickham T J. Adenoviral vectors for gene transfer. Curr Opin Biotechnol 1997; (8): 583-589.

Krix M, Weber M A, Krakowski-Roosen H, Huttner H B, Delorme S, Kauczor H U, Hildebrandt W. Assessment of skeletal muscle perfusion using contrast-enhanced ultrasonography. J Ultrasound Med 2005; (24): 431-441.

Kuukasjarvi P, Salenius J P, Riekkinen H. [Acute ischemia of lower extremities]. Duodecim 1997; (113): 31-37.

Lahteenvuo J E, Lahteenvuo M T, Kivela A, Rosenlew C, Falkevall A, Klar J, Heikura T, Rissanen T T, Vahakangas E, Korpisalo P, Enholm B, Carmeliet P, Alitalo K, Eriksson U, Yla-Herttuala S. Vascular Endothelial Growth Factor-B Induces Myocardium-Specific Angiogenesis and Arteriogenesis via Vascular Endothelial Growth Factor Receptor-1- and Neuropilin Receptor-1-Dependent Mechanisms. Circulation 2009; (119): 845-856.

Laitinen M, Pakkanen T, Donetti E, Baetta R, Luoma J, Lehtolainen P, Viita H, Agrawal R, Miyanohara A, Friedmann T, Risau W, Martin J F, Soma M, Ylä-Herttuala S. Gene transfer into the carotid artery using an adventitial collar: comparison of the effectiveness of the plasmid- liposome complexes, retroviruses, pseudotyped retroviruses, and adenoviruses. Hum Gene Ther 1997; (8): 1645-1650.

Lange T, Guttmann-Raviv N, Baruch L, Machluf M, Neufeld G. VEGF162, a new heparin-binding vascular endothelial growth factor splice form that is expressed in transformed human cells. J Biol Chem 2003; (278): 17164-17169.

Langille B L, O'Donnell F. Reductions in arterial diameter produced by chronic decreases in blood flow are endothelium-dependent. Science 1986; (231): 405-407.

LaRochelle W J, Jeffers M, McDonald W F, Chillakuru R A, Giese N A, Lokker N A, Sullivan C, Boldog F L, Yang M, Vernet C, Burgess C E, Fernandes E, Deegler L L, Rittman B, Shimkets J, Shimkets R A, Rothberg J M, Lichenstein H S. PDGF-D, a new protease-activated growth factor. Nat Cell Biol 2001; (3): 517-521.

Laurema A, Heikkila A, Keski-Nisula L, Heikura T, Lehtolainen P, Manninen H, Tuomisto T T, Heinonen S, Yla-Herttuala S. Transfection of oocytes and other types of ovarian cells in rabbits after direct injection into uterine arteries of adenoviruses and plasmid/liposomes. Gene Ther 2003; (10): 580-584.

Lederman R J, Mendelsohn F O, Anderson R D, Saucedo J F, Tenaglia A N, Hermiller J B, Hillegass W B, Rocha-Singh K, Moon T E, Whitehouse M J, Annex B H. Therapeutic angiogenesis with recombinant fibroblast growth factor-2 for intermittent claudication (the TRAFFIC study): a randomised trial. Lancet 2002; (359): 2053-2058.

Lehrman S. Virus treatment questioned after gene therapy death. Nature 1999; (401): 517-518.

Leung D W, Cachianes G, Kuang W J, Goeddel D V, Ferrara N. Vascular endothelial growth factor is a secreted angiogenic mitogen. Science 1989; (246): 1306-1309.

Levanon K, Varda-Bloom N, Greenberger S, Barshack I, Goldberg I, Orenstein A, Breitbart E, Shaish A, Harats D. Vascular wall maturation and prolonged angiogenic effect by endothelial-specific platelet-derived growth factor expression. Pathobiology 2006; (73): 149-158.

Li H, Fredriksson L, Li X, Eriksson U. PDGF-D is a potent transforming and angiogenic growth factor. Oncogene 2003; (22): 1501-1510.

Li X, Ponten A, Aase K, Karlsson L, Abramsson A, Uutela M, Backstrom G, Hellstrom M, Bostrom H, Li H, Soriano P, Betsholtz C, Heldin C H, Alitalo K, Ostman A, Eriksson U. PDGF-C is a new protease-activated ligand for the PDGF alpha-receptor. Nat Cell Biol 2000; (2): 302-309.

Li X, Tjwa M, Van H, I, Enholm B, Neven E, Paavonen K, Jeltsch M, Juan T D, Sievers R E, Chorianopoulos E, Wada H, Vanwildemeersch M, Noel A, Foidart J M, Springer M L, von Degenfeld G, Dewerchin M, Blau H M, Alitalo K, Eriksson U, Carmeliet P, Moons L. Reevaluation of the role of VEGF-B suggests a restricted role in the revascularization of the ischemic myocardium. Arterioscler Thromb Vasc Biol 2008; (28): 1614-1620.

Libby P. Inflammation in atherosclerosis. Nature 2002; (420): 868-874.

Lindahl P, Johansson B R, Leveen P, Betsholtz C. Pericyte loss and microaneurysm formation in PDGF-B-deficient mice. Science 1997; (277): 242-245.

Lindblom P, Gerhardt H, Liebner S, Abramsson A, Enge M, Hellstrom M, Backstrom G, Fredriksson S, Landegren U, Nystrom H C, Bergstrom G, Dejana E, Ostman A, Lindahl P, Betsholtz C. Endothelial PDGF-B retention is required for proper investment of pericytes in the microvessel wall. Genes Dev 2003; (17): 1835-1840.

Lobov I B, Brooks P C, Lang R A. Angiopoietin-2 displays VEGF-dependent modulation of capillary structure and endothelial cell survival in vivo. Proc Natl Acad Sci U S A 2002; (99): 11205-11210.

Luttun A, Tjwa M, Carmeliet P. Placental growth factor (PlGF) and its receptor Flt-1 (VEGFR-1): novel therapeutic targets for angiogenic disorders. Ann N Y Acad Sci 2002; (979:80-93.): 80-93.

Lymboussaki A, Olofsson B, Eriksson U, Alitalo K. Vascular endothelial growth factor (VEGF) and VEGF-C show overlapping binding sites in embryonic endothelia and distinct sites in differentiated adult endothelia. Circ Res 1999; (85): 992-999.

Lyttle D J, Fraser K M, Fleming S B, Mercer A A, Robinson A J. Homologs of vascular endothelial growth factor are encoded by the poxvirus orf virus. J Virol 1994; (68): 84-92.

Maglione D, Guerriero V, Viglietto G, Delli-Bovi P, Persico M G. Isolation of a human placenta cDNA coding for a protein related to the vascular permeability factor. Proc Natl Acad Sci U S A 1991; (88): 9267-9271.

Maglione D, Guerriero V, Viglietto G, Ferraro M G, Aprelikova O, Alitalo K, Del Vecchio S, Lei K J, Chou J Y, Persico M G. Two alternative mRNAs coding for the angiogenic factor, placenta growth factor (PlGF), are transcribed from a single gene of chromosome 14. Oncogene 1993; (8): 925-931.

Maisonpierre P C, Suri C, Jones P F, Bartunkova S, Wiegand S J, Radziejewski C, Compton D, McClain J, Aldrich T H, Papadopoulos N, Daly T J, Davis S, Sato T N, Yancopoulos G D. Angiopoietin-2, a natural antagonist for Tie2 that disrupts in vivo angiogenesis. Science 1997; (277): 55-60.

Makinen K, Manninen H, Hedman M, Matsi P, Mussalo H, Alhava E, Yla-Herttuala S. Increased Vascularity Detected by Digital Subtraction Angiography after VEGF Gene Transfer to Human Lower Limb Artery: A Randomized, Placebo-Controlled, Double-Blinded Phase II Study. Mol Ther 2002; (6): 127-133.

Makinen T, Veikkola T, Mustjoki S, Karpanen T, Catimel B, Nice E C, Wise L, Mercer A, Kowalski H, Kerjaschki D, Stacker S A, Achen M G, Alitalo K. Isolated lymphatic endothelial cells transduce growth, survival and migratory signals via the VEGF-C/D receptor VEGFR-3. EMBO J 2001; (20): 4762-4773.

Miller J C, Pien H H, Sahani D, Sorensen A G, Thrall J H. Imaging angiogenesis: applications and potential for drug development. J Natl Cancer Inst 2005; (97): 172-187.

Millette E, Rauch B H, Defawe O, Kenagy R D, Daum G, Clowes A W. Platelet-derived growth factor-BB-induced human smooth muscle cell proliferation depends on basic FGF release and FGFR-1 activation. Circ Res 2005; (96): 172-179.

Mould A W, Tonks I D, Cahill M M, Pettit A R, Thomas R, Hayward N K, Kay G F. Vegfb gene knockout mice display reduced pathology and synovial angiogenesis in both antigen-induced and collagen-induced models of arthritis. Arthritis Rheum 2003; (48): 2660-2669.

Murakami M, Simons M. Fibroblast growth factor regulation of neovascularization. Curr Opin Hematol 2008; (15): 215-220.

Muruve D A. The innate immune response to adenovirus vectors. Hum Gene Ther 2004; (15): 1157-1166.

Nauck M, Karakiulakis G, Perruchoud A P, Papakonstantinou E, Roth M. Corticosteroids inhibit the expression of the vascular endothelial growth factor gene in human vascular smooth muscle cells. Eur J Pharmacol 1998; (341): 309-315.

Neufeld G, Kessler O, Herzog Y. The interaction of Neuropilin-1 and Neuropilin-2 with tyrosine-kinase receptors for VEGF. Adv Exp Med Biol 2002; (515:81-90.): 81-90.

Norgren L, Hiatt W R, Dormandy J A, Nehler M R, Harris K A, Fowkes F G, Bell K, Caporusso J, Durand-Zaleski I, Komori K, Lammer J, Liapis C, Novo S, Razavi M, Robbs J, Schaper N, Shigematsu H, Sapoval M, White C, White J, Clement D, Creager M, Jaff M, Mohler E, III, Rutherford R B, Sheehan P, Sillesen H, Rosenfield K. Inter-Society Consensus for the Management of Peripheral Arterial Disease (TASC II). Eur J Vasc Endovasc Surg 2007; (33 Suppl 1:S1-75. Epub;2006 Nov 29.): S1-75.

Ny A, Autiero M, Carmeliet P. Zebrafish and Xenopus tadpoles: small animal models to study angiogenesis and lymphangiogenesis. Exp Cell Res 2006; (312): 684-693.

Odorisio T, Schietroma C, Zaccaria M L, Cianfarani F, Tiveron C, Tatangelo L, Failla C M, Zambruno G. Mice overexpressing placenta growth factor exhibit increased vascularization and vessel permeability. J Cell Sci 2002; (115): 2559-2567.

Ogawa S, Oku A, Sawano A, Yamaguchi S, Yazaki Y, Shibuya M. A novel type of vascular endothelial growth factor, VEGF-E (NZ-7 VEGF), preferentially utilizes KDR/Flk-1 receptor and carries a potent mitotic activity without heparin-binding domain. J Biol Chem 1998; (273): 31273-31282.

Olofsson B, Pajusola K, Kaipainen A, von Euler G, Joukov V, Saksela O, Orpana A, Pettersson R F, Alitalo K, Eriksson U. Vascular endothelial growth factor B, a novel growth factor for endothelial cells. Proc Natl Acad Sci U S A 1996a; (93): 2576-2581.

Olofsson B, Pajusola K, von Euler G, Chilov D, Alitalo K, Eriksson U. Genomic organization of the mouse and human genes for vascular endothelial growth factor B (VEGF-B) and characterization of a second splice isoform. J Biol Chem 1996b; (271): 19310-19317.

Orden M R, Jurvelin J S, Kirkinen P P. Kinetics of a US contrast agent in benign and malignant adnexal tumors. Radiology 2003; (226): 405-410.

Orecchia A, Lacal P M, Schietroma C, Morea V, Zambruno G, Failla C M. Vascular endothelial growth factor receptor-1 is deposited in the extracellular matrix by endothelial cells and is a ligand for the alpha 5 beta 1 integrin. J Cell Sci 2003; (116): 3479-3489.

Ornitz D M, Xu J, Colvin J S, McEwen D G, MacArthur C A, Coulier F, Gao G, Goldfarb M. Receptor specificity of the fibroblast growth factor family. J Biol Chem 1996; (271): 15292-15297.

Oura H, Bertoncini J, Velasco P, Brown L F, Carmeliet P, Detmar M. A critical role of placental growth factor in the induction of inflammation and edema formation. Blood 2003; (101): 560-567.

Ozawa C R, Banfi A, Glazer N L, Thurston G, Springer M L, Kraft P E, McDonald D M, Blau H M. Microenvironmental VEGF concentration, not total dose, determines a threshold between normal and aberrant angiogenesis. J Clin Invest 2004; (113): 516-527.

Pajusola K, Aprelikova O, Korhonen J, Kaipainen A, Pertovaara L, Alitalo R, Alitalo K. FLT4 receptor tyrosine kinase contains seven immunoglobulin-like loops and is expressed in multiple human tissues and cell lines. Cancer Res 1992; (52): 5738-5743.

Pajusola K, Kunnapuu J, Vuorikoski S, Soronen J, Andre H, Pereira T, Korpisalo P, Yla-Herttuala S, Poellinger L, Alitalo K. Stabilized HIF-1alpha is superior to VEGF for angiogenesis in skeletal muscle via adeno-associated virus gene transfer. FASEB J 2005; (19): 1365-1367.

Papapetropoulos A, Fulton D, Mahboubi K, Kalb R G, O'Connor D S, Li F, Altieri D C, Sessa W C. Angiopoietin-1 inhibits endothelial cell apoptosis via the Akt/survivin pathway. J Biol Chem 2000; (275): 9102-9105.

Partanen T A, Arola J, Saaristo A, Jussila L, Ora A, Miettinen M, Stacker S A, Achen M G, Alitalo K. VEGF-C and VEGF-D expression in neuroendocrine cells and their receptor, VEGFR-3, in fenestrated blood vessels in human tissues. FASEB J 2000; (14): 2087-2096.

Pettersson A, Nagy J A, Brown L F, Sundberg C, Morgan E, Jungles S, Carter R, Krieger J E, Manseau E J, Harvey V S, Eckelhoefer I A, Feng D, Dvorak A M, Mulligan R C, Dvorak H F. Heterogeneity of the angiogenic response induced in different normal adult tissues by vascular permeability factor/vascular endothelial growth factor. Lab Invest 2000; (80): 99-115.

Phillips P, Gardner E. Contrast-agent detection and quantification. Eur Radiol 2004; (14 Suppl 8:P4-10.): 4-10.

Pipp F, Heil M, Issbrucker K, Ziegelhoeffer T, Martin S, Van Den H J, Weich H, Fernandez B, Golomb G, Carmeliet P, Schaper W, Clauss M. VEGFR-1-selective VEGF homologue PlGF is arteriogenic. Evidence for a monocyte-mediated mechanism. Circ Res 2003; (92): 378-385.

Poltorak Z, Cohen T, Sivan R, Kandelis Y, Spira G, Vlodavsky I, Keshet E, Neufeld G. VEGF145, a secreted vascular endothelial growth factor isoform that binds to extracellular matrix. J Biol Chem 1997; (272): 7151-7158.

Purhonen S, Palm J, Rossi D, Kaskenpaa N, Rajantie I, Yla-Herttuala S, Alitalo K, Weissman I L, Salven P. Bone marrow-derived circulating endothelial precursors do not contribute to vascular endothelium and are not needed for tumor growth. Proc Natl Acad Sci U S A 2008; (105): 6620-6625.

Rafii S, Lyden D. Therapeutic stem and progenitor cell transplantation for organ vascularization and regeneration. Nat Med 2003; (9): 702-712.

Rajagopalan S, Trachtenberg J, Mohler E, Olin J, McBride S, Pak R, Rasmussen H, Crystal R. Phase I study of direct administration of a replication deficient adenovirus vector containing the

vascular endothelial growth factor cDNA (CI-1023) to patients with claudication. Am J Cardiol 2002; (90): 512-516.

Raty J K, Lesch H P, Wirth T, Yla-Herttuala S. Improving safety of gene therapy. Curr Drug Saf 2008; (3): 46-53.

Rehman J, Li J, Orschell C M, March K L. Peripheral blood "endothelial progenitor cells" are derived from monocyte/macrophages and secrete angiogenic growth factors. Circulation 2003; (107): 1164-1169.

Richardson T P, Peters M C, Ennett A B, Mooney D J. Polymeric system for dual growth factor delivery. Nat Biotechnol 2001; (19): 1029-1034.

Risau W. Mechanisms of angiogenesis. Nature 1997; (386): 671-674.

Risau W, Flamme I. Vasculogenesis. Annu Rev Cell Dev Biol 1995; (11:73-91): 73-91.

Rissanen T T, Markkanen J E, Arve K, Rutanen J, Kettunen M I, Vajanto I, Jauhiainen S, Cashion L, Gruchala M, Narvanen O, Taipale P, Kauppinen R A, Rubanyi G M, Yla-Herttuala S. Fibroblast growth factor-4 induces vascular permeability, angiogenesis, and arteriogenesis in a rabbit hind limb ischemia model. FASEB J 2003a; (17): 100-102.

Rissanen T T, Markkanen J E, Gruchala M, Heikura T, Puranen A, Kettunen M I, Kholova I, Kauppinen R A, Achen M G, Stacker S A, Alitalo K, Yla-Herttuala S. VEGF-D is the strongest angiogenic and lymphangiogenic effector among VEGFs delivered into skeletal muscle via adenoviruses. Circ Res 2003b; (92): 1098-1106.

Rissanen T T, Vajanto I, Hiltunen M O, Rutanen J, Kettunen M I, Niemi M, Leppanen P, Turunen M P, Markkanen J E, Arve K, Alhava E, Kauppinen R A, Yla-Herttuala S. Expression of Vascular Endothelial Growth Factor and Vascular Endothelial Growth Factor Receptor-2 (KDR/Flk-1) in Ischemic Skeletal Muscle and Its Regeneration. Am J Pathol 2002; (160): 1393-1403.

Rissanen T T, Yla-Herttuala S. Current status of cardiovascular gene therapy. Mol Ther 2007; 1233-1247.

Ross R. Mechanisms of disease - Atherosclerosis - An inflammatory disease. N Engl J Med 1999; (340): 115-126.

Ross R, Glomset J, Kariya B, Harker L. A platelet-dependent serum factor that stimulates the proliferation of arterial smooth muscle cells in vitro. Proc Natl Acad Sci U S A 1974; (71): 1207-1210.

Roy H, Bhardwaj S, Babu M, Kokina I, Uotila S, Ahtialansaari T, Laitinen T, Hakumaki J, Laakso M, Herzig K H, Yla-Herttuala S. VEGF-A, VEGF-D, VEGF receptor-1, VEGF receptor-2, NF-kappaB, and RAGE in atherosclerotic lesions of diabetic Watanabe heritable hyperlipidemic rabbits. FASEB J 2006; (20): 2159-2161.

Ruhrberg C, Gerhardt H, Golding M, Watson R, Ioannidou S, Fujisawa H, Betsholtz C, Shima D T. Spatially restricted patterning cues provided by heparin-binding VEGF-A control blood vessel branching morphogenesis. Genes Dev 2002; (16): 2684-2698.

Rutanen J, Leppanen P, Tuomisto T T, Rissanen T T, Hiltunen M O, Vajanto I, Niemi M, Hakkinen T, Karkola K, Stacker S A, Achen M G, Alitalo K, Yla-Herttuala S. Vascular endothelial growth factor-D expression in human atherosclerotic lesions. Cardiovasc Res 2003; (59): 971-979.

Rutanen J, Rissanen T T, Markkanen J E, Gruchala M, Silvennoinen P, Kivela A, Hedman A, Hedman M, Heikura T, Orden M R, Stacker S A, Achen M G, Hartikainen J, Yla-Herttuala S. Adenoviral Catheter-Mediated Intramyocardial Gene Transfer Using the Mature Form of Vascular Endothelial Growth Factor -D Induces Transmural Angiogenesis in Porcine Heart. Circulation 2004; (109): 1029-1035.

Saharinen P, Tammela T, Karkkainen M J, Alitalo K. Lymphatic vasculature: development, molecular regulation and role in tumor metastasis and inflammation. Trends Immunol 2004; (25): 387-395.

Saltin B, Radegran G, Koskolou M D, Roach R C. Skeletal muscle blood flow in humans and its regulation during exercise. Acta Physiol Scand 1998; (162): 421-436.

Schaper W, Scholz D. Factors regulating arteriogenesis. Arterioscler Thromb Vasc Biol 2003; (23): 1143-1151.

Scholte op Reimer WJM, Gitt AK, Boersma E, Simoons ML (eds.). Cardiovascular Diseases in Europe, Euro Heart Survey. Sophia Antipolis; European Society of Cardiology 2006.

Senger D R, Galli S J, Dvorak A M, Perruzzi C A, Harvey V S, Dvorak H F. Tumor cells secrete a vascular permeability factor that promotes accumulation of ascites fluid. Science 1983; (219): 983-985.

Shalaby F, Rossant J, Yamaguchi T P, Gertsenstein M, Wu X F, Breitman M L, Schuh A C. Failure of blood-island formation and vasculogenesis in Flk-1-deficient mice. Nature 1995; (376): 62-66.

Shim W S, Teh M, Bapna A, Kim I, Koh G Y, Mack P O, Ge R. Angiopoietin 1 promotes tumor angiogenesis and tumor vessel plasticity of human cervical cancer in mice. Exp Cell Res 2002; (279): 299-309.

Shyu K G, Manor O, Magner M, Yancopoulos G D, Isner J M. Direct intramuscular injection of plasmid DNA encoding angiopoietin-1 but not angiopoietin-2 augments revascularization in the rabbit ischemic hindlimb. Circulation 1998; (98): 2081-2087.

Skobe M, Hawighorst T, Jackson D G, Prevo R, Janes L, Velasco P, Riccardi L, Alitalo K, Claffey K, Detmar M. Induction of tumor lymphangiogenesis by VEGF-C promotes breast cancer metastasis. Nat Med 2001; (7): 192-198.

Sleeman M, Fraser J, McDonald M, Yuan S, White D, Grandison P, Kumble K, Watson J D, Murison J G. Identification of a new fibroblast growth factor receptor, FGFR5. Gene 2001; (271): 171-182.

Soker S, Takashima S, Miao H Q, Neufeld G, Klagsbrun M. Neuropilin-1 is expressed by endothelial and tumor cells as an isoform-specific receptor for vascular endothelial growth factor. Cell 1998; (%20;92): 735-745.

Soriano P. Abnormal kidney development and hematological disorders in PDGF beta-receptor mutant mice. Genes Dev 1994; (8): 1888-1896.

Soriano P. The PDGF alpha receptor is required for neural crest cell development and for normal patterning of the somites. Development 1997; (124): 2691-2700.

Sorlie D, Straume B, Grimsgaard C, Johnsrud N K. Arterial collateral vessels in legs with obliterative arteriosclerosis. Scand J Clin Lab Invest 1978; (38): 361-367.

Stacker S A, Caesar C, Baldwin M E, Thornton G E, Williams R A, Prevo R, Jackson D G, Nishikawa S S, Kubo H, Achen M G. VEGF-D promotes the metastatic spread of tumor cells via the lymphatics. Nat Med 2001; (7): 186-191.

Stacker S A, Stenvers K, Caesar C, Vitali A, Domagala T, Nice E, Roufail S, Simpson R J, Moritz R, Karpanen T, Alitalo K, Achen M G. Biosynthesis of vascular endothelial growth factor-D involves proteolytic processing which generates non-covalent homodimers. J Biol Chem 1999; (274): 32127-32136.

Stavri G T, Zachary I C, Baskerville P A, Martin J F, Erusalimsky J D. Basic fibroblast growth factor upregulates the expression of vascular endothelial growth factor in vascular smooth muscle cells. Synergistic interaction with hypoxia. Circulation 1995; (92): 11-14.

Stewart D J, Hilton J D, Arnold J M, Gregoire J, Rivard A, Archer S L, Charbonneau F, Cohen E, Curtis M, Buller C E, Mendelsohn F O, Dib N, Page P, Ducas J, Plante S, Sullivan J, Macko J, Rasmussen C, Kessler P D, Rasmussen H S. Angiogenic gene therapy in patients with nonrevascularizable ischemic heart disease: a phase 2 randomized, controlled trial of AdVEGF(121) (AdVEGF121) versus maximum medical treatment. Gene Ther 2006; (13): 1503-1511.

Storkebaum E, Lambrechts D, Carmeliet P. VEGF: once regarded as a specific angiogenic factor, now implicated in neuroprotection. Bioessays 2004; (26): 943-954.

Su E J, Cioffi C L, Stefansson S, Mittereder N, Garay M, Hreniuk D, Liau G. Gene therapy vector-mediated expression of insulin-like growth factors protects cardiomyocytes from apoptosis and enhances neovascularization. Am J Physiol Heart Circ Physiol 2003; (284): H1429-H1440.

Suri C, McClain J, Thurston G, McDonald D M, Zhou H, Oldmixon E H, Sato T N, Yancopoulos G D. Increased vascularization in mice overexpressing angiopoietin-1. Science 1998; (282): 468-471.

Svet-Moldavsky G J, Chimishkyan K L. Tumour angiogenesis factor for revascularisation in ischaemia and myocardial infarction. Lancet 1977; (1): 913.

Sylven C, Sarkar N, Insulander P, Kenneback G, Blomberg P, Islam K, Drvota V. Catheter-based transendocardial myocardial gene transfer. J Interv Cardiol 2002; (15): 7-13.

Tallquist M, Kazlauskas A. PDGF signaling in cells and mice. Cytokine Growth Factor Rev 2004; (15): 205-213.

Terman B I, Dougher-Vermazen M, Carrion M E, Dimitrov D, Armellino D C, Gospodarowicz D, Bohlen P. Identification of the KDR tyrosine kinase as a receptor for vascular endothelial cell growth factor. Biochem Biophys Res Commun 1992; (187): 1579-1586.

Thijssen J M, de Korte C L. Modeling ultrasound contrast measurement of blood flow and perfusion in biological tissue. Ultrasound Med Biol 2005; (31): 279-285.

Thurston G, Suri C, Smith K, McClain J, Sato T N, Yancopoulos G D, McDonald D M. Leakage-resistant blood vessels in mice transgenically overexpressing angiopoietin-1. Science 1999; (286): 2511-2514.

Tille J C, Wood J, Mandriota S J, Schnell C, Ferrari S, Mestan J, Zhu Z, Witte L, Pepper M S. Vascular endothelial growth factor (VEGF) receptor-2 antagonists inhibit VEGF- and basic fibroblast growth factor-induced angiogenesis in vivo and in vitro. J Pharmacol Exp Ther 2001; (299): 1073-1085.

Tischer E, Mitchell R, Hartman T, Silva M, Gospodarowicz D, Fiddes J C, Abraham J A. The human gene for vascular endothelial growth factor. Multiple protein forms are encoded through alternative exon splicing. J Biol Chem 1991; (266): 11947-11954.

Topsakal R, Eryol N K, Abaci A, Oymak S, Ozdogru I, Yilmaz Y, Seyfeli E, Oguzhan A, Ergin A. The relation between chronic obstructive pulmonary disease and coronary collateral vessels. Angiology 2005; (56): 651-656.

Toyoda M, Takayama H, Horiguchi N, Otsuka T, Fukusato T, Merlino G, Takagi H, Mori M. Overexpression of hepatocyte growth factor/scatter factor promotes vascularization and granulation tissue formation in vivo. FEBS Lett 2001; (509): 95-100.

Vajanto I, Rissanen T T, Rutanen J, Hiltunen M O, Tuomisto T T, Arve K, Narvanen O, Manninen H, Rasanen H, Hippelainen M, Alhava E, Yla-Herttuala S. Evaluation of angiogenesis and side effects in ischemic rabbit hindlimbs after intramuscular injection of adenoviral vectors encoding VEGF and LacZ. J Gene Med 2002; (4): 371-380.

Visconti R P, Richardson C D, Sato T N. Orchestration of angiogenesis and arteriovenous contribution by angiopoietins and vascular endothelial growth factor (VEGF). Proc Natl Acad Sci U S A 2002; (99): 8219-8224.

Wahlberg E. Angiogenesis and arteriogenesis in limb ischemia. J Vasc Surg 2003; (38): 198-203.

Wang L, Dutta S K, Kojima T, Xu X, Khosravi-Far R, Ekker S C, Mukhopadhyay D. Neuropilin-1 modulates p53/caspases axis to promote endothelial cell survival. PLoS ONE 2007; (2): e1161.

Wang L, Zeng H, Wang P, Soker S, Mukhopadhyay D. Neuropilin-1-mediated vascular permeability factor/vascular endothelial growth factor-dependent endothelial cell migration. J Biol Chem 2003; (278): 48848-48860.

Watson J D, Gilman M, Witkowski J, Zoller M. Recombinant DNA. Scientific American books, Inc., New York 1992.

White F C, Bloor C M, McKirnan M D, Carroll S M. Exercise training in swine promotes growth of arteriolar bed and capillary angiogenesis in heart. J Appl Physiol 1998; (85): 1160-1168.

Whitlock P R, Hackett N R, Leopold P L, Rosengart T K, Crystal R G. Adenovirus-mediated transfer of a minigene expressing multiple isoforms of VEGF is more effective at inducing angiogenesis than comparable vectors expressing individual VEGF cDNAs. Mol Ther 2004; (9): 67-75.

Wickham T J. Targeting adenovirus. Gene Ther 2000; (7): 110-114.

Wickham T J, Mathias P, Cheresh D A, Nemerow G R. Integrins alpha v beta 3 and alpha v beta 5 promote adenovirus internalization but not virus attachment. Cell 1993; (73): 309-319.

Wise L M, Veikkola T, Mercer A A, Savory L J, Fleming S B, Caesar C, Vitali A, Makinen T, Alitalo K, Stacker S A. Vascular endothelial growth factor (VEGF)-like protein from orf virus NZ2 binds to VEGFR2 and neuropilin-1. Proc Natl Acad Sci U S A 1999; (96): 3071-3076.

Yablonka-Reuveni Z, Seger R, Rivera A J. Fibroblast growth factor promotes recruitment of skeletal muscle satellite cells in young and old rats. J Histochem Cytochem 1999; (47): 23-42.

Yamada Y, Nezu J, Shimane M, Hirata Y. Molecular cloning of a novel vascular endothelial growth factor, VEGF-D. Genomics 1997; (42): 483-488.

Yamazaki Y, Takani K, Atoda H, Morita T. Snake venom vascular endothelial growth factors (VEGFs) exhibit potent activity through their specific recognition of KDR (VEGF receptor 2). J Biol Chem 2003; (278): 51985-51988.

Yamazaki Y, Tokunaga Y, Takani K, Morita T. Identification of the heparin-binding region of snake venom vascular endothelial growth factor (VEGF-F) and its blocking of VEGF-A165. Biochemistry 2005; (44): 8858-8864.

Yang W, Ahn H, Hinrichs M, Torry R J, Torry D S. Evidence of a novel isoform of placenta growth factor (PlGF-4) expressed in human trophoblast and endothelial cells. J Reprod Immunol 2003; (60): 53-60.

Yla-Herttuala S, Alitalo K. Gene transfer as a tool to induce therapeutic vascular growth. Nat Med 2003; (9): 694-701.

Ylä-Herttuala S, Lipton B A, Rosenfeld M E, Särkioja T, Yoshimura T, Leonard E J, Witztum J L, Steinberg D. Expression of monocyte chemoattractant protein 1 in macrophage- rich areas of human and rabbit atherosclerotic lesions. Proc Natl Acad Sci U S A 1991; (88): 5252-5256.

Yla-Herttuala S, Rissanen T T, Vajanto I, Hartikainen J. Vascular endothelial growth factors: biology and current status of clinical applications in cardiovascular medicine. J Am Coll Cardiol 2007; (49): 1015-1026.

Yuan L, Moyon D, Pardanaud L, Breant C, Karkkainen M J, Alitalo K, Eichmann A. Abnormal lymphatic vessel development in neuropilin 2 mutant mice. Development 2002; (129): 4797-4806.

Zheng Y, Murakami M, Takahashi H, Yamauchi M, Kiba A, Yamaguchi S, Yabana N, Alitalo K, Shibuya M. Chimeric VEGF-E(NZ7)/PlGF promotes angiogenesis via VEGFR-2 without significant enhancement of vascular permeability and inflammation. Arterioscler Thromb Vasc Biol 2006; (26): 2019-2026.

# Kuopio University Publications G. - A.I.Virtanen Institute

**G 51. Keinänen, Riitta et al. (eds.).** The first annual post-graduate symposium of the graduate school of molecular medicine: winter school 2007.
2007. 65 p. Abstracts.

**G 52. Vartiainen, Suvi.** Caenorhabditis elegans as a model for human synucleopathies.
2007. 94 p. Acad. Diss.

**G 53. Määttä, Ann-Marie.** Development of gene and virotherapy against non-small cell lung cancer.
2007. 75 p. Acad. Diss.

**G 54. Rautsi, Outi.** Hurdles and Improvements in Therapeutic Gene Transfer for Cancer.
2007. 79 p. Acad. Diss.

**G 55. Pehkonen, Petri.** Methods for mining data from genome wide high-throughput technologies.
2007. 91 p. Acad. Diss.

**G 56. Hyvönen, Mervi T.** Regulation of spermidine/spermine N'-acetyltransferase and its involvement in cellular proliferation and development of acute pancreatitis.
2007. 79 p. Acad. Diss.

**G 57. Gurevicius, Kestutis.** EEG and evoked potentials as indicators of interneuron pathology in mouse models of neurological diseases.
2007. 76 p. Acad. Diss.

**G 58. Leppänen, Pia.** Mouse models of atherosclerosis, vascular endothelial growth factors and gene therapy.
2007. 91 p. Acad. Diss.

**G 59. Keinänen, Riitta et al.** The second annual post-graduate symposium of the graduate school of molecular medicine: winter school 2008.
2008. 57 p. Abstracts.

**G 60. Koponen, Jonna.** Lentiviral vector for gene transfer: a versatile tool for regulated gene expression, gene silencing and progenitor cell therapies.
2008. 71 p. Acad. Diss.

**G 61. Ahtoniemi, Toni.** Mutant Cu,Zn superoxide dismutase in amyotrophic lateral sclerosis: molecular mechanisms of neurotoxicity.
2008. 107 p. Acad. Diss.

**G 62. Purhonen, Anna-Kaisa.** Signals arising from the gastointestinal tract that affect food intake.
2008. 86 p. Acad. Diss.

**G 63. Kaikkonen, Minna.** Engineering baculo- and lentiviral vectors for enhanced and targeted gene delivery.
2008. 109 p. Acad. Diss.

**G 64. Gureviciene, Irina.** Changes in hippocampal synaptic plasticity in animal models of age-related memory impairment. 2
2008. 106 p. Acad. Diss.

**G 65. Oikari, Sanna.** Evaluation of phenotypic changes of Acyl-CoA binding protein / diazepam binding inhibitor overexpression in transgenic mice and rats.
2008. 79 p. Acad. Diss.

**G 66. Laurema, Anniina.** Adenoviral gene therapy and fertility: distribution studies in reproductive organs and risk of vertical transmission in female rabbits and rats.
2008. 79 p. Acad. Diss.