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JOHANNA LÄHTEENVUO (née MARKKANEN)

Gene Therapy for Ischemic Diseases

VEGFs in Myocardial and Peripheral Ischemia

Doctoral dissertation

To be presented by permission of the Faculty of Medicine of the University of Kuopio for public examination in Auditorium, Mediteknia building, University of Kuopio, on Friday 11th September 2009, at 12 noon

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

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ISBN 978-951-27-1133-8
ISBN 978-951-27-1114-7 (PDF)
ISSN 1458-7335

Kopijyvä
Kuopio 2009
Finland

Lähteenvuo, Johanna. Gene Therapy for Ischemic Diseases. VEGFs in Myocardial and Peripheral Ischemia. Kuopio University Publications G. – A.I.Virtanen Institute for Molecular Sciences 74. 2009. 103 p.
ISBN 978-951-27-1133-8
ISBN 978-951-27-1114-7 (PDF)
ISSN 1458-7335

ABSTRACT

Gene therapy is a promising new treatment option for ischemic heart disease and peripheral arterial disease. Viral vectors are used to deliver therapeutic genes to ischemic tissue, and transduced cells begin to produce therapeutic proteins locally. Ischemia is a result of insufficient blood flow, and induction of blood vessel growth in the ischemic tissue is a potential treatment option for ischemic diseases. Vascular endothelial growth factors (VEGFs) regulate blood and lymphatic vessel growth both during development and as an endogenous response to ischemia. Viral delivery of VEGFs could therefore be used to treat ischemic diseases.

Vascular endothelial growth factors mediate their effects via VEGF receptors (VEGFR-1-3) and their effects vary from angiogenic to lymphangiogenic responses. In this study we first compared these growth factors by adenovirally expressing them in a rabbit model of hind limb ischemia. VEGF-$D^{\Delta N \Delta C}$ was the most potent angiogenic factor and VEGF-D^{full} produced the strongest lymphangiogenic response. Angiogenic factors VEGF-A and VEGF-$D^{\Delta N \Delta C}$ increased vascular permeability and edema as a side effect. VEGF-C^{full}, VEGF-C_{156S} and VEGF-D^{full} only induced growth of lymphatic vessels.

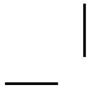
The effects of VEGFR-2 ligands VEGF-A and VEGF-$D^{\Delta N \Delta C}$ were studied in normoxic pig myocardium. Adenoviruses were injected intramyocardially via a NOGA® injection catheter. Both VEGF-A and VEGF-$D^{\Delta N \Delta C}$ induced angiogenesis in a dose-dependent manner and increased perfusion as measured by contrast-enhanced ultrasound imaging. Both growth factors also increased plasma protein extravasation, and larger doses led to accumulation of pericardial effusion.

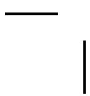
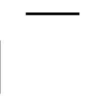
VEGFR-1 ligands VEGF-A and PlGF induced angiogenesis both in skeletal muscle and myocardium via a VEGFR-2 dependent pathway, while VEGF-B induced blood vessel growth only in the myocardium. This tissue-specific angiogenic response was mediated via a new VEGFR-1 and Nrp-1 dependent pathway. VEGF-B also induced growth of collateral arteries in the infarction border zone and improved left ventricular ejection fraction. In addition, VEGF-B induced metabolic changes in the myocardial cells and inhibited apoptosis in the infarction border zone.

As increased vascular permeability, edema and accumulation of pericardial effusion are serious side effects of angiogenic gene therapy, we also explored the possibility to alleviate edema by induction of lymphatic vessel growth. Combination gene therapy with VEGF-$D^{\Delta N \Delta C}$ and VEGF-D^{full} increased angiogenesis but decreased vascular permeability by 60% as compared to VEGF-$D^{\Delta N \Delta C}$ monotherapy in rabbit skeletal muscles.

In conclusion, adenoviral delivery of VEGFs is a potent tool to treat ischemia both in myocardium and peripheral muscles. VEGFs can also be used to alleviate the side effects of angiogenic gene therapy by induction of lymphatic vessel growth.

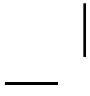
National Library of Medicine Classification: WG 300, WG 104, QU 107, QW 165.5.A3
Medical Subject Headings: Gene Therapy; Myocardial Ischemia; Peripheral Vascular Diseases; Angiogenesis Inducing Agents; Vascular Endothelial Growth Factors; Receptors, Vascular Endothelial Growth Factor; Neuropilins; Adenoviridae; Rabbits; Swine

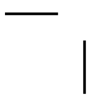
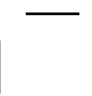


“By three methods we may learn wisdom: First, by reflection, which is noblest; second, by imitation, which is easiest; and third, by experience, which is the bitterest."

Kung Fu-tse
(ca. 551—478 BCE)



ACKNOWLEDGEMENTS

This study was carried out at the Department of Biotechnology and Molecular Medicine, A.I.Virtanen Institute, University of Kuopio during the years 2001-2009. I want to acknowledge the people who have made this study possible.

I've had the great privilege to work in Seppo Ylä-Herttuala's group. Seppo's enthusiasm, optimism and endless trust on PhD students' scientific capabilities have created a unique environment for learning independent research. I wish to thank my other supervisor docent Marja Hedman for her friendly help and the clinical perspective she has provided.

I thank the official reviewers of this thesis MD, PhD Mikko Savontaus and PhD Marika Kärkkäinen for the comments and suggestions that greatly helped to improve this thesis. Professor Kirsi Vähäkangas gave me wise suggestions in the beginning of the writing process.

I have been privileged to collaborate with true experts in growth factor biology, Dr. Marc Achen, professor Kari Alitalo and Dr. Peter Carmeliet. I'm deeply grateful for the expert help of Dr. Carolina Rosenlew and professor Ulf Eriksson on VEGF-B work.

I'm deeply grateful for all the great people I've worked with over these years. Tuomas Rissanen and Juha Rutanen hired me and introduced me to the field of gene therapy. Tuomas' critical thinking and ambitious attitude have taught me how science should be done and Juha showed that science doesn't need to be dead serious. Tommi Heikura has been a great colleague, invaluable source of information and skillful animal surgeon over the years. I thank Antti Kivelä for interesting conversations and for teaching me invasive cardiology. I wish to thank all the wonderful summer workers Antti Puranen, Johanna Kilpijoki, Johanna Ylönen, Teemu Takalo, Atte Räty, Anssi Uutela, Tessa Heino, Valtteri Reinman and Mikko Taina for their contribution to my projects. Maarit Kosonen, Essi Kattainen and Krista Honkonen not only made it through the summer but also have joined our team, thank you!

I want to thank the past and present members of the SYH group for a wonderful working environment, all the help in the lab and great time after work. I acknowledge Seija Sahrio, Tiina Koponen, Sari Järveläinen and Mervi Nieminen for their technical assistance. This thesis wouldn't have been possible without the expert help of the skillful staff at NLAC. I warmly thank Heikki Pekonen and Heikki Karhunen for their expertise, invaluable help and all the long days in the animal facilities. Helena Pernu and Marja Poikolainen have kindly and patiently helped me with the wonders of university bureaucracy.

The years at AIVI have taught me to love science, but most importantly, I've met there many of the dearest people in my life. Elisa Vähäkangas has kept me sane through these years. Our conversations, both scientific and not so scientific and your great company at and outside work have been invaluable. Sanna-Kaisa Häkkinen has taught me to love wine and the therapeutic value of whining and complaining. Your well-hidden optimism surfaces when truly needed. Tuomas Mäntylä and Suvi Jauhiainen have been important friends through these years.

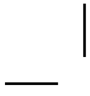
I want to thank my family, mother Mallu, father Tapani and brother Lauri for their love and support. You've taught me how full of interesting things the world is, the joy of learning and critical thinking. I thank my sister-in-law in training Katri for the bit of spice you add to the family, it has been great to get to know you. I thank my parents-in-law Raija and Risto for their hospitality and relaxing weekends. My warmest thanks and deepest gratitude go to Markku, my husband and co-author. Your invaluable contributions to our scientific projects, your encouragement and support have motivated me through the hard times. I admire your great mind and optimism. Thank you for sharing my world.

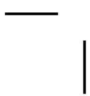
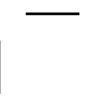
Kuopio, August 2009

This study was supported by grants from the Finnish Medical Foundation, Maud Kuistila Foundation, Aarne Koskelo Foundation, Instrumentariun Foundation, Aarne and Aili Turunen Foundation, Orion-Farmos Research Foundation, Eemil Aaltonen Foundation, Kuopio University Foundation, Ida Montini Foundation and European Union (LSHG-CT-2004-503573)

ABBREVIATIONS

Ad	adenoviral
AMI	acute myocardial infarction
Ang	angiopoietin
ATP	adenosine triphosphate
α-SMA	alpha smooth muscle actin
β-gal	β-galactosidase
BM	bone marrow
CAR	coxsackie and adenovirus receptor
COUP-TFII	COUP transcription factor 2 (NR2F2 nuclear receptor subfamily 2, group F, member 2)
EC	endothelial cell
ECG	electrocardiogram
ELISA	enzyme-linked immunosorbent assay
Flk-1	fetal liver kinase-1 (VEGFR-2)
Flt-1	fms-like tyrosine kinase-1 (VEGFR-1)
Flt-4	fms-like tyrosine kinase-4 (VEGFR-3)
GAIP	GIPC interacting protein
GIPC	G-protein interacting protein
GLUT	glucose transporter
GT	gene transfer
HIF	hypoxia-inducible factor
i.m.	intramuscular or intramyocardial
LAD	left anterior descending coronary artery
LCX	left circumflex coronary artery
LDL	low density lipoprotein
L-NAME	Lω-Nitro-L-arginine methyl ester
LYVE-1	lymphatic vessel endothelial hyaluronan receptor-1
MRI	magnetic resonance imaging
NfκB	nuclear factor kappa-B
NO	nitric oxide
Nrp	neuropilin receptor
PC	proprotein convertase
PDGF	platelet derived growth factor
PlGF	placental growth factor
p.o.	peroral
Prox1	prospero- related homeobox transcription factor-1
RCA	right coronary artery
RT-PCR	reverse transcriptase polymerase chain reaction
Slp76	lymphocyte cytosolic protein 2, SH2 domain containing leukocyte protein of 76kDa
SMC	smooth muscle cell
Tie	tyrosine kinase with immunoglobulin and epidermal growth factor homology
VEGF	vascular endothelial growth factor
VEGFR	vascular endothelial growth factor receptor
vp	viral particles



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I. Rissanen TT*, Markkanen JE*, Gruchala M, Heikura T, Puranen A, Kettunen MI, Kholová I, Kauppinen RA, Achen MG, Stacker SA, Alitalo K, Ylä-Herttuala S.
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* equal contribution

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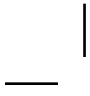
Circulation. 2004 Mar 2;109(8):1029-35. Epub 2004 Feb 16.

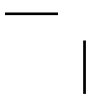
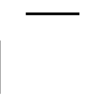
* equal contribution

III. Lähteenvuo JE, Lähteenvuo MT, Kivelä A, Rosenlew C, Falkevall A, Klar J, Heikura T, Rissanen TT, Vähäkangas E, Korpisalo P, Enholm B, Carmeliet P, Alitalo K, Eriksson U, Ylä-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 Feb 17;119(6):845-56. Epub 2009 Feb 2.

IV. Lähteenvuo JE, Lähteenvuo MT, Puranen A, Honkonen K, Rissanen TT, Alitalo K, Ylä-Herttuala S
Lymphangiogenic and angiogenic combination gene therapy reduces edema induced by therapeutic angiogenesis.
Manuscript 2009



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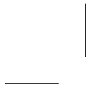
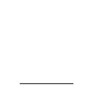
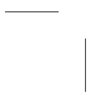




INTRODUCTION

Cardiovascular diseases are the leading cause of death in industrialized countries. Efficient primary prevention, early diagnosis and efficient treatment methods have increased the prevalence. Patients are older, and often have undergone several revascularization procedures. Conventional treatment methods are not suitable or feasible for all patients, and a growing number of patients fall into this “no option” category. Therapeutic angiogenesis, stimulation of blood vessel growth to salvage ischemic tissue was first proposed as a new treatment method for cardiovascular diseases over thirty years ago (Svet-Moldavsky and Chimishkyan, 1977). Discovery of a growth factor inducing blood vessel growth (angiogenesis) in tumors (VEGF) and development of viral gene transfer techniques enabled the first gene transfer experiment to ischemic myocardium in experimental animals in 1996 (Giordano et al., 1996) and the first adenoviral gene transfer to human myocardium in 1999 (Rosengart et al., 1999).

Despite the early optimism the development of therapeutic angiogenesis has been slow. Instead of being a simple angiogenic factor VEGFs have proven to be a large family of growth factors inducing various effects from blood and lymphatic vessel growth to metabolic effects, regulation of blood vessel permeability and inflammatory responses. It has become evident that development of a safe and efficient angiogenic therapy requires thorough knowledge of the biological function of these growth factors, localized and efficient gene transfer techniques and clinically applicable experiments in large animal models and finally a series of clinical phase I studies.

REVIEW OF THE LITERATURE

GENE THERAPY

In gene therapy the treated tissue is stimulated to produce therapeutic agents locally. Most potential advantages of gene therapy are long term expression of the therapeutic agent and the possibility to target the treatment to the desired tissue (Ylä-Herttuala and Alitalo, 2003). The therapeutic approach has to be designed to best suit the treated condition. The combination of viral vector, delivery method and choice of the therapeutic gene define where and how long the therapeutic protein is produced. While some diseases require a life long production of the drug, for some conditions weeks or months of production of the therapeutic agent is sufficient (Kay et al., 2001).

Expression time is dependent on the vector used: vectors integrating into the host genome produce the therapeutic product for the lifespan of the transduced cell while some viruses only produce the transgene for a few weeks (Kay et al., 2001). Locality of the treatment depends on the delivery method, and the qualities of the vector carrying the transgene. The range of the cells transduced depends on the natural tropism of the virus used, but can be modified by several targeting methods. Modifications of the virus surface, selective promoters to limit the transgene expression to a desired cell type or targeting the transgene product itself have been used (Rots et al., 2003). The properties of the therapeutic protein, such as solubility, interactions with endogenous molecules and immunogenicity further shape the therapeutic response.

Non-viral vectors

Non-viral vectors have been proposed to provide a safe method for gene transfer. In early experiments in mouse skeletal muscles, high transduction efficiency and transgene expression of several months were reported (Wolff et al., 1992). However, transduction efficiency was considerably lower in non-human primates (Jiao et al., 1992). Naked plasmids are easy to produce in high concentrations (Wolff and Budker, 2005), but due to lack of specific cell entry mechanisms, instability in extracellular milieu, tendency to be targeted to intracellular degradation pathways and lack of effective transport into the nucleus transduction efficiencies have been low *in vivo* (Lu et al., 2003). Although no severe side effects have been observed in clinical trials, transient fever has been observed after naked plasmid gene transfer (Hedman et al., 2003). Although considered a safe gene transfer method, widespread inflammation has been observed after intramuscular injection of naked plasmids in experimental animal models (McMahon et al., 1998) and possible formation of anti-DNA antibodies in autoimmune conditions (MacColl et al., 2001). Marked increases in transduction efficiency have been published by using ultrasound to permeabilize endothelial cells or by using cationic liposomes as carriers (Lawrie et al., 2000).

Adenoviral vectors

Adenoviral (Ad) vectors have high transduction efficiency and express the transgene for 1-2 weeks (Yang et al., 1994). Adenoviruses are capable of transducing both dividing and quiescent cells in a wide variety of tissues (Horwitz, 1996). Wild type

adenoviruses cause respiratory and gastrointestinal infections and myocarditis (Martin et al., 1994), and have tropism for liver but transduce a wide variety of cell types. The cell entry of most commonly used serotypes is mediated via coxsackie and adenovirus receptor (CAR, CD46) (Bergelson et al., 1997), and also heparin sulphate proteoglycans and integrins are involved in virus binding and internalization (Wickham et al., 1993; Dechecchi et al., 2001). Several targeting approaches have been used to increase transduction efficiency in treated tissues and to limit side effects, including modifications of the receptor binding sites on cell surface, antibody mediated targeting and tissue-specific promoters (Baker, 2004). Adenovirus has a limited transgene capacity of approximately up to 8kb, but it can be produced in high titers (Kovesdi et al., 1997). Adenoviruses have been used for gene therapy for decades, and adenoviruses are the most commonly used vectors in gene therapy trials (http://www.wiley.co.uk/genetherapy/clinical/). Adenoviruses trigger both innate and acquired immune responses, and production of neutralizing antibodies prevents repeated administration of the therapeutic agent (St George, 2003). This problem has been circumvented in animal models by the use of another adenoviral serotype for the second gene transfer (Parks et al., 1999). Most humans have naturally been exposed to the adenovirus serotypes used most commonly as gene transfer vectors (Horwitz, 1996), possibly preventing efficient transduction. Despite the immunologic reactions, only mild fever and a transient rise in liver transaminases has been observed in human trials, and a large number of clinical trials have been done without any serious adverse effects connected to the virus (Ylä-Herttuala et al., 2007).

CARDIOVASCULAR SYSTEM

Anatomy and function of the cardiovascular system

The cardiovascular system delivers oxygen and nutrients to tissues, provides a route for transport of chemical messengers throughout the body and is essential for immune defense and removal of waste products. The heart pumps blood to the systemic circulation and maintains a blood pressure sufficient to deliver blood throughout the body. The left ventricle pumps oxygenated blood to peripheral tissues and the right ventricle pumps oxygen poor blood to the lungs. Contraction of the heart, the systole, pushes blood out from the ventricle through the aortic valve, and blood flows into the heart during the relaxation of the heart muscle, the diastole. The amount of blood pumped out during each systole is expressed as stroke volume (Guyton and Hall, 2000).

The contraction of the individual cardiomyocytes has to be tightly controlled to achieve coordinated, rhythmic contraction of the heart. Specialized myocytes in the sinoatrial node initiate a wave of depolarization of the atrial cardiomyocytes which is further conducted to the ventricles via the atrioventricular node. Depolarization spreads to the ventricles via the fast-conducting atrioventricular bundle (bundle of His), which divides into the left and right bundle branches. Purkinje fibers, chords of specialized myocardial cells, spread the depolarization to contracting cardiomyocytes. Cardiomyocytes are tightly connected to the surrounding cells via gap junctions, electrically conducting openings that readily distribute the depolarization throughout the myocardium (Katz, 2005).

Arteries are thick-walled vessels that transport blood away from the heart. Arteries have muscle coated walls to sustain high pressure and flow. The elastic wall of the vessel pulsates with the blood flow, and evens out the blood flow in the smaller vessels. Blood pressure in the ascending aorta is the same as in the left ventricle, and is gradually lower in smaller vessels, reaching an average pressure of 17mmHg in the capillaries. Arteries branch into smaller arterioles and finally form capillary vessels. Arterioles control blood

flow and pressure by contracting or dilating according to the needs of the tissue (Guyton and Hall, 2000). Blood flow in a vessel is directly proportional to the fourth power of the radius of the vessel. Thus small differences in the diameter of the artery greatly influence the amount of blood flow in the tissue (Heil and Schaper, 2004). In metarterioles or terminal arterioles the continuous smooth muscle layer is replaced by patchy pericyte coverage of the endothelial cell (EC) layer. Capillaries form an interface for oxygen and nutrient delivery to the tissues (Figure 1). The diameter of a capillary vessel is approximately equal to the diameter of a red blood cell, which facilitates the diffusion of oxygen through the capillary endothelium. Capillary walls are also highly permeable to water and nutrients. Transport of water and nutrients mainly occurs via intercellular clefts. A smooth muscle cell (SMC) in the end of the metarteriole controls the blood flow to a capillary. Function of this precapillary sphincter is controlled by local oxygen tension in the tissue, and thus each tissue environment controls its own blood flow (Guyton and Hall, 2000).

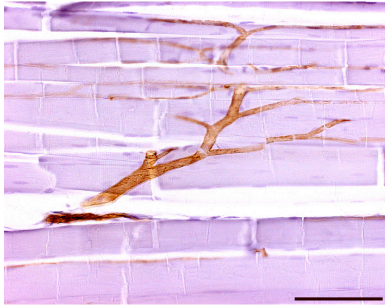


Figure 1. Microvessels in skeletal muscle. Larger vessels branch to form capillaries running parallel to muscle fibers. CD31 staining, magnification 200X, scale bar 100µm. Lähteenvuo et al., unpublished result.

From capillaries blood flows to venules, thin walled small veins that function as an entry point for inflammatory cells into the tissues (Zarbock and Ley, 2008). Venules connect to form larger veins. Blood pressure in the veins is low, and unidirectional flow is maintained by presence of valves preventing back flow. In the limbs contractions of the surrounding muscles push blood towards the heart. Veins have large luminal volume and are able to control the amount of blood available for tissues by constricting or enlarging. Veins connect and finally form the inferior and superior vena cavas, large veins that deliver oxygen poor blood back to the right heart to be reoxygenated in the lungs.

Hierarchy and structure of the vascular network are tightly regulated in normal adult tissues. Specific mechanisms prevent formation of arterio-venous shunts, excess arterialisation of the vessels and connections between lymphatic vessels and veins. These regulatory systems are likely highly dependent on level blood flow and pressure within the vessels after embryogenesis (Swift and Weinstein, 2009). In pathological situations such as tumor growth these mechanisms are disturbed creating non-hierarchical, immature vessels (Cao, 2009).

Blood vessels of the heart

Coronary arteries originate from two openings in the ascending aorta, right above the aortic valve cusps. The left main coronary artery divides to form left anterior descending artery (LAD) and left circumflex artery (LCX). The right coronary artery (RCA) originates from the second orifice. The anatomy of the coronary arteries varies between individuals, but generally LAD is responsible for the circulation in the anterior wall of the left ventricle and septum, LCX for the lateral wall and RCA for most of the right ventricle and posterior wall of the left ventricle. LAD further divides to diagonal branches that supply the left ventricular wall, septal branches that supply the anterior part of the intraventricular septum and right ventricular branches. (Katz, 2005)

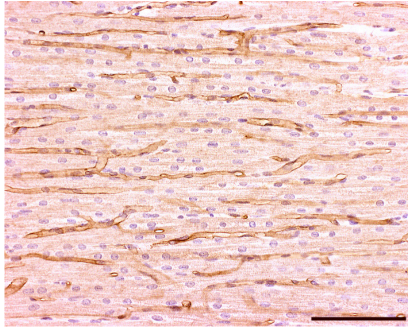


Figure 2. Microvessels in the heart. Capillaries form a dense network between muscle fibers. CD31 staining, magnification 200X, scale bar 100µm. Lähteenvuo et al., unpublished result.

Coronary arteries run on the surface of the heart and branch into smaller arteries that penetrate deeper into the myocardium.The heart has a dense network of capillaries (approximately one capillary per cardiomyocyte) (Kumar V. et al., 2005)(Figure 2). The innermost layer of the myocardium (endocardium) also receives some blood directly form the ventricles. The coronary veins run alongside the arteries, and venous blood returns to the right atrium via the sinus coronarius located on the posterior side of the heart. Blood flow into the capillaries occurs mostly during the diastole when the intramyocardial pressure is on its lowest level. The heart receives 5-6% of the blood pumped from the left ventricle at rest, and the flow can rise up to 3-4-fold at stress. Increased demand for oxygen and energy induces relaxation of the coronary arterioles increasing the flow (Guyton and Hall, 2000). Human myocardium contains collateral vessels, small connecting arterioles between conducting arteries (Fulton, 1963a). Collaterals are present in all individuals, but their number, size and anatomy vary considerably. Epicardial collateral vessels are smaller, 20-200µm in diameter, and are unlikely to significantly contribute to cardiac circulation under normal conditions while endocardial collateral network consists of larger, 100-200µm vessels. Due to a shorter period of blood flow in the endocardial vessels due to high pressure in the ventricles endocardial collateral vessels may play a role in normal perfusion of the myocardium (Fulton, 1963b).

Metabolism of the heart

At rest the heart utilizes 70% of the oxygen in the arterial blood and cardiomyocytes use fatty acids as the main source of energy (Guyton and Hall, 2000). Cells in the conduction system of the heart, on the other hand, partially rely on anaerobic energy production (Henry and Lowry, 1983). Metabolism is regulated by the availability of oxygen and substrates for energy metabolism, dietary status, age, hormonal status, and work load of the heart. Fatty acids are the prime source of energy in the fasting individual, and fatty acid metabolism itself inhibits glycolysis (Mäki, 1998). Fatty acid metabolism is dependent on continuous delivery of oxygen, and when myocardium becomes ischemic metabolism shifts towards glycolysis (Neely and Morgan, 1974). Ischemia induces recruitment of glucose transporter GLUT4 to plasma membranes, and glucose moves down the concentration gradient across the cell membrane (Sun et al., 1994). Insulin increases glucose uptake via the same mechanism (Manchester et al., 1994). In addition to glucose uptake, ischemia also leads to breakdown of stored glycogen in the cardiomyocytes (Lopaschuk and Stanley, 1997).

CARDIOVASCULAR DISEASES

Atherosclerosis

Atherosclerosis is the common pathogenic process behind ischemic diseases. Although the initial cause of the process remains unclear, subsequent steps include accumulation of lipids into the vascular wall, accumulation of inflammatory cells to the plaque and formation of neovasculature within the plaque. Changes in the vessel wall gradually occlude the vascular lumen and reduce the blood flow. A plaque becomes hemodynamically significant at stress when it occludes 75% of the vessel lumen and when the occlusion exceeds 90% it disturbs blood flow also at rest (Kumar V. et al., 2005). Threrefore the first manifestation of atherosclerosis is often ischemic pain during exercise. As the disease progresses tissues suffer from insufficient delivery of oxygen and nutrients also at rest.

Peripheral arterial disease

Peripheral arterial disease manifests as intermittent pain during exercise. In chronic lower limb ischemia atherosclerotic plaques gradually occlude the arteries (Kumar V. et al., 2005). In critical limb ischemia complications such as ulcers or gangrene have developed as a result of ischemia. In acute limb ischemia, a vessel is suddenly occluded as a result of a thromboembolic event and tissue necrosis results within hours (Minar, 2009). Exercise is the only efficient non-invasive treatment method, and treatment includes risk factor management and in some cases medication. Invasive treatment options include percutaneous transluminal angioplasty and by-pass grafting (Slovut and Sullivan, 2008). However, 20-30% of these patients cannot be treated with conventional therapies and amputation is the only treatment option (Dormandy and Rutherford, 2000).

Myocardial ischemia

Myocardial ischemia is caused by a lack of oxygen in the heart. In chronic myocardial ischemia blood flow is reduced as a result of the atherosclerotic process. Developing atherosclerotic plaques gradually occlude the vessel lumen. 90% of the vessel lumen can be occluded without reduction in blood supply at rest, while 75% reduction in the vessel diameter causes significant reduction of blood flow in stress (Kumar V. et al., 2005). Gradual occlusion of the coronary arteries directs blood to collateral arteries that respond by increasing their diameter both by dilation and cell proliferation. The ability to develop these collateral arteries is individual, but in optimal situation collateral flow can be sufficient to replace the flow of a whole main coronary vessel at rest (Schaper, 2004).

Acute myocardial ischemia (AMI) is the result of a sudden blockage of a coronary vessel (Boersma et al., 2003). Acute myocardial infraction is thought to be caused by rupture of an atherosclerotic plaque or by endothelial damage. Damage or rupture of the endothelial cell layer reveals a thrombogenic surface and a blood clot comprising of thrombocytes and fibrin may develop. Alternatively, plaque rupture may release plaque contents to the circulation causing a thrombus downstream or form a mechanical block at the rupture site (Kumar V. et al., 2005). Compensatory arterial growth is too slow to

restore the circulation in AMI, and within hours a permanent infarction scar starts to develop. Cardiomyocyte damage starts within minutes after cessation of oxygen supply and contraction ceases within 60 seconds and cardiomyocytes begin to die after 15-40 minutes (Katz, 2005).

If circulation is not restored, irreversible damage occurs. In the areas where blood supply is cut off completely cells begin to die by coagulation necrosis (Kumar V. et al., 2005). Blood vessels in the border of the ischemic area become leaky, and inflammatory cells infiltrate into the forming scar. Within the first couple of days inflammatory cells are mostly neutrophils, but around day 7 monocytes/macrophages become predominant. Necrotic cardiomyocytes are first replaced by granulation tissue which starts to remodel into a fibrotic scar. Fibroblasts proliferate and secrete collagen, and both resident and bone marrow (BM) derived cells begin to differentiate into myofibroblasts. The myocardial infarction scar is completely formed approximately 2 months after AMI (Frangogiannis et al., 2002).

If a significant portion of the myocardial wall is damaged, myocardial function is affected and complications may develop. The infarction scar does not contract and is mechanically weaker than normal myocardial wall. Fibrosis occurs throughout the myocardium. Fibrotic myocardium is electrically unstable because the normal conduction of the depolarization wave is disturbed by the electrically inactive fibrotic scar increasing the risk of fatal ventricular arrythmias (Rothman et al., 1997). The healthy part of the myocardial wall begins to hypertrophy to compensate for the impaired function of the scar area, and the ventricle starts to dilate leading to cardiac dysfunction and eventually cardiac failure. Initially hypertrophy is accompanied by capillary growth, but eventually hypertrophy proceeds without compensatory growth of new blood vessels.

Metabolic adaptation to ischemia

In the border zone of the infarction area where some but insufficient perfusion remains, cardiomyocytes assume a hibernating phenotype. They cease to contract to save energy and convert their metabolism towards less oxygen demanding glycolysis. Under normal conditions cardiomyocytes use 75% of their oxygen consumption for contraction (McKeever et al., 1958; Izzi et al., 1991) and therefore only a low level of perfusion is required to maintain cell viability in resting cardiomyocytes (Gewirtz et al., 1994). During longer periods of insuffient blood flow cardiomyocytes may also dedifferentiate to increase their resistance to ischemia. They accumulate glycogen in their cytoplasm, and start to express fetal type cardiomyocyte markers such as alpha smooth muscle actin (α-SMA) and desmin (Dispersyn et al., 2002). The function of the hibernating myocardium may recover if blood flow is restored, but responses between patients vary considerably.

Metabolic changes in the ischemic myocardium have been extensively studied for a century. Interpretation of these results is complicated by different criteria to identify ischemic and hibernating areas of the myocardium and by use of different animal models and clinical settings. However, the switch to glucose metabolism seems to be a double-edged sword. If the period of ischemia is brief and blood flow is restored shortly, or a low level of perfusion is maintained, glycolysis has a beneficial effect. Glycolysis requires less oxygen per mole of adenosine triphosphate (ATP) created than fatty acid metabolism, and can be maintained also under anaerobic conditions. However, if glycolysis is carried out in anaerobic conditions, lactate starts to build up in the tissue, pH becomes acidic and cellular damage results. Restoration of tissue blood flow is therefore essential to save myocardial cells and rescue myocardial function.

GROWTH OF BLOOD VESSELS

Formation of the cardiovascular system

The formation of the cardiovascular system begins by the formation of an immature vascular plexus that gives rise to the dorsal aorta, cardinal vein and vessels of the yolk sac. Immature hemangioblasts differentiate to form both blood vessels and blood cells (Swift and Weinstein, 2009). After initial formation of the primitive vascular plexus, subsequent blood vessel growth is stimulated by hypoxia in the growing embryo, and primitive blood vessels sprout to invade developing organs (Risau, 1997).

Arteries and veins have different functions, and they specialize to meet the physiological requirements. Arteries acquire a thicker muscle layer, and arterial endothelial cells express specific surface markers such as ephrinB2 while veins are more permeable, have a thinner muscle layer and express ephrinB4 (Gerety et al., 1999). Angioblasts commit to arterial and venous lineages before the heart starts to pump blood. Notch pathway regulates this lineage commitment and bidirectional ephrin signaling maintains the separation and guides blood vessel branching. Also Nrp-1 has been suggested to be involved in vascular hierarchy formation in the retina through different VEGF-A isoforms (Stalmans et al., 2002). Arterio-venous identity is reprogrammable even in the adult and is regulated by Notch signaling and orphan nuclear receptor CoupTFII (You et al., 2005; Roca and Adams, 2007) and by adaptation to alterations in blood flow (Garcia-Cardena et al., 2001; le Noble et al., 2004).

Growth of blood vessels in adult

Physiological angiogenesis in adults is practically limited to blood vessel growth in the endometrium during every menstrual cycle. Blood vessel growth occurs in many common pathological conditions, such as wound healing, psoriasis and rheumatoid arthritis (Carmeliet, 2003). Angiogenesis is also required for tumor growth. Tumors both recruit blood vessels from surrounding tissues and stimulate blood vessel growth by producing angiogenic growth factors (Folkman and Shing, 1992). Angiogenesis is an endogenous repair mechanism also in adult heart and skeletal muscles both in acute infarction and in chronic ischemia.

Blood vessel growth in myocardial ischemia

Growth of new blood vessels is an endogenous response to ischemia. Maintaining the normal function of the myocardial tissue requires both growth of large arteries to replace the occluded coronary vessel (arteriogenesis) and growth of capillary vessels to deliver oxygen and nutrients to the myocardial cells (angiogenesis) (Scholz et al., 2001). Collateral arteries are small arteries that form connections between conducting arteries. When a coronary artery occludes, pressure in collateral arteries rises and they dilate within seconds. If flow remains high, collaterals begin to grow. As a result, blood flow in collateral arteries can double in 2-3 days (Heil and Schaper, 2004). Thus forming collateral circulation can at least partially compensate the diminished blood flow in the periphery. The potency to form collateral arteries varies between individuals, and requires weeks to months to reach blood flow sufficient to compensate the flow of the original artery even at

rest. According to the Poiseuille's law, flow is proportional to the 4th potency of the vessel diameter and hence a large number of smaller arteries are required to replace a large diameter coronary artery (Heil and Schaper, 2004).

Cells in the ischemic myocardium start to produce angiogenic growth factors such as VEGF-A (Li et al., 1996) (Figure 3), and inflammatory cells present in the infarction border zone are shown to be able to produce a wide variety of growth factors. Blood vessels dilate and start to grow towards the angiogenic stimulus to increase blood flow in the ischemic area. Endogenously growing capillary network is not able to keep up with the developing tissue damage and later tissue growth due to ventricular remodeling, and apoptotic cell death continues for weeks after the ischemic event (Narula et al., 1996; Cheng et al., 1996).

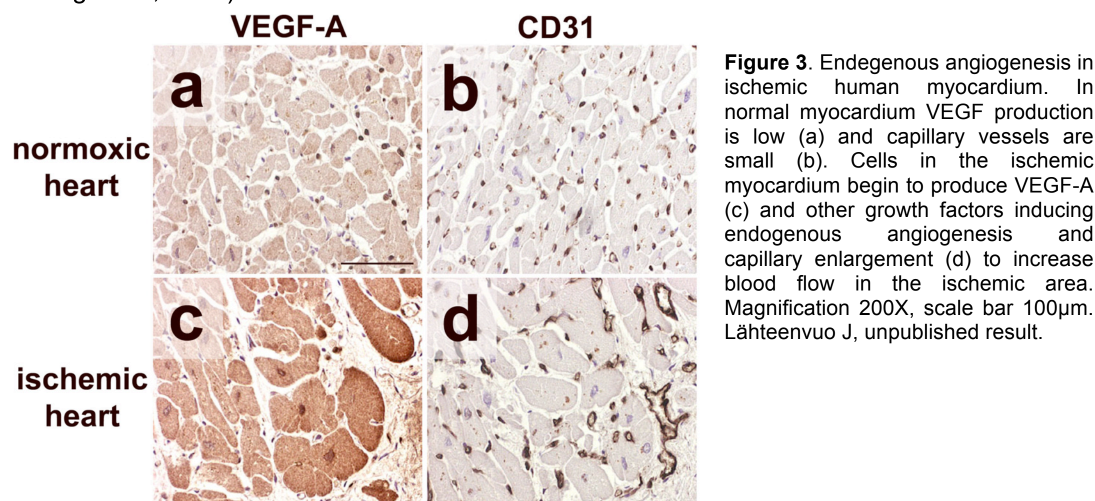


Figure 3. Endegenous angiogenesis in ischemic human myocardium. In normal myocardium VEGF production is low (a) and capillary vessels are small (b). Cells in the ischemic myocardium begin to produce VEGF-A (c) and other growth factors inducing endogenous angiogenesis and capillary enlargement (d) to increase blood flow in the ischemic area. Magnification 200X, scale bar 100µm. Lähteenvuo J, unpublished result.

MECHANISMS OF BLOOD VESSEL GROWTH

Vasculogenesis and stem cells in blood vessel growth

Vasculogenesis is growth of blood vessels from vascular progenitor cells. It is the first step in the formation of the cardiovascular system in the embryo. Blood vessel formation and maintenance have been suggested to require vasculogenesis also in adult tissues, but the biological significance and mechanism remain unclear (Sieveking and Ng, 2009). Incorporation of bone marrow (BM) derived cells to normal myocardium and blood vessels has been demonstrated in humans (Gunsilius et al., 2000; Quaini et al., 2002). Several studies have also provided indirect evidence on their role in endogenous repair mechanisms. Cells are recruited from the BM after vascular injury and by expression of several cytokines including VEGF (Gill et al., 2001; Kalka et al., 2000). Acute myocardial infarction increases plasma levels of VEGF and increases mobilization of BM derived cells resembling ECs *in vitro* (Shintani *et al.*, 2001). The amount of circulating progenitor cells has also been inversely correlated with known clinical risk factors for cardiovascular diseases and endothelial function (Hill *et al.*, 2003). Although the recruitment of bone marrow derived cells to peripheral circulation has been observed in various situations, the role of these cells in vascular repair *in vivo* has not been convincingly demonstrated.

Several populations of multipotent BM derived cells have been identified, and their potency in blood vessel regeneration has been tested in several preclinical and clinical studies (Rafii and Lyden, 2003). However, varying markers to identify and enrich the cell populations, different disease models and human applications the cells have been studied in, different delivery methods and end point measurements have produced conflicting results.

Bone-marrow derived stem cells have been reported to incorporate directly into angiogenic vessels forming both endothelial and smooth muscle cells during physiological and pathological angiogenesis and to improve the function of ischemic tissues (Gunsilius et al., 2000; Kocher et al., 2001; Orlic et al., 2001). However, the capacity of bone-marrow derived cells to incorporate into vessel structures and to differentiate into mature endothelial or myocardial cells has been questioned (Ziegelhoeffer et al., 2004; Murry et al., 2004; Balsam et al., 2004).

Several studies have proposed that instead of incorporation into blood vessels BM derived cells may facilitate neovessel growth by homing into angiogenic tissue and producing angiogenic factors (Kinnaird et al., 2004a). Growth factor -producing mononuclear cells have been shown to be essential for collateral formation, and several studies have demonstrated the presence of BM derived cells in perivascular position in developing blood vessels but no incorporation to vascular endothelium (Ziegelhoeffer et al., 2004; Kinnaird et al., 2004b). BM derived cells may also fuse into cells in host tissues. Thus far cell fusion appears to be a rare phenomenon *in vivo*, and the biological role of the cell fusion demonstrated *iv vitro* remains unclear (Terada et al., 2002; Ying et al., 2002).

In addition to BM derived cells, adult tissues have been suggested to contain resident progenitor cells that can contribute to vasculogenesis and regeneration of ischemic tissues. Skeletal muscles contain populations of precursor cells capable of transdifferentiating into endothelial cells and smooth muscle cells (Majka *et al.*, 2003) but not cardiomyocytes (Reinecke et al., 2002). The presence of myocardial progenitor cells has been demonstrated in humans and their ability to differentiate into cardiomyocytes, endothelial cells, smooth muscle cells and to integrate into ischemic myocardium has been shown in experimental animal models (Bearzi et al., 2007; Beltrami et al., 2003).

Reprogramming adult cells to pluripotent stem cells (iPS) is a new method to create stem cell populations. iPS can be derived from the skin and differentiated into multiple cell types including endothelial cells, cardiomyocytes and smooth muscle cells (Tulloch et al., 2008).

The use of BM or tissue derived stem cells for treatment of ischemic diseases poses several safety issues. Unselected populations of BM-derived cells may induce inflammation and produce excessive amounts of growth factors inducing poorly organized vascular structures, vascular leakage and edema. Transplanted cells may fail to differentiate and incorporate into host tissue, possibly leading to tumor formation or disturbances in host tissue function, such as arrythmias in the myocardium (Villa et al., 2007; Fernandes et al., 2006). BM transplantation has also been shown to lead to tissue calcification and fibrosis (Yoon et al., 2004; Wang et al., 2001b). Lack of specific markers and incomplete understanding of the functions of different cell populations may lead to selection of suboptimal cell fractions. The majority of the progenitor cell experiments have been performed on young, healthy animals, and progenitor cell number is lower and function is possibly impaired in elderly, hypercholesterolemic and diabetic individuals (Moresi et al., 2005; Krankel et al., 2005; Chen et al., 2004; Vasa et al., 2001).

Angiogenesis

Angiogenesis is the growth of new capillary vessels by endothelial cell (EC) proliferation and migration from pre-existing vessels. Endogenous angiogenesis is driven by hypoxia. Lack of oxygen stimulates expression of hypoxia-sensitive factors e.g. hypoxia-inducible factor 1α (Hif1-α) expression, and this in turn activates production of angiogenic growth factors (Marti, 2005; Fong, 2008). During embryogenesis, angiogenesis is the mechanism by which blood vessel growth keeps up with the growth of organs. Angiogenesis is also the main mechanism of blood vessel growth in the adult (Risau, 1997).

Angiogenesis is a multi-step process. In quiescent state ECs are tightly attached by intercellular junctions and the turnover rate of ECs is low (Ahn et al., 2008). The angiogenic process is initiated by vessel dilation, increase in vascular permeability, disattachment of pericytes, degradation of basement membrane and release of proteolytic factors altering the interstitial environment and releasing angiogenic factors from the extracellular matrix (Jain, 2003). Leakage of plasma proteins creates a matrix for endothelial cell migration rich in growth factors and proteases. Proliferation and migration of ECs produce first chords of ECs that connect to other blood vessels and then develop a lumen.

Immature neovessel network is then remodeled to respond to the metabolic needs of the tissue. Continuing hypoxia induces production of growth factors, while cessation of growth factor production in normoxic or hyperoxic tissue leads to pruning of the vascular network and vessel maturation (Dor et al., 2001). If a vessel is not needed, tissue regulation decreases blood flow eventually leading to EC apoptosis (Sho et al., 2001). Blood vessels acquire pericyte coverage, either by proliferation of pre-existing pericytes or by differentiation of fibroblasts into myofibroblasts and further to mural cells (Chambers et al., 2003; Jain, 2003). Pericyte coverage has been proposed to be a hallmark of vessel stabilization (Benjamin et al., 1998), but further maturation may be required as vessels with apparent pericyte coverage have been shown to regress (Rissanen et al., 2005). Finally, new basement membrane is secreted and blood vessels assume quiescent state (Abtahian et al., 2003).

Arteriogenesis

Arteriogenesis is the growth of collateral arteries. Collateral arteries in healthy tissues are small, thin walled vessels forming anastomoses between arteries. Collateral growth has been thought to be dependent on pre-existing collateral channels found widely in different species and tissues. (Helisch 2003.) The human heart was demonstrated to have arterial anastomoses almost a hundred years ago (Gross, 1921). Unlike angiogenesis, arteriogenesis is not controlled by hypoxia (Deindl 2001). When an artery is occluded, blood pressure distally from the occlusion falls, and a steep pressure gradient develops (Helisch 2003). As a result, flow is increased in pre-existing collateral arteries increasing fluid shear stress, and pressure-related forces on the vessel wall (Heil 2004). Endothelial cells respond within hours, shown by ultrastructural changes. Nitric oxide released by the ECs induces dilation of the vessels, further increasing flow and relaxing distal arterioles (Nishida et al., 1992). Adhesion molecule expression is up-regulated within 12 hours, and monocytes adhere to endothelium and start to accumulate in the vessel wall (Scholz 2000). Monocytes have been shown to be important mediators of collateral growth, possibly via production of proteases and growth factors. Endothelial and smooth muscle cell proliferation start 12-24 hours after the initiation of the growth process.

Proliferation has been suggested to be stimulated by either local production of growth factors, or by direct pressure stimulation of the cells. The internal elastic lamina is partially degraded by proteases, allowing direct contact between endothelial cells and smooth muscle cells and smooth muscle cell migration (Scholz 2000).

Collateral growth continues until pressure and shear stress in the growing collateral returns to normal level. Collateral growth has been shown to compensate diminished blood flow in humans. Well-developed collaterals can replace the flow of one of the main coronary arteries, and collateral flow correlated with clinical symptoms and outcome of patients suffering from myocardial ischemia in a 10-year follow-up study (Rockstroh and Brown, 2002). Arteries formed by collateral growth are ultrastructurally normal, apart from increased amount of collagen between the smooth muscle cells (Scholz et al., 2000).

Blood vessel growth and vascular permeability

Growth of blood vessels is accompanied by an increase in the permeability of the vessels. ECs in their quiescent state are tightly attached to each other and form a continuous layer in the vessel wall. EC contacts induce signals to maintain this quiescent state and inhibit proliferation (Holash et al., 1999). Angiogenesis is initiated by disattachment of the pericytes surrounding the capillary and by breakdown of the basement membrane by proteases. ECs are able to proliferate after intercellular contacts have been disrupted. This disruption of normal vessel structure leads to leakiness of the growing vessels. Other mechanisms are also involved in the plasma protein leakage observed after growth factor administration. Vascular endothelial growth factor (formerly vascular permeability factor, Vpf) is a potent inducer of vascular permeability. Vascular leakiness is induced within seconds after administration, at a concentration 1/50 000 compared to that of histamine (Senger et al., 1983). This effect is likely due to increased permeability at the intracellular junctions of the ECs. In addition, mechanisms involving transcellular transport through ECs have been proposed (Bates and Harper, 2002; Nagy et al., 2008).

Several approaches have been published to inhibit vascular permeability. Some growth factors have been published to induce angiogenesis without vessel leakiness but results in large animal models have been in conflict with this data (Luttun et al., 2002). Also combination treatments with either factors naturally involved in the signaling of the quiescent state (Ang-1) (Thurston et al., 2000) or with factors stimulating pericytes and therefore stabilizing the vessel wall (PDGF) have been proposed. As attempts to uncouple angiogenesis and vascular permeability have not been successful in large animal models (Lähteenvuo and Rissanen, unpublished results) and uncoupling these events seems conceptually challenging, other approaches such as reduction of tissue edema by inducing lymphatic vessel wall should be explored.

MOLECULES REGULATING BLOOD VESSEL GROWTH

Growth of blood vessels is a multi-step process induced by orchestrated function of growth factors and their receptors. Blood vessel growth can be triggered by hypoxia, increased flow in the vessel or by production of angiogenic growth factors. To produce a balanced growth of functional blood vessels requires coordinated expression of several

factors and responses in several cell types. The focus is on VEGFR-1, VEGFR-2 and Nrp-1 receptors and their ligands (Figure 4).

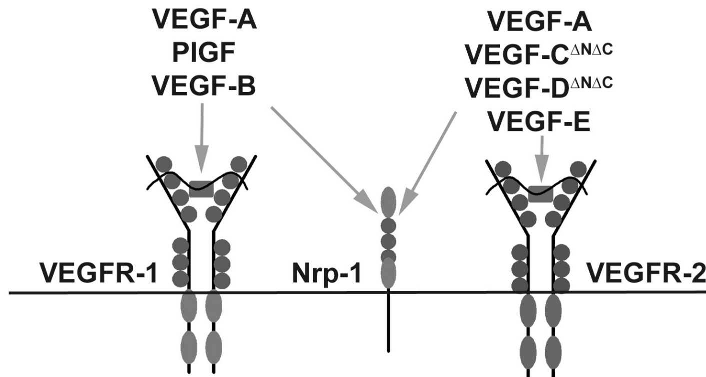


Figure 4. Vascular endothelial growth factors and receptors regulating angiogenesis.

Vascular endothelial growth factor receptor-1 (VEGFR-1)

VEGFR-1 (Flt-1) is required for the development of the cardiovascular system. VEGFR1 null mice die by embryonic day 9 due to lack of formation of organized blood vessels (Fong *et al.*, 1995). However, only the extracellular part of the receptor seems to be required for the normal development, as mice lacking only the intracellular part of the receptor are healthy and fertile (Hiratsuka *et al.*, 1998). VEGFR-1 is expressed in ECs, osteoblasts, monocytes/macrophages, pericytes, placental trophoblasts, renal cells and some hematopoietic stem cells (Ferrara *et al.*, 2003). VEGFR-1 is up-regulated in hypoxic environment directly by a hypoxia responsive element (Marti and Risau, 1998; Gerber et al., 1997) and in angiogenic vessels (Plate et al., 1993). Interestingly, in hypoxia VEGFR-1 is diffusely up-regulated in both ECs and cardiomyocytes (Marti and Risau, 1998; Carmeliet et al., 2001) and on coronary smooth muscle cells (Ishida et al., 2001). In addition, in the ischemic myocardium, an increased proportion of VEGFR-1 is the membrane-bound form suggesting a role for intracellular signaling via VEGFR-1 (Carmeliet et al., 2001).

VEGFR-1 binds VEGF-A, Placental Growth factor (PlGF) and VEGF-B (de et al., 1992; Park et al., 1994; Olofsson et al., 1998). VEGF-A and PlGF have been shown to phosphorylate different residues in VEGFR-1, thus possibly inducing ligand-specific functions via the same receptor (Autiero et al., 2003). VEGFR-1 has been suggested to be involved in several pathological conditions, and inhibition of VEGFR-1 has been shown to inhibit tumor growth, atherosclerotic plaque growth and vulnerability, and arthritic joint destruction (Luttun et al., 2002). During angiogenesis VEGFR-1 has been suggested to be involved in blood vessel sprouting and EC migration (Kearney et al., 2004). In addition, VEGFR-1 stimulation induces monocyte chemotaxis (Clauss et al., 1996). Monocytes/macrophages have been shown to be essential for arteriogenesis, and accordingly VEGFR-1 ligands have been shown to induce collateral artery growth (Pipp et al., 2003).

VEGFR-1 is naturally expressed also in a soluble form. The soluble form of VEGFR-1 may function as a decoy receptor to limit the bioavailability of its ligands (Hiratsuka et al., 1998) and to prevent blood vessel growth in avascular tissues such as the cornea (Ambati et al., 2006). Since VEGFR-1 has a relatively weak tyrosine kinase activity, it was suggested to function as a decoy receptor sequestering VEGF-A and thus

inhibiting VEGFR-2 activation (Park et al., 1994). However*,* *in vitro* studies suggest that the soluble form of VEGFR-1 may mediate migration and branching via binding to extracellular matrix components (Orecchia et al., 2003; Kappas et al., 2008). Although healthy and fertile, mice only expressing the sVEGFR-1 have impaired monocyte migration in response to VEGF (Hiratsuka et al., 1998).

Placental growth factor (PlGF)

PlGF is expressed only in low levels in developing embryo but is expressed in the placenta, heart, brain, skeletal muscle and lungs in the adult (Persico et al., 1999) and in both endothelial cells and in lower levels in pericytes (Zhao et al., 2004). PlGF deficient mice are healthy and fertile, but they recover poorly after experimental myocardial infarction or hindlimb ischemia (Carmeliet *et al.*, 2001). PlGF is expressed in three isoforms, PlGF-1, -2 and -3 (Cao et al., 1997; Park et al., 1994). PlGFs binds to VEGFR-1 (Park et al., 1994; Sawano et al., 1996) and PlGF-2 also binds to heparin sulphates and is a ligand for Nrp-1 receptor (Migdal *et al.*, 1998). PlGF forms biologically active heterodimers with VEGF-A that are able to bind VEGFR-2 (Cao et al., 1996a). Interestingly, also PlGF homodimers have been shown to up-regulate (Odorisio et al., 2002) and phosphorylate VEGFR-2 (Autiero et al., 2003) although no direct binding is observed.

PlGF is not up-regulated by hypoxia in transcriptional level, but increases in protein levels of both PlGF/VEGF heterodimer and PlGF homodimer suggest post-transcriptional regulation (Cao et al., 1996b). In addition, VEGF-A and hyperglycemia up-regulate PlGF in ECs *in vitro* (Zhao et al., 2004).

PlGF has been reported to be a weak EC mitogen *in vitro* (Park et al., 1994; Sawano et al., 1996) but to induce EC proliferation and angiogenesis *in vivo* (Ziche et al., 1997). The effect of PlGF on vascular permeability remains controversial. PlGF was originally shown to be a weak inducer of acute vascular permeability (Park et al., 1994), but was later shown to increase protein extravasation and edema after both acute and prolonged expression (Odorisio et al., 2002; Oura et al., 2003). PlGF induced angiogenesis in mouse myocardial infarction, hindlimb ischemia and skin angiogenesis models without increasing vascular permeability or edema (Luttun et al., 2002). PlGF induces monocyte migration via membrane-bound form of VEGFR-1 (Hiratsuka et al., 1998), and has been reported to induce arteriogenesis via monocyte recruitment (Pipp et al., 2003).

Due to conflicting results, weak signaling via VEGFR-1 and weak potency *in vitro*, several indirect mechanisms of function have been suggested for PlGF. PlGF has been suggested to potentiate the effects of VEGF-A (Park et al., 1994), function via PlGF/VEGF heterodimer formation (Cao et al., 1996a) or by modulation of VEGFR-1/VEGFR-2 interactions (Autiero et al., 2003).

Vascular endothelial growth factor-B (VEGF-B)

VEGF-B is produced in two isoforms, VEGF-B_{167} and VEGF-B_{186} that are further proteolytically processed (Olofsson et al., 1996b). VEGF-B_{186} is readily soluble, while VEGF-B_{167} binds heparan sulphate proteoglycans. VEGF-B binds to VEGFR-1 and Nrp-1 (Olofsson et al., 1998; Mäkinen et al., 1999). VEGF-B_{167} is the predominant form in adult tissues, and it is expressed in myocardium, in a subset of skeletal muscle fibers and the pancreas (Olofsson et al., 1996a). VEGF-B expression in the embryonic and postnatal

myocardium coincides with coronary vessel and capillary development in the ventricles, but is mainly seen in the myocytes (Bellomo et al., 2000). VEGF-B was also shown to be up-regulated in ischemic hind limb muscles in mice (Silvestre et al., 2003). In one genetic backround VEGF-B deficient mice have smaller hearts and they recover poorly after experimental myocardial infarction (Bellomo *et al.*, 2000), while in other genetic background mice have ECG abnormalities (Aase *et al.*, 2001) but in both cases animals are viable and fertile.

VEGF-B increases EC proliferation (Olofsson et al., 1996a) and naked plasmid transduction of both VEGF-B_{167} and VEGF-B_{186} was shown to induce angiogenesis in mouse hindlimbs in a nitric oxide dependent manner (Silvestre *et al.*, 2003). In several other settings and tissue environments, such as in the adventitia (Bhardwaj et al., 2003) and skin (Kärpänen et al., 2008), VEGF-B has been shown to be a weak or inefficient inducer of angiogenesis. Both prolonged and transient adenoviral VEGF-B expression have been shown to induce cardiac hypertrophy without attenuating the heart function (Tirziu et al., 2007; Kärpänen et al., 2008).

VEGF-B has recently been suggested to have metabolic functions. Over-expression of VEGF-B leads to cardiac hypertrophy and alterations in lipid metabolism (Kärpänen et al., 2008). In addition, VEGF-B may function as a survival factor. It has been shown to inhibit apoptosis (Li et al., 2008) and to protect both brain and myocardium from ischemic injury (Sun et al., 2004; Kärpänen et al., 2008).

Vascular endothelial growth factor receptor-2 (VEGFR-2)

VEGFR-2 (Flk-1) is essential for the formation of the cardiovascular system. VEGFR-2 null mice die on embryonic day (E)8.5 due to defects in the hematopoietic and EC precursor differentiation (Shalaby et al., 1995). VEGFR-2 is expressed on ECs, neuronal cells, osteoblasts, pancreatic duct cells, megacaryocytes and hematopoietic stem cells (Shibuya and Claesson-Welsh, 2006). VEGFR-2 expression is autoregulated by its ligands VEGF-A, VEGF-C and VEGF-D (Tammela et al., 2005a). VEGFR-2 has been shown to be up-regulated in ischemic tissues in vivo, for example in ischemic myocardium (Li et al., 1996). Since no hypoxia responsive element has been identified, regulation has been suggested to be indirect, either via a paracrine mechanism (Brogi et al., 1996) or post-transcriptional mechanisms such as RNA stabilization (Waltenberger et al., 1996). VEGFR-2 and VEGFR-1 are capable of forming dimers, and dimer formation may mediate ligand-specific functions of these receptors. Prostaglandin production and more efficient arteriogenic response have been suggested to be functions of this receptor dimer (Neagoe et al., 2005).

Several phosphorylation sites and signaling pathways have been identified for VEGFR-2 (Shibuya and Claesson-Welsh, 2006). VEGFR-2 mediates EC migration, proliferation, vascular permeability and angiogenesis (Gille et al., 2001; Ferrara et al., 2003). Nitric oxide has been shown to be a mediator of many of these functions (Zachary and Gliki, 2001).

Vascular endothelial growth factor-A (VEGF-A)

VEGF-A was identified from ascites fluid as a factor inducing vascular permeability (Senger *et al.*, 1983). VEGF-A is required for vascular development. VEGF-A null mice die on E8-9 due to defects in blood island formation, EC development and vascular formation (Ferrara et al., 1996). Even deletion of a single VEGF allele leads to death at E11-12 due to defects in cardiovascular formation (Carmeliet et al., 1996; Ferrara et al., 1996). VEGF-

A is produced in at least six different isoforms due to alternative splicing producing proteins containing 121, 145, 165, 183 or 189 amino acids. The isoforms vary in their solubility: the shortest isoform $VEGF_{121}$ is relatively freely soluble, about half of $VEGF_{165}$ bind to heparin sulphate proteoglycans and $VEGF_{189}$ is tightly bound in extracellular matrix (Tammela et al., 2005a). VEGF-A isoforms are capable of forming growth factor gradients guiding blood vessel growth due to varying solubility properties of the different isoforms. In addition, proteins bound to the extracellular matrix can be mobilized via proteolysis and thus heparin binding may regulate the bioavailability of VEGFs via activation of matrix-bound proteins by proteolytic cleavage (Houck *et al.*, 1992). In addition, different VEGF-A isoforms have different receptor binding profiles. For example, VEGF-A_{121} only binds Nrp-1 (Pan et al., 2007) while VEGF-A_{165} binds to both Nrp-1 and Nrp-2 (Ferrara et al., 2003). VEGF-A expression is increased in hypoxic conditions by hypoxia inducible factor (HIF) (Shweiki et al., 1992). VEGF-A induces proliferation of both EC and smooth muscle cells (Parenti et al., 2002). VEGF-A has been shown to regulate the expression of glutein transporter GLUT1 implicating a role in metabolic regulation in ischemia (Pekala et al., 1990).

Vascular endothelial growth factor-D (VEGF-D)

VEGF-D is produced and secreted as a full length protein dimer. After secretion proprotein convertases (PC) furin, PC5 and PC7 and plasmin cleave the C- and N-terminal propeptides increasing the receptor binding affinity to VEGFR-2 by 17 000 –fold (McColl et al., 2003; McColl et al., 2007). The proteolytic processing is therefore required for the angiogenic activity of VEGF-D (Stacker et al., 1999). The proteolytically processed form of VEGF-D, VEGF-$D^{\Delta N \Delta C}$ has been shown to induce angiogenesis in cornea and skeletal muscle, while adenoviral over-expression of VEGF-$D^{\Delta N \Delta C}$ in the skin induced both blood and lymphatic vessel growth (Byzova et al., 2002). VEGF-D null mice are healthy and fertile and do not display any cardiovascular deformities (Baldwin *et al.*, 2005). In adult tissues VEGF-D is mainly expressed in the heart, lung, skeletal muscles, in the intestine (Achen et al., 1998) and in both normal and atherosclerotic arteries (Rutanen et al., 2003). Although VEGF-D is not required for embryonic development of blood vessels, it was shown to be up-regulated in hypoxia-driven angiogenesis *in vitro* (Nilsson *et al.*, 2004) and in skeletal muscles of diabetic mice in vivo (Kivela et al., 2007). In vitro also hypoxia and cell-cell contacts have been shown to up-regulate VEGF-D (Teng et al., 2002; Orlandini and Oliviero, 2001). Transgenic over-expression of VEGF-$D^{\Delta N \Delta C}$ increases capillary density in the heart and skeletal muscles and improves recovery after ischemia (Kärkkäinen et al., 2009). VEGF-D over-expression had also been shown to promote tumor growth and metastasis (Kopfstein et al., 2007; Kärkkäinen et al., 2009).

Vascular endothelial growth factor-E (VEGF-E)

VEGF-E is a homologue of VEGF-A expressed in the poxvirus orf virus (Lyttle et al., 1994). It binds to VEGFR-2 (Meyer et al., 1999) and Nrp-1 (Wise et al., 1999). VEGF-E has been shown to induce angiogenesis via NO production (Cudmore et al., 2006) and to improve wound healing (Zheng et al., 2007). Transgenic over-expression of VEGF-E was shown to induce blood vessel growth without increasing vascular permeability (Kiba et al., 2003).

Neuropilin-1 (Nrp-1)

Neuropilin-1 (Nrp-1) binds both class 3 semaphorins and VEGFs. It is involved in axonal guidance during development of the nervous system. In adult tissues Nrp-1 was shown to be expressed in the placenta, myocardium and in arterial endothelium and to a lesser extent in kidney, skeletal muscle and pancreas by Northern blotting (Soker *et al.*, 1998). Nrp-1 also plays a role in the formation of the cardiovascular system in the embryo. Nrp-1 null mice display defects in the heart development, formation of large arteries and vascular networks in the yolk sac (Kawasaki *et al.*, 1999), whereas over-expression leads to increase in vessel density, formation of dilated hemorrhagic vessels and cardiac malformations (Kitsukawa *et al.*, 1995). Nrp-1 is also over-expressed in regions of physiological and pathological angiogenesis, suggesting a role in growth of blood vessels. In addition, manipulation of flow also alters neuropilin expression patterns (le Noble et al., 2004).

Nrp-1 is a transmembrane glycoprotein that has only a short cytoplasmic domain. Only the extracellular domain of Nrp-1 is required in the neuronal guidance (Nakamura *et al.*, 1998) and enhancement of VEGFR-2 phosphorylation (Yamada *et al.*, 2001), while intracellular signaling events seem to be essential in vessel formation at least during embryogenesis (Wang *et al.*, 2006). Nrp-1 signaling is poorly understood, but recent findings suggest that Nrp-1 transduces biological signals via NIP (neuropilin interacting protein, GIPC) and GAIP (Gα interacting protein) leading to G-protein mediated signaling. GIPC binds to C-terminal three amino acids of Nrp-1. This complex formation has been shown to be essential for EC migration *in vitro* and angiogenesis *in vivo* (Wang *et al.*, 2006). Further, GIPC has been shown to bind to GAIP, a member of RGS (regulator of G-protein signaling) family suggesting a link to G-protein mediated intracellular signaling events (De *et al.*, 1998).

Nrp-1 binds several VEGF family members: VEGF-A_{165}, PIGF-2, VEGF-E, VEGF-B_{167}, VEGF-B_{186} after proteolytical activation of the protein, VEGF-C (Ober et al., 2004) and partially processed form of VEGF-D (Soker et al., 1998; Mäkinen et al., 1999; Migdal et al., 1998; Kärpänen et al., 2006a). Binding of several VEGFs is dependent on heparin, but in addition heparin may induce multimerization of the receptor, possibly altering its function (Fuh et al., 2000). Nrp-1 co-expression enhances VEGF-A_{165} binding to VEGFR-2 (Soker *et al.*, 1998) but the biological role of PIGF-2 binding remains unclear (Migdal *et al.*, 1998).

Several different splice forms are generated by alternative splicing of the Nrp-1 gene, including naturally occurring soluble monomeric forms that function as natural inhibitors. Soluble Nrp-1 is expressed in heart, placenta and by some tumor cell lines (Gagnon *et al.*, 2000). Soluble Nrp-1 dimer can partially restore the function of Nrp-1, indicating that receptor complexes can form between soluble and cell membrane bound receptors (Yamada *et al.*, 2001). Accordingly, VEGFR-2 and Nrp-1 can form complexes via bridging with VEGF-A_{165} even when these two receptors are expressed by different cells (Soker et al., 2002) or when Nrp-1 is in soluble, dimeric form (Yamada *et al.*, 2001).

Other molecules regulating blood vessel growth

VEGFR-3 has been suggested to be involved in blood vessel growth. VEGFR-3 null embryos die on E9.5 due to disturbances in blood vessel development (Dumont *et al.*, 1998). VEGFR-3 is expressed on both blood and lymphatic vessel endothelium from E8 onward, but its expression becomes mostly limited to lymphatic ECs in midgestation (Kaipainen *et al.*, 1995). A subset of fenestrated capillary endothelial cells expresses VEGFR-3 also in adult (Partanen *et al.*, 2000). Tumor blood vessels have been shown to express VEGFR-3 (Valtola et al., 1999). VEGFR-3 was also shown to be involved in hypoxia-driven angiogenesis in an *in vitro* model using embryonic bodies (Nilsson *et al.*, 2004).

Angiopoietins (Ang) have been shown to be involved in both blood vessel growth and stabilization (Holash et al., 1999), platelet-derived growth factors (PDGFs) participate in angiogenesis by regulating mural cell proliferation (Andrae et al., 2008) and recruitment and fibroblast growth factors have been shown to induce both angiogenesis and arteriogenesis (Murakami and Simons, 2008).

Summary of blood vessel growth

Formation of mature blood vessel network requires coordinated function of several growth factors and receptors (Figure 5). Growth factor expression proceeds in a stepwise manner. Briefly, Hif1-α and its downstream target VEGF-A are essential in the initiation of blood vessel growth. VEGF-induced nitric oxide (NO) production dilates pre-existing vessels, VEGF-A increases permeability and co-expression of Ang-2 induces sprouting of the ECs (Lobov et al., 2002). VEGF expression induced EC proliferation and migration forming immature vascular networks. Remodeling of the neovessels adjusts the vascular network to metabolic needs of the tissue. Ang-2 in the absence of VEGF leads to EC apoptosis (Lobov et al., 2002), while sustained flow and expression of PDGFs stimulate pericyte proliferation and attachment to ECs leading to stabilization of vessels. Ang-1 has been also suggested to be involved in stabilization of the vessels and signaling in the quiescent state (Holash et al., 1999).

As briefly described, coordinated function of several growth factors are needed to produce mature, functional vessels. In angiogenic gene therapy approaches usually only one growth factor is over-expressed. Fortunately, once blood vessel growth is initiated mechanical factors largely regulate the maturation process (Rissanen et al., 2005) and for example VEGF-A is capable of up-regulating factors required in the subsequent steps. Endogenous vascular network pruning and maturation after cessation of the growth factor production may also help to produce functional, mature vessels with single growth factor gene therapy.

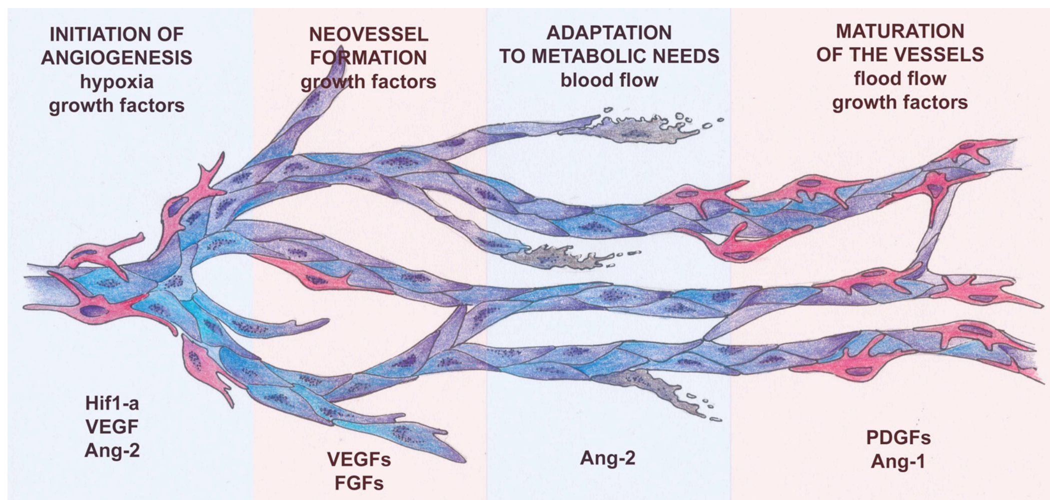


Figure 5. Blood vessel growth is a multi-step process. Coordinated function of different growth factors and mechanical factors produce a hierarchical, functional vascular network. Modified from Markkanen et al. Cardiovascular Research, 2005.

GENE THERAPY FOR CARDIOVASCULAR DISEASES

Cardiovascular diseases are challenging targets for gene therapy approaches. Ischemic disease is a complex process involving a large number of genes and environmental factors, possibly beginning early in the childhood. Several different treatment targets and approaches have been published since the concept of therapeutic angiogenesis was introduced (Svet-Moldavsky and Chimishkyan, 1977). Pathogenesis of the atherosclerotic process has been targeted by lowering blood cholesterol level, inhibiting harmful modification of blood lipids and several other approaches. Angiogenic gene therapy focuses on the treatment of the complications of the ischemic disease. Atherosclerosis occludes blood vessels, and in acute infarction blood flow is blocked completely. Neovessel growth is induced to grow blood vessels around the block in the artery and to improve delivery of oxygen and nutrients to the ischemic tissue.

Therapeutic approach has to be chosen according to the therapeutic goal. Treatment of familial hypercholesterolemia requires life-long production of the low density lipoprotein (LDL) receptor in the hepatic cells, while a week of growth factor production may be sufficient to jump start angiogenesis in the border of a myocardial infarction scar (Markkanen et al., 2005).

Cardiomyocytes as a target for gene therapy

Fatty acid metabolism requires a continuous supply of oxygen and removal of waste products to avoid lactate metabolism and a drop in the pH level. Cardiac muscle is capable of endogenously adapting to low levels of oxygen. Altering the cardiomyocyte metabolism to make cells more resistant to ischemia may be a potential treatment method for myocardial ischemia, at least in combination with angiogenic approaches. Production of new vascular cells requires approximately 24h and growth of new blood vessels takes from days to weeks (Schaper and Ito, 1996), and therefore fast alterations in metabolism may provide the extra time cardiomyocytes need for the neovessels to recover the blood supply. Maintaining myocyte vitality is essential, as 70% of the cells need to be viable in a segment of myocardium for it to be able to regain contractile function (deSilva et al., 1992).

In addition, scattered viable myocytes within the infarction scar may be more detrimental than beneficial by initiating ventricular arrythmias (Rothman et al., 1997).

Cardiac remodeling after myocardial infarction leads to fibrous tissue accumulation, compensatory hypertrophy with inadequate accompanying angiogenesis and ultimately to heart failure (Pfeffer et al., 1991; Colucci, 1997). Inhibiting cardiomyocyte apoptosis and negative remodeling events may improve the clinical outcome of the patients with AMI.

Blood vessel growth as a target for gene therapy

Blood vessel growth is an endogenous response to ischemia. However, endogenous repair attempts often fall short and the need to develop therapeutic interventions persists. Endogenous collateral growth in humans occurs within a period of months to years, while ischemia often occurs acutely. Endogenous collateral artery growth can be sufficient to replace blood supply of one of the main coronary vessels at rest, but is usually not sufficient during stress. Despite new treatment methods for ischemic diseases, not all patients are suitable for conventional treatments. Diffuse coronary artery disease limits the possibilities of percutaneous coronary interventions (PCI) and some patients are in too poor general condition to undergo coronary bypass grafting. Angiogenic gene therapy is a promising new treatment for these patients.(Ylä-Herttuala et al., 2007)

Induction of blood vessel growth has two, partially separate goals: to increase capillary vessel area and density in the ischemic area and to grow arteries to supply blood into these capillaries (Markkanen et al., 2005). Angiogenesis in the ischemic area can improve transport of oxygen and nutrients to the ischemic area. Growth of these capillaries can be directly induced by over-production of angiogenic growth factors. Capillaries are thin-walled, and their ECs are separated from the tissue interstitium only by a discontinuous layer of pericytes and a thin basement membrane. In addition, capillaries are specialized to exchange molecules with the surrounding tissue. Capillary growth is therefore a feasible target of growth factor gene therapy.

New capillaries need blood supply from up-stream arteries, and increase in local blood supply requires growth of the arteries supplying the area. Arterial endothelium is surrounded by a tight internal elastic lamina, layers of smooth muscle cells, an outer elastic membrane and an adventitia. Although many growth factors induce proliferation of smooth muscle cells, their delivery to correct microenvironment within the vessel wall poses a challenge. Further, unbalanced proliferation of the cells in the vessel wall may lead to obstruction of the vessel lumen rather than flow-increasing growth of the vessel caliber. Several studies including our own work, however, have reported collateral artery growth after growth factor delivery (Rissanen et al., 2003a). This may be due to hemodynamic alterations: increased total capillary vessel area downstream increases flow in the supplying arteries, leading to endogenous arteriogenesis stimulated by increased shear stress (Rissanen et al., 2005).

In addition to myocardial ischemia caused by atherosclerosis, therapeutic angiogenesis may provide a tool to treat pathologic cardiac hypertrophy and prevent cardiac failure. In pathologic hypertrophy initially beneficial adaptive process leads to ischemia due to imbalance in increases of capillaries and cardiomyocyte size (Tomanek, 1990). This may be due to too rapid hypertrophy of the cardiomyocytes or by attenuated angiogenic responses in the pressure-overloaded heart (Choi et al., 2008). Eventually this imbalance leads to heart failure (Shiojima et al., 2005).

Safety issues in angiogenic gene therapy

Safety in adenoviral angiogenic gene therapy experiments consists of two main parts, the safety issues related to the viral construct and safety issues related to the transgene. Adenoviruses trigger both rapid innate and adaptive immune responses when viral particles are in contact with blood (St George, 2003). In addition, 98% of adenoviral genomes were associated with blood cells after *in vivo* gene transfer in humans. Interestingly, such interactions were not observed with mouse blood cells highlighting the possibility of species-specific responses (Lyons et al., 2006). Contact with reticuloendothelial system induces release and production of several proinflammatory cytokines possibly causing systemic responses (St George, 2003). However, in both experimental and clinical settings therapeutic adenoviral doses have been well tolerated. Toxicity associated with adenoviral vectors is dependent on the dose and route of administration. If adenoviral vector is delivered locally to a tissue permissive for adenoviral transduction, systemic spread and contact with blood cells can be significantly reduced (Baker et al., 2007).

Adenoviral particles are eliminated from the blood stream within 3 minutes of i.v. administration (Seshidhar et al., 2003). Adenovirus does not integrate into the host DNA and transgene expression time is limited to a few weeks thus limiting the side effects of the therapeutic product. However, if foreign proteins are used, the protein itself may trigger the immunological responses. In case of VEGFs, the most relevant safety issues can be controlled by correct dosing, delivery method and expression time of the growth factor: vascular permeability leading to edema (discussed elsewhere), excessive blood vessel growth, stimulation of blood vessel in unwanted areas. Adenoviruses have natural tropism for liver, and systemic expression in a variety of tissues was reported after intravenous delivery (Hiltunen et al., 2000b). Physical targeting of the virus by selective delivery methods is therefore required to minimize the systemic effects (Simons et al., 2000; Ylä-Herttuala and Alitalo, 2003).

Too high local concentration or prolonged expression time of angiogenic growth factors may induce chaotic growth of blood vessels and formation of glomerular bodies, disorganized vascular structures (Schwarz et al., 2000; Lee et al., 2000). These may develop to hemangiomas, vascular tumors, or disturb tissue blood flow by either stealing blood from other areas or fail to deliver blood and nutrients to tissues due to slow and turbulent blood flow (Carmeliet, 2000). Natural pruning process of the vascular network limits the possibility of these side effects. Although expression level of growth factors is high after adenoviral delivery, expression time is limited. If excessive growth of blood vessels occurs in the initial phase, cessation of the growth factor production and distribution of blood flow according to the needs of the tissue allow the remodeling of the vascular network to meet the demand (Sho et al., 2001). Cessation of the VEGF production may also allow maturation of the vessels, as VEGF-A has recently been shown to prevent EC-pericyte interactions (Greenberg et al., 2008).

Administration of VEGF protein has been shown to induce vasodilatation via VEGFR-2 (Li et al., 2002), and intracoronary delivery of large dose of VEGF recombinant protein induced severe hypotension in pigs (Hariawala et al., 1996), limiting the safe therapeutic dose. Transient tachycardia, hypotension and decreased cardiac output were observed in rats after i.v. administration (Yang et al., 1996). These side effects are likely avoided by local delivery and production of growth factors, resulting in much lower serum levels of growth factors. In addition, after adenoviral delivery protein production starts slowly and compensatory mechanisms may hamper these effects.

Blood vessel growth is required for a tumor to grow beyond the diameter of 1-2 mm (Carmeliet, 2005). Tumor cells are capable of producing a wide variety of angiogenic

growth factors. The local concentration of these factors determines a tumorigenic switch, a critical level of growth factor in the microenvironment to induce angiogenesis. Millions of microtumors form and are destroyed during a lifetime. Exogenous stimulation of angiogenic growth factor production may increase the systemic levels of growth factors and facilitate growth of these dormant tumors (Bergers and Benjamin, 2003).

Angiogenesis occurs also in atherosclerotic plaques, and the effect of exogenous growth factor delivery on the progression of the disease remains unclear (Khurana et al., 2005). Growth factor production and neovessel formation have been shown to destabilize plaques making them vulnerable to rupture and thus increasing the risk of a tromboembolic event (Hayden and Tyagi, 2004). Thus increase in systemic levels of angiogenic factors could contribute to an increase in cardiovascular events. However, over-expression of VEGFs was not shown to be associated with increased atherosclerosis in mice (Leppanen et al., 2005) and no such side effects have been observed in clinical studies (Mäkinen et al., 2002).

Short half lives of VEGFs limit their systemic effects. As measured by ELISA, the half lives of VEGF-A and VEGF-D in the plasma are approximately 8 and 15 minutes, respectively. Growth factors are cleared form the circulation completely by 30 min after recombinant protein administration likely due to clearance by the liver, spleen and the kidneys (Veikkola et al., 2001). The production of the growth factors by the transduced cells is however continuous, and significant concentrations can be released to circulation in therapeutic approaches (Rissanen et al., 2003b). Growth factors differ in their solubility, which determines how easily they are transported from the transduced tissue to the systemic circulation. Therefore also matrix-binding properties of the growth factors can be exploited to control the spread of the therapeutic protein.

LYMPHATIC SYSTEM

Anatomy and function of lymphatic vessels

Lymphatic vessels regulate tissue fluid balance by transporting proteins and extravasated fluid from the interstitial space. In addition, lymphatics transport inflammatory cells and antigens to lymph nodes and transport absorbed triglycerides from intestinal wall to blood circulation. Lymphatic vessels play an active role in the maintenance of fluid balance. Water and proteins are filtered to the interstitial space in the arterial end of the capillaries. Under normal conditions, the majority of the filtered fluid reenters the circulation in the postcapillary venules, and only a small portion of the fluid is removed via lymphatic capillaries. In pathological conditions when permeability of the capillaries in increased more fluid enters the interstitial space. Lymphatic vessels can increase their capacity by 3-4-fold, but if this capacity is surpassed fluid and protein accumulation leads to tissue edema (Guyton and Hall, 2000).

Lymphatic vessels start as blind-ended capillaries. They have discontinuous basement membrane and have no pericyte coverage. Lymphatic ECs have loose intracellular connections and are attached to interstitial collagen and elastin via anchoring filaments. Via these, an increase in interstitial volume pulls open the interendothelial valves and expands the lymphatic lumen (Kärpänen and Alitalo, 2008). In this manner a combined increase in hydraulic conductivity and pressure gradient facilitates lymph formation. The collecting lymphatics have an intrinsically contractile smooth muscle layer as part of their vessel wall. Collecting lymphatic vessels unite to form larger collecting

trunks and finally ductus thoracicius, a lymphatic trunk that delivers lymph to the venous circulation (Guyton and Hall, 2000). Anastomoses between lymphatic vessels and veins have been shown to exist at least in the kidneys, adrenal glands and in the liver. Lymphaticovenous anastomoses also develop in several conditions where lymphatic flow is disturbed (Aalami et al., 2000).

All vascular tissues excluding the brain contain also lymphatic vessels. In skeletal muscles, lymphatic vessels are mainly located in the connective tissue between muscle bundles (Figure 6). In the myocardium, lymphatic capillaries drain to collecting lymphatic vessels located mainly on the epicardial surface.

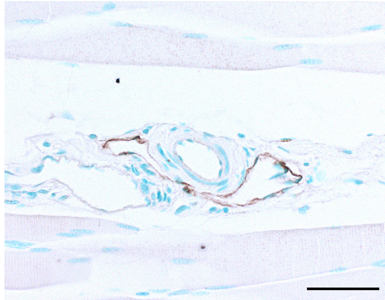


Figure 6. Lymphatic vessels in the skeletal muscle are located in connective tissue between the muscle bundles. Vessels run both parallel and across the direction of the muscle fibers. LYVE-1 staining, magnification 200X, scale bar 100µm. Lähteenvuo et al., unpublished results.

GROWTH OF LYMPHATIC VESSELS

Formation of the lymphatic system

Lymphatic vessels begin to develop after the initial blood vessels have formed. Endothelial cells in the anterior cardinal vein start to express lymphatic vessel hyaluronan receptor 1 (LYVE-1), and a few hours later expression of transcription factor prospero-related homeobox 1 (Prox1) starts. Prox1 is required for formation of the lymphatic system (Wigle and Oliver, 1999) and for lymphatic differentiation of the ECs essential for further development of the lymphatic system (Wigle *et al.*, 2002). Differentiating lymphatic ECs bud from the cardinal vein and migrate to form primitive lymph sacs in response to VEGF-C expression (Kärkkäinen et al., 2004). Lymphatic vasculature forms from centrifugal sprouting from these primitive lymphatic structures (Hong et al., 2004).

Lymphatic cell phenotype remains adjustable. Venous endothelial cells are capable of transdifferentiating into lymphatic cells (Hong et al., 2002; Petrova et al., 2002) and blood ECs are able to assume a lymphatic phenotype. Both of these processes are controlled by prox1 transcription factor activity which is required for maintenance of the lymphatic phenotype (Johnson *et al.*, 2008). Separation of lymphatic and blood vascular networks is maintained by tyrosine kinase Syk and adaptor protein Slp76. Interestingly, these factors are not expressed on lymphatic endothelium and separation of these vascular beds may require presence of circulating blood cells (Abtahian et al., 2003).

Lymphatic vessel development continues during the postnatal period. Collecting lymphatic vessels both sprout to produce superficial lymphatic capillaries and mature to conducting lymphatic vessels by acquiring a smooth muscle cell layer. Lymphatic vessels remain dependent on VEGFR-3 activation for several weeks after birth after which lymphatic vessels become resistant to VEGFR-3 inhibition (Kärpänen et al., 2006b). Further hierarchical differentiation may also be dependent on flow in the lymphatic vessels (Ng et al., 2004).

Growth of lymphatic vessels in adult

Endogenous stimuli induce lymphangiogenesis in chronic inflammation. Lymphatic vessel growth is observed in kidney transplant rejection, chronic airway inflammation and in psoriasis (Alitalo et al., 2005). Inflammation induces lymphangiogenesis via several mechanisms. Macrophages secrete lymphangiogenic growth factors VEGF-C and VEGF-D and may even be able to transdifferentiate into lymphatic ECs. Proinflammatory cytokines such as nuclear factor kappa-B (NFκB) up-regulate VEGF-C that is able to induce lymphatic vessel growth in adult tissues (Watari et al., 2008).

Lymphangiogenesis also occurs side by side with endogenous angiogenesis at least in wound healing and in forming myocardial infarction scar (Paavonen et al., 2000; Ishikawa et al., 2007). Inhibition of lymphangiogenesis has been shown to inhibit and over-expression of lymphatic growth factors to improve wound healing (Saaristo et al., 2006).

Lymphatic vessel growth in adult occurs mainly via sprouting from pre-existing lymphatic vessels (Paavonen *et al.*, 2000). Lymphatic endothelial precursor cells have been shown to exist in peripheral blood (Salven et al., 2003) and to emerge in *in vitro* models (Kreuger *et al.*, 2006), but their role in lymphatic neovessel growth *in vivo* has not yet been clearly demonstrated. In renal transplants lymphatic progenitor cells have been suggested to be incorporated into forming lymphatic vessels (Kerjaschki *et al.*, 2006) while no incorporation of bone marrow derived precursors was observed in tumor xenografts (He et al., 2004). Also other cell types such as macrophages have been shown to incorporate into lymphatic vessels (Maruyama et al., 2005).

MOLECULES REGULATING LYMPHATIC VESSEL GROWTH

Many molecules connected to lymphatic vessel growth have been identified. The most important regulators are VEGFR-3 and its ligands both during embryonic development and in adult tissues. Also Nrp-2 has recently been identified as a regulator of lymphatic vessel growth (Figure 7).

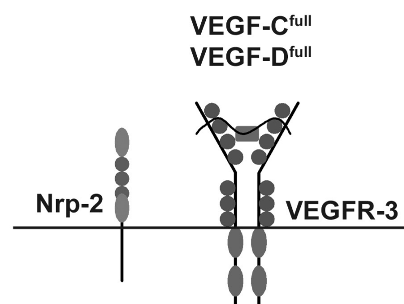


Figure 7. Vascular endothelial growth factors and their receptors regulating lymphatic vessel growth.

Vascular endothelial growth factor receptor-3 (VEGFR-3)

In early development VEGFR-3 (Flt-4) is expressed by both lymphatic and blood vessel endothelial cells. During development its expression becomes restricted to the lymphatic capillary endothelium and some expression is detected in specialized fenestrated blood capillaries (Kaipainen et al., 1995; Partanen et al., 2000). VEGFR-3 signaling is required for development and maintenance of lymphatic vessels during embryonic development and for two weeks after birth in mice (Kärpänen et al., 2006b). VEGFR-3 null mice die *in utero* due to malformations in the cardiovascular system (Dumont et al., 1998). Experiments in morpholino knock down tadpoles supports the role of VEGFR-3 in developmental lymphangiogenesis, although blood vascular malformations

occur also in this model (Ny *et al.*, 2008). VEGFR-3 is also expressed in the adult lymphatic endothelium, and VEGFR-3 stimulation is sufficient to induce lymphangiogenesis (Veikkola *et al.*, 2001).

Vascular endothelial growth factor-C (VEGF-C)

VEGFR-3 ligand VEGF-C was the first identified VEGFR-3 ligand and its expression pattern suggested a role in lymphatic vascular development (Joukov et al., 1996; Kukk et al., 1996). VEGF-C null mice lack lymphatic vasculature and die on embryonic day 15 and lack of one VEGF-C allele results in lymphedema. VEGF-C is essential for the initial sprouting of differentiating lymphatic ECs during embryogenesis (Kärkkäinen et al., 2004). VEGF-C is produced as a full length protein, and further proteolytically processed by intracellular proprotein convertases furin, PC and PC7 and the secreted protein is further processed extracellularly by plasmin (Joukov et al., 1996; McColl et al., 2003; Siegfried et al., 2003). Proteolytic processing of VEGF-C increases its affinity to VEGFR-2 and during early embryonic development VEGF-C may induce its effects on cardiovascular development via VEGFR-2. Proteolytic processing also likely increases the angiogenic functions of VEGF-C in inflammatory situations and in tumor environment where proteolytic molecules are abundant. VEGF-C is expressed in multiple adult tissues, but the strongest expression is detected in the heart, placenta, skeletal muscle, ovary and small intestine (Joukov *et al.*, 1996).

VEGF-C_{156S} is an artificial VEGFR-3 specific ligand. Replacing the second of the eight cystine residues with a serine residue limits the binding of the growth factor solely to VEGFR-3 (Joukov et al., 1998). Its transgenic expression in the skin induces lymphangiogenesis but no induction of blood vessel growth (Veikkola *et al.*, 2001).

Vascular endothelial growth factor-D (VEGF-D)

VEGF-D is not required for lymphatic vessel development, as the only defect in Vegfd null mice is a slight reduction of lymphatic vessel density around the bronchioles (Baldwin *et al.*, 2005). However, VEGF-D may amplify the effects of other lymphangiogenic molecules, as morpholino knockdown experiments in tadpoles suggest an effect on lymphatic EC sprouting and migration, but not on function of lymphatic vessels (Ny *et al.*, 2008). VEGF-D is produced as a full length protein that binds preferentially to VEGFR-3. Proteolytic processing of VEGF-D by plasmin increases its affinity to VEGFR-2 and increases its angiogenic potency (McColl *et al.*, 2007). However, even the processed form has a higher affinity towards VEGFR-3. VEGF-D is naturally expressed most tissues, but the highest expression is detected in the lungs and skin during embryogenesis (Yamada et al., 1997) and heart, lung, skeletal muscle, colon, and small intestine in the adult (Achen et al., 1998). Smooth muscle cells of human arteries contain large amounts of the unprocessed form of VEGF-D (Rutanen et al., 2003).

The role of VEGF-D in adult remains unclear. Wide expression adult tissues, high conservation of the structure and *in vivo* processing of the protein suggest a biological role for VEGF-D and similarities of structure and function with VEGF-C further imply a role in lymphatic vessels. VEGF-D is involved in blood vessel growth and transgenic over-expression of VEGF-D was shown to facilitate recovery after peripheral ischemia (Kärkkäinen et al., 2009). Over-expression of VEGF-D induces lymphatic vessel growth in the adult (Veikkola et al., 2001). The role of VEGF-D might be to induce lymphatic vessel

growth in inflammatory situations to alleviate edema. Macrophages present in inflamed tissues and in edematous angiogenic tissue produce VEGF-D (Watari et al., 2008). Therefore, lymphangiogesis induced by VEGFR-1 and VEGFR-2 ligands may be an indirect effect induced by macrophage recruitment (Murakami et al., 2008). Inflammation provides both a stimulus for macrophage recruitment and presence of proteases to activate the protein. VEGF-D expression has also been connected to lymphatic metastasis and leukocyte infiltration possibly connected to cancer angiogenesis (Bouma-ter Steege et al., 2004). Emphasizing the role of VEGF-D in cancer, transgenic over-expression has been shown to induce spontaneous tumor formation and to promote the growth of tumor xenografts (Kärkkäinen et al., 2009).

Neuropilin-2 (Nrp-2)

Nrp-2 is expressed in embryonic veins and later in lymphatic ECs. Nrp-2 null mice lack small lymphatic vessels and capillaries at birth but larger collecting lymphatic vessels developed normally and animals were not edematous (Yuan et al., 2002). Lymphatic vessel growth was rescued postnatally in surviving mice, but postnatally developed lymphatic vessels were abnormally patterned especially in the heart and in the intestine. Nrp-2 may therefore be required for sprouting of lymphatic capillaries from pre-existing lymphatic vessels.

Nrp-2 expression is also detected in adult lymphatic vessels. Nrp-2 binds VEGF-C in a heparin-independent and VEGF-D in a heparin-dependent manner (Kärkkäinen et al., 2001; Kärpänen et al., 2006a). Nrp-2 is capable of forming a complex with VEGFR-3 and is internalized with VEGFR-3 after VEGF-C and VEGF-D stimulation suggesting that it might modulate VEGFR-3 signaling (Kärpänen et al., 2006a). Nrp-2 inducess also migration of cultured lymphatic ECs (Kärpänen et al., 2006a).

Also angiogenic factors VEGF-A_{145}, VEGF-A_{165} and PlGF bind to Nrp-2 (Gluzman-Poltorak et al., 2000; Neufeld et al., 2002). However, the biological role of this binding remains unclear, as the binding of VEGF-A_{165} to Nrp-2 does not induce internalization of Nrp-2 or interaction with VEGFR-2 (Kärpänen et al., 2006a). Furthermore, although Nrp-2 is expressed in venous endothelium, veins developed normally in Nrp2- null mice (Yuan *et al.*, 2002).

Other molecules regulating lymphatic vessel growth

VEGFR-2 has been suggested to play a role in lymphangiogenesis. VEGFR-2 is expressed on collecting lymphatic vessels and in growing lymphatic capillaries (Jeltsch et al., 1997; Veikkola et al., 2001). Orf virus derived selective VEGFR-2 ligand VEGF-E has been shown to induce lymphatic vessel dilation and several publications have reported induction of lymphangiogenesis by VEGFR-2 ligand VEGF-A (Wirzenius et al., 2007). However, the role of inflammation, up-regulation of endogenous lymphangiogenic growth factors and edema has not fully been addressed. In embryonic bodies cultured *in vitro* VEGF-A did not induce differentiation of lymphatic ECs or lymphatic vessel sprouting, possibly due to lack of blood flow, vascular leakage and edema in this model (Kreuger *et al.*, 2006).

Several integrins are also expressed in lymphatic ECs and function as ligands for lymphangiogenic growth factors. Integrin $\alpha_9\beta_1$ has been shown to bind VEGF-C and VEGF-D (Vlahakis et al., 2005) and integrin $\alpha_5\beta_1$ participates in VEGFR-3 activation by a

VEGFR-3 specific ligand VEGF-C_{156S} (Zhang et al., 2005). Integrin β1 stimulation by fibronectin and collagen has been shown to lead to tyrosine phosphorylation of VEGFR-3 (Wang et al., 2001a).

Angiopoietin receptors Tie1 and Tie2 are expressed in lymphatic ECs. Tie2 receptor has two known ligands, Ang-1 and Ang-2. Their functions differ in blood and lymphatic vessels. In lymphatic cells both ligands seem to have agonistic functions. Ang-2 deficient mice have disorganized lymphatic vasculature and lymphatic vessel dysfunction (Gale *et al.*, 2002). Ang-1 is capable of rescuing this phenotype and its over-expression has been shown to induce lymphatic vessel growth in adult tissues (Tammela et al., 2005b). EphrinB2 has been shown to be involved in lymphatic vessel maturation. Deletion of PDZ binding site of EphrinB2 lead to formation of pericyte-covered lymphatic capillaries and disturbances in lymph flow (Mäkinen et al., 2005).

LYMPHATIC VESSEL GROWTH AS A THERAPEUTICAL TARGET

Lymphedema

Lymphedema is a chronic disturbance of lymph flow leading to progressive swelling of the affected tissues. In hereditary forms the underlying cause is a gene mutation leading to abnormal lymphatic vessel development (Kärkkäinen et al., 2000; Irrthum et al., 2000; Finegold et al., 2001). Radiotherapy, trauma or surgical damage to lymph vessels can lead to acquired lymphedema. 20-30% of surgically treated breast cancer patients who undergo axillary lymphnode evacuation suffer from lymphedema, and no efficient treatments are yet available for this condition. Induction of lymphatic vessel growth is therefore a promising approach for these patients. Promising results have been obtained in small animal models. (Tammela et al., 2007)

Lymphatic vessels in reduction of edema

Lymphatic vessels regulate interstitial fluid and protein concentrations. In angiogenic gene therapy the most important side effect is an increase in vascular permeability, leakage of plasma proteins to interstitial space and subsequent edema. Edema in peripheral muscle compartments can lead to compartment syndrome and in myocardium to the accumulation of pericardial fluid and even tamponation of the heart. Endogenous blood vessel growth in inflammation, wound healing and in healing myocardial infarction scars is accompanied by lymphangiogenesis (Paavonen et al., 2000; Ishikawa et al., 2007). Promoting lymphatic vessel growth may therefore help to balance the blood and lymphatic vessel growth responses and reduce the side effects of angiogenic gene therapies.

Safety issues concerning lymphangiogenic gene therapy

Lymphatic vessels are the most important route of metastasis for many solid tumors (Achen and Stacker, 2008). Many tumors produce lymphangiogenic growth factors such as VEGF-C and VEGF-D, and blocking VEGFR-3 has been shown to inhibit lymphatic vessel growth in tumors and to prevent metastasis (He et al., 2002). Recently

lymphangiogenesis has been shown to occur in sentinel lymph nodes before tumor cells have metastasized suggesting that secreted lymphangiogenic factors reach concentrations sufficient to induce lymphatic vessel growth (Das and Skobe, 2008).

Over-expression of lymphatic growth factors may promote subclinical microtumor metastasis (Achen and Stacker, 2008). Processed forms of VEGF-C and VEGF-D are readily soluble and were secreted to circulation after adenoviral delivery. Transgenic over-expression of VEGF-D was shown to promote tumor growth in mice (Kärkkäinen et al., 2009). While no promotion of spontaneous tumor growth has been reported after delivery of lymphangiogenic factors, systemic concentration of these factors, local delivery methods and limiting expression to targeted cell type are important considerations in lymphangiogenic gene therapy.

ANIMAL MODELS IN GENE THERAPY RESEARCH

Studies on growth factor biology and receptor kinetics have been largely done *in vitro*. However, animal models are required to further elucidate their biological function. Biological responses often require interplay between several cell types, and possibly components of the extracellular environment. Such interactions, although often possible to reproduce also in *in vitro* settings, can be difficult to predict without *in vivo* data. Further, biodistribution of therapeutic substances and systemic effects such as effects on blood pressure can only be studied *in vivo.* Effects of growth factors on different diseases can only be performed in animal models, and data obtained from transgenic animals is essential to study their biological relevance. Use of animal models is thus essential to develop new therapies.

Molecules may have slightly different functions in different species, and for example different receptor binding profiles have been demonstrated for human and mouse VEGF-D (Baldwin et al., 2001). Mechanisms of blood vessel growth vary between small and large animals. Angiogenesis in the skeletal muscles in response to VEGF is mainly enlargement in rabbits (Rissanen et al., 2003a) but increase in vessel density in mice (Kholova et al., 2007). Rabbit collaterals can increase in diameter from 50µm up to 400µm, while collaterals in mice only increase their diameter 2.5-fold and acquire only 1-2 layers of smooth muscle cells (Scholz et al., 2000). Large animal models that resemble human as closely as possible are thus invaluable in increasing safety before clinical trials are initiated. VEGFs in general are highly conserved but differences between species exist. For example, comparisons of mouse, rabbit and pig VEGF-A and VEGFR-2 homologies are presented in tables 1 and 2.

Table 1. Homologies as compared to human VEGF-A (NM_001025366.1)

Species	Gene ID	Query coverage	Max identity
Mouse (mus musculus)	NT_039649.7	92%	97%
Rabbit (oryctolagus cuniculus)	AY196796.1	9%	91%
Pig (sus scrofa)	CT009664.17	93%	100%

Table 2. Homologies as compared to human VEGFR-2 (NM_002253.2)

Species	Gene ID	Query coverage	Max identity
Mouse (mus musculus)	NM_010612.2	83%	82%
Rabbit (oryctolagus cuniculus)	AB017155.1	8%	88%
Pig (sus scrofa)	EU14326	99%	90%

The pig genome is only partially known, and relevant comparisons to all VEGFs and receptors cannot yet be made based on published data. However, high homologies of the known members of the VEGF family suggest that pig is a relevant model to study the functions of human growth factors. The metabolism and ability to tolerate toxins varies between species, and thus doses of therapeutic agents cannot be directly extrapolated to humans from rodents. Delivery methods effect the local concentration of therapeutic agent achieved, and methods relevant for clinical applications may be difficult to reproduce in small animal models. Finally, methods to study and monitor the effects of therapies need to be validated in animal models prior to clinical use.

AIMS OF THE STUDY

The aim of this study was to develop a safe and efficient angiogenic gene therapy approach for the treatment of myocardial and peripheral ischemia. The goal was to find the most efficient VEGF family growth factor for angiogenic and lymphangiogenic therapies and to minimize the side effects. Clinical applications as a target, animal models, gene delivery methods and end point measurements were chosen to best reflect the clinical potency of the growth factor studied.

The individual aims of the original publications were as follows:

I

The aim of this study was to compare different VEGFs side by side to choose optimal growth factors for the treatment of ischemia. Vascular endothelial growth factors mediate angiogenesis via VEGFR-2 and lymphangiogenesis via VEGFR-3 while the role of VEGFR-1 remains unclear. Experiments studying the effects of different VEGFs have been performed in various *in vivo* and *in vitro* systems and in different species complicating the interpretation of their relative contributions to these effects. Direct comparison of angiogenic and lymphangiogenic properties and side effects in the same animal model using the same dose and route of delivery would allow *in vivo* profiling of VEGFs for therapeutic applications.

II

The aim of the second part of the study was to find optimal vector and dose of AdVEGF-D$^{\Delta N\Delta C}$ for angiogenic gene therapy in the myocardium. The goal was to induce neovessel growth in normal pig myocardium and to increase blood flow in the treated area. Also the safety and feasibility of this approach was assessed.

III

The aim of the third study was to explore the angiogenic potency and mechanism of the different VEGFR-1 ligands in both normoxic and ischemic skeletal muscles and in myocardium. Different aspects of myocardial infarction scar healing, apoptosis, cell proliferation and metabolic effects were addressed. Both rabbit and pig tissues were studied to reduce the possibility of species-specific functions of human growth factors.

IV

The aim of the fourth part of this study was to develop a combination gene therapy to minimize the side effects of angiogenic gene therapy by simultaneously inducing lymphatic vessel growth. The aim was also to elucidate the role of lymphatic vessel growth in the resolution of edema in the endogenous angiogenic response to ischemia.

METHODS

ANIMAL MODELS

Gene transfers (GT) were done both to normoxic and ischemic rabbit skeletal muscles, normoxic rabbit heart, normoxic pig skeletal muscles and both normoxic and ischemic pig heart. Normoxic tissues were used for comparisons of different growth factors and mechanistic studies to avoid interference from endogenously up-regulated growth factors and receptors. Therapeutic potential of the most efficient growth factors were assessed in ischemia models. All animal experiments were approved by the Experimental Animal Committee, University of Kuopio.

Rabbit hind limb ischemia model (I, III)

Rabbit model of hind limb ischemia was used to study the angiogenic potency of VEGFs in peripheral muscles. Ischemia was induced by excising the superficial femoral artery. Re-entry branches for collaterals growing from the lateral circumflex and deep femoral arteries were ligated to limit the collateral growth to internal iliac artery (Rissanen et al., 2003a). In original publication III, one more re-entry branch from the lateral side of the knee was ligated to induce ischemia also in the distal thigh.

Pig model of acute myocardial ischemia (III)

Myocardial ischemia was induced in domestic pigs by occluding the left anterior descending coronary artery (LAD) using a VortX-18 occlusion coil (Boston Scientific). Coronary vasculature was first visualized by angiography via a 7F catheter and a platinum occlusion coil was placed in the LAD vessel. Complete occlusion of the vessel was confirmed by angiography, movement defect in the anterior wall of the left ventricle by intracardiac ultrasound imaging (Acunav, Acuson Sequioia, Siemens) and by ECG changes. Infarction was allowed to stabilize for 30min before gene transfer.

GENE TRANSFER METHODS

First generation replication deficient serotype 5 adenoviruses were used as gene transfer vectors. Adenoviruses expressing LacZ reporter gene, VEGF-A_{165}, PlGF-2, VEGF-B_{167}, VEGF-B_{186}, VEGF-E, VEGF-C^{full}, VEGF-C_{156S}, VEGF-$D^{\Delta N\Delta C}$, VEGF-D^{full}, soluble (s) VEGFR-1, sVEGF-2 and sNrp-1 under CMV promoter were used. The functionality of viral constructs was confirmed by RT-PCR, Western blot, ELISA, immunohistochemistry and confirmation of biological activity as presented in table 3. Virus dosing and delivery for VEGFs is presented in table 4.

Table 3. Functionality of viral constructs.

construct	**method**	**reference**
AdLacZ	X-gal staining for B-galactosidase	(Vajanto et al., 2002)
AdVEGF-A	RT-PCR Western blot ELISA Immunohistochemistry Biological activity	(Bhardwaj et al., 2003) (Hiltunen et al., 2000a) (Vajanto et al., 2002; Viita et al., 2008) (Bhardwaj et al., 2003) (Vajanto et al., 2002)
AdVEGF-B_{167}	RT-PCR Western blot ELISA Immunohistochemistry Biological activity	(Bhardwaj et al., 2003) Dijkstra M., unpublished (Leppanen et al., 2005) (Bhardwaj et al., 2003) (Bhardwaj et al., 2003)
AdVEGF-B_{186}	Western blot Immunohistochemistry	Dijkstra M., unpublished Lähteenvuo J., unpublished
AdPlGF	RT-PCR ELISA Biological activity	(Viita et al., 2008) (Viita et al., 2008) (Roy et al., 2005)
AdVEGF-C^{full}	RT-PCR Western blot ELISA Immunohistochemistry Biological activity	(Bhardwaj et al., 2003) (Hiltunen et al., 2000a; Saaristo et al., 2002) (Leppanen et al., 2005) (Bhardwaj et al., 2003) (Saaristo et al., 2002)
AdVEGF-C_{156S}	Western blot Biological activity	(Saaristo et al., 2002) (Saaristo et al., 2002)
AdVEGF-$D^{\Delta N \Delta C}$	RT-PCR Western blot ELISA Immunohistochemistry Biological activity	(Bhardwaj et al., 2003) Jauhiainen S., unpublished (Leppanen et al., 2005) (Bhardwaj et al., 2003) (Bhardwaj et al., 2003)
AdVEGF-D^{full}	RT-PCR Western blot Immunohistochemistry Biological activity	(Bhardwaj et al., 2003) (McColl et al., 2007) (Bhardwaj et al., 2003) (Bhardwaj et al., 2003)
AdVEGF-E	Western blot ELISA	(Zheng et al., 2007) (Zheng et al., 2007)
AdsVEGFR-1	RT-PCR Western blot ELISA Biological activity	(Sallinen et al., 2009) (Sallinen et al., 2009) (Sallinen et al., 2009) (Roy et al., 2005) (Sallinen et al., 2009)
AdsVEGFR-2	RT-PCR Western blot ELISA Biological activity	(Sallinen et al., 2009) (Sallinen et al., 2009) (Sallinen et al., 2009) (Roy et al., 2005; Sallinen et al., 2009)
AdsNrp-1	Western blot	Jauhiainen S., unpublished

Table 4. Tissues, delivery methods, virus concentrations in viral particles (vp), total doses and injection volumes used in gene transfer experiments.

Species	tissue	Delivery method	Virus concentration	Total dose	volume
rabbit	skeletal muscle	Intramuscular (i.m.) injection (25G needle)	$1x10^{11}$vp/ml	$1x10^{11}$vp	10x100µl (1ml)
rabbit	normal heart	Intramyocardial Injection	$1x10^{12}$vp/ml	$5x10^{11}$vp	5x100µl
pig	skeletal muscle	Intramuscular injection (20G needle)	$1x10^{11}$vp/ml	$1x10^{12}$vp	10x1ml (10ml)
pig	normal heart	Catheter-mediated intramyocardial Injection	$1x10^{12}$vp/ml	$2x10^{12}$vp	10x200µl (2ml)
pig	ischemic heart	Catheter-mediated intramyocardial Injection	$5x10^{11}$vp/ml	$1x10^{12}$vp	10x200µl (2ml)

For soluble receptor blocking experiments (III), larger virus concentrations were used to achieve 2-3 fold over-expression of the soluble receptor as compared to the growth factor studied (table 5).

Table 5. Tissues, total doses of viruses encoding growth factors and injection volumes used in soluble receptor gene transfer experiments.

Tissue	Growth factor (total dose)	Soluble receptor (total dose)	volume
Rabbit skeletal muscle	$1x10^{11}$vp	$3x10^{11}$vp	1ml
Normal pig heart	$2x10^{12}$vp	$4x10^{12}$vp	2ml
Ischemic pig heart	$1x10^{12}$vp	$2\text{-}3x10^{12}$vp	2ml

To study the role of nitric oxide, L-NAME (Lω-Nitro-L-arginine methyl ester, Sigma) was administered twice a day (50mg/kg) p.o. for the duration of the experiment to a subset of animals.

ASSESSMENT OF BLOOD VESSEL GROWTH

Histology (I, II, III, IV)

Tissues were perfusion fixed with 1%PFA in citrate buffer (pH 3.5). Tissue samples were further immersion fixed in 4%PFA sucrose, embedded in paraffin and cut to 7µm sections. Immunohistochemical stainings were performed to identify cell types, vessel structures, growth factors, receptors and components of intracellular signaling cascades. Avidin-biotin-HRP system with 3'-5'-diaminobenzidine (DAB, Zymed) and alkaline phosphatase system (Vector Laboratories) with Vector Blue (Vector laboratories) were used as color substrates. Tyramide amplification system (TSA-kit, NEN Lifescience) was used for VEGFR-2 and $\alpha_v\beta_3$ antibodies. Antibodies used are presented in table 6.

Table 6. Primary antibodies.

Structure stained	Antibody	Manufacturer	publication
Endothelial cells (rabbit)	CD31	DAKO	I, III, IV
Endothelial cells (pig)	Pecam-1	Santa Cruz biotechnology	III, IV, unpublished results
Smooth muscle cells	α-SMA (1A4)	Sigma Adrich	I, II, III, IV
Lymphatic endothelial cells	LYVE-1	R&D systems	IV, unpublished results
Proliferating cells	BrdU (Bu20a)	DAKO	I, IV
Proliferating cells	PCNA	Promega	III
Apoptotic cells	TUNEL	Chemicon	III, IV
VEGF-A	VEGF (C-1)	Santa Cruz biotechnology	II, unpublished results
VEGFR-2	Flk-1	Santa Cruz biotechnology	I
Integrin $\alpha_v\beta_3$	$\alpha_v\beta_3$	Biogenesis	I
Nrp-1	Nrp-1 (C-19)	Santa Cruz biotechnology	III
GIPC	GIPC (N-19)	Santa Cruz biotechnology	III
GAIP	GAIP (N-17)	Santa Cruz biotechnology	III
Transduced cells	β-galactosidase (β-gal)	Promega	Unpublished results

Blood and lymphatic vessel measurements (I, II, III, IV)

Mean blood vessel area (μm^2) was measured from CD31 immunostained sections of rabbit skeletal muscles at 200X magnification. Pig tissues were stained with Pecam-1 antibody and vessels from normal myocardium were analyzed at 200X magnification and the infarction edge samples were analyzed at 100X magnification. Lymphatic vessels were measured from CD31+α-SMA stained sections (I) or from LYVE-1 stained sections (IV and unpublished results) at 100X magnification. All measurements were performed with AnalySIS software (Soft Imaging System) in a blinded manner from 5-10 different randomly selected fields from each section. Means of the measurements were reported.

Microspheres (I, II, III)

Fluorescent microsphere particles were used to measure tissue perfusion. Red fluorescent microspheres ($1x10^6$ in rabbits, $5x10^6$ in pigs) were injected into the left ventricle and samples were collected from transduced and control tissues. Tissue samples were lysated in ethanol-potassium hydroxide solution (48h in +60C˚) and microspheres were isolated according to manufacturer’s instructions. 10 000 yellow-green microspheres were added to each sample as a reference in hind limb perfusion measurements. In myocardium, red microspheres were injected at rest, and pharmacological stress was induced using dobutamine infusion. Dobutamine infusion was gradually increased from 5μg/kg until heart rate reached 200 beats per minute. Yellow-green microspheres were

then injected to measure perfusion during stress. Results were expressed as a ratio between transduced and control samples.

Angiography (III)

Angiography was used to visualize collateral growth in hind limb and in myocardium. Digital subtraction angiography of the rabbit hind limbs was performed before sacrifice. Last image before venous filling was observed and chosen for analysis. Coronary angiographies were done *in vivo* before induction of AMI, and to confirm complete closure of the LAD, and *ex vivo* after the animals were sacrificed. Angiographies were performed with Siemens Siremobil (Siemens, Germany) using a 7F AR2 catheter (Cordis) and Gadolinium contrast agent.

Ultrasound imaging and ejection fraction (II, III)

In normoxic myocardium, pericardial effusion was visualized from long axis projections with a 3V2c transducer (Acuson Sequoia, Siemens). Perfusion was assessed using myocardial contrast echocardiography. Contrast agent ($2x10^8$ bubbles/ml, SonoVue, Bracco) was injected via the ear vein, bubbles were destructed with high energy Doppler wave, and short axis view images were recorded from the mid-papillary level at the time of maximal refilling during systole.

In ischemic myocardium, echocardiography was performed at the baseline, after AMI and before sacrifice with Acunav catheter inserted into the right atrium. Longitudinal 2-chamber views are shown to visualize pericardial effusion six days after the gene transfer. Cardiac ultrasound imaging was performed by using intracardiac ultrasound transducer (Acunav®, Acuson Sequoia, Siemens). Ejection fraction was measured from longitudinal two-chamber views using Acuson software (modified Simpsons formula). Two measurements from two different ultrasound recordings were done when possible.

CEU perfusion measurement was done in skeletal muscle as described (Rissanen et al., 2003b) after a bolus injection of a second generation contrast agent ($2x10^8$ bubbles/ml, mean diameter 2.5 µm, SonoVue, Bracco, Italy) via the ear vein.

PROTEIN ANALYSES

Immunoprecipitation and Western blot (III)

Immunoprecipitation was performed to study ligand binding and receptor activation of Nrp-1. Protein lysates from transduced myocardium were incubated for 8h with Nrp-1 antibody (C-19, Santa Cruz biotechnology), protein A sepharose beads (Amersham Biosciences) were added and samples were incubated in +4C over night. Sepharose beads were then carefully washed with 0.2% Tween 20 in PBS and electrophoresis was performed. Western blot analyses were then performed with VEGF-B antibody (MAB 3373, R&D biosystems) to confirm ligand binding and with GIPC antibody (N-19, Santa Cruz biotechnology) to show receptor activation. GAIP (N-17, Santa Cruz biotechnology) expression was studied from native protein lysates with Western blot.

ELISA (II)

Enzyme linked immuno sorbent assay (ELISA) was used to measure local and systemic protein concentration after intramyocardial gene transfers. Proteins were isolated from snap frozen tissue samples using T-Per protein isolation buffer (Pierce), protease inhibitor was added (11697498, Roche) and total protein concentration of the samples were measured. ELISA kits for VEGF-A (DVE00) and VEGF-D (DVED00) (R&D biosystems) were used according to manufacturer's instructions.

VASCULAR PERMEABILITY

Modified Miles assay (I, II, III, IV)

Vascular permeability of plasma proteins was measured by modified Miles assay. Evans blue dye (E2129, Sigma) was diluted to saline (30mg/ml, 1ml/kg) and injected intravenously 30 minutes before sacrifice. Evans blue binds to plasma proteins. Animals were perfusion fixed with 1%PFA in citrate buffer, pH 3.5 to clear remaining dye from blood vessels and to enhance dye binding to the tissues. Samples were incubated in formamide for 48 hours in +60C° to extract the dye from tissue samples and the amount of Evans blue was quantified with spectrophotometer at 610nm. Results were expressed as ratios between treated samples and intact controls.

Magnetic resonance imaging (I)

Magnetic resonance imaging (MRI) was performed six days after the gene transfer to assess edema in the transduced rabbit skeletal muscles. Imaging was performed with a Varian ^{UNITY}inova (Varian Inc.) imaging console interfaced to a 4.7T horizontal magnet (Magnex Scientific Ltd.) Data were aquired 3min after gadodiamide contrast agent (Omniscan, Nycomed) with a custom built surface coil using a 3D flow compensated T_2* weighted gradient echo sequence (FOV 6x8x6cm, matrix 256x128x64, tr=25ms, te=80ms) (Rissanen et al., 2003a).

CELL CULTURE EXPERIMENTS (III)

bEnd3 cells (ATCC) were cultured in high glucose DMEM (Gibco) supplemented with 10% FCS. Murine HL-1 cardiomyocyte cell line was kindly provided by Dr. W.C. Claycomb (Louisiana State University Medical Center)(4) and were cultured in Claycomb medium (JRH Biosciences Ltd.) supplemented with 10% FCS according to the instructions of Dr. Claycomb. mVEGF-B186, mVEGF-B167, mPlGF2 and sVEGFR1 were purchased from R&D systems (Minneapolis, MN). For the treatment of HL-1 and bEnd3 cells with VEGF-B or PlGF2, cells were starved in Claycomb medium containing 0.5% FCS for 4 h followed by addition of 100 ng/ml of the growth factors, or 1 µg/ml of sVEGFR1, and further incubated for 20-25 h. For experiments with conditioned media, the media from

growth factor treated bEnd3 cells was transferred to pre-starved HL-1 cells, and the HL-1 cells were further incubated for 8h. Total RNA fractions were isolated, reverse transcribed and analyzed by real-time RT-PCR. All experiments were performed in triplicates, and were repeated three times. Primers for the analyzed genes are shown below (Table 7).

Table 7. Primer sequences. All sequences are written 5'→ 3'

	Fwd primer	Rev primer
mL19	GGTGACCTGGATGAGAAGGA	TTCAGCTTGTGGATGTGCTC
mFatp1	TCAATGTACCAGGAATTACAGAAGG	GAGTGAGAAGTCGCCTGCAC
mFatp4	GCAAGTCCCATCAGCAACTG	GGGGGAAATCACAGCTTCTC
ssFatp4	TGGTCCGTGTCAACGAGG	GGTGGACACGTTCTCGCC
ssBeta-Actin	ATGGAATCCTGCGGCATC	CTTGCTGATCCACATCTGC

Expression levels in cultured cells were normalized to the expression of mL19, whereas beta-actin was used as normalization gene for pig samples. For detection of Mouse BH3-only genes (Bik1, Bmf1 and Bad1), Qiagen QuantiTect specific primers were used (Qiagen, Germany). m, Mouse; ss, Sus scrofa.

STATISTICAL ANALYSES

Results are expressed as mean ±SEM. Statistical significance was evaluated using one-way ANOVA or Kruskal-Wallis followed by Mann-Whitney U test where appropriate in original publications I and II. In original publication III and unpublished work, statistical significance was evaluated using Linear Mixed Models (SPSS version 14.0) which is based on generalized estimating equations (GEE) or by ANOVA followed by Students t-test. $P<0.05$ (marked * in the figures) was considered statistically significant. Symbols ** and *** were used for significances of <0.01 and <0.001, respectively.

RESULTS AND DISCUSSION

Selected results from the original publications and unpublished data are presented. New viral constructs, staining methods and slowly accumulating data from experimental animal work has enabled new analyses and comparisons that were not available at the time of publication of the original articles. Therefore, the data presented does not follow the order of the original publications. For published data, the number of original publication is indicated in the figure legend.

EFFICACY OF ADENOVIRAL GENE TRANSFER IN SKELETAL MUSCLE AND MYOCARDIUM

AdLacZ marker gene was used as a negative control for all gene transfer experiments. Transduction efficiencies were studied from β-galactosidase (β-gal) stained tissue sections (Figure 8). In skeletal muscles β-gal positive nuclei were observed both in the connective tissue between the muscle bundles and in the skeletal myocytes. Transduction efficiency was quite low (only a few percent of the cells), but positive nuclei were found thoughout the transduced muscle. Only occasional positive ECs were observed (a). In myocardium most of the transduced cells were cardiomyocytes (b). Approximately 50% of the cardiomyocytes were β-gal positive in the vicinity of the needle tracks. Transduction efficiency was higher on the epicardial side, forming a triangular pattern extending towards the epicardial surface from the injection site.

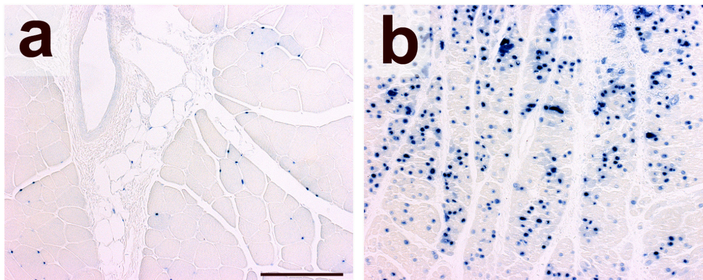


Figure 8. β-gal staining (blue) for AdLacZ transduced cells six days after the gene transfer in rabbit skeletal muscle (a) and pig myocardium (b), magnification 100X, scale bar 200µm. Unpublished results.

Adenoviral transduction is dependent on the presence of specific molecules required for virus entry, mainly CAR receptor expression on the cell surface (Bergelson et al., 1997). Mechanical factors control the spread of the virus. Local virus concentration, rather than total viral dose, is more vital for the transduction efficiency. Physical qualities of the target tissue also play a role. Intramyocardial pressure is higher in the endocardium (Katz, 2005), and the pressure gradient across the myocardial wall may push the virus towards the epicardium. Cardiomyocytes are tightly attached to one another and no large extracellular spaces exist. This may limit the local spread of the virus. In skeletal muscles, loose connective tissue separates skeletal muscle bundles forming a route for wider spread of the virus.

ECs are the target of angiogenic gene therapy, but in neither tissue environment are the endothelial cells transduced efficiently. This mimics growth factor production in endogenous angiogenesis, where cells in the hypoxic area secrete VEGF to stimulate directed blood vessel growth (Shweiki et al., 1992). The growth factor gradient is essential

for migration of ECs (Gerhardt et al., 2003; Barkefors et al., 2008), and growth factor production in the ECs may lead to formation of glomerular structures due to lack of directional stimulus. The lack of EC transduction may also limit the systemic spread of the growth factor. Transduced cells secrete the growth factors, and as ECs form the interface between local tissue environment and systemic circulation, lack of EC transduction may limit the systemic concentrations of the transduced growth factors. Epicardial spread of the virus on the other hand may increase the side effects. Epicardial cells are readily transduced, and production of angiogenic factors may lead to growth of aberrant blood vessels in the connective tissue, possible leading to the accumulation of pericardial effusion.

Biodistribution

Biodistribution of the adenoviral vector was studied from AdLacZ transduced animals (Figure 9). High transduction efficiency was achieved in the myocardium (a). A few transduced, β-gal positive cells were found from the spleen (c), lung (d) and a single positive cell also from the connective tissue in the ovary (f). No positive cells were observed in the liver (b) or in the kidneys (e).

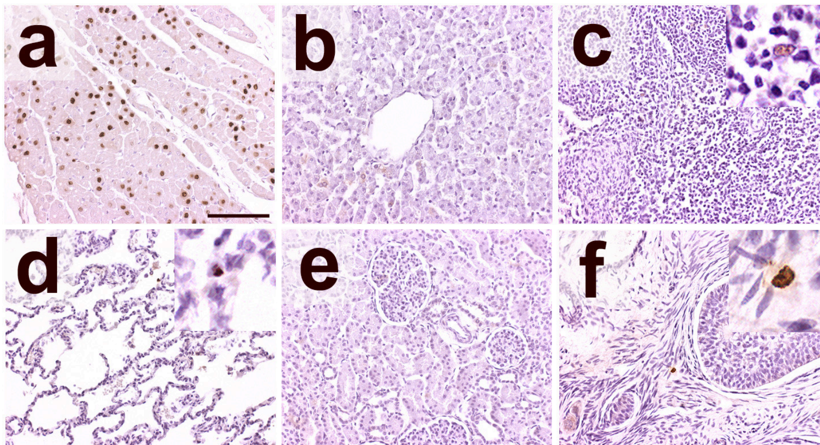


Figure 9. β-galactosidase staining for AdLacZ transduced cells in pig tissues, magnification 200X, scale bar 100µm. Single β-galactosidase positive nuclei are shown in higher magnification in the inserts in figures c, d and f. Unpublishd results.

Biodistridution of the therapeutic protein was assessed from the pigs that received intramyocardial AdVEGF-A or AdVEGF-D$^{\Delta N \Delta C}$ GT (Figure 10). ELISA analysis showed high protein expression in the transduced myocardium in AdVEGF-D$^{\Delta N \Delta C}$ transduced animals (a) and human VEGF-D was also detected in the plasma six days after the GT (b). In AdVEGF-A transduced tissues no elevated VEGF-D protein levels were detected (b). ELISA analysis of the safety tissues revealed protein levels barely above the detection limit in some of the liver and spleen samples (data not shown).

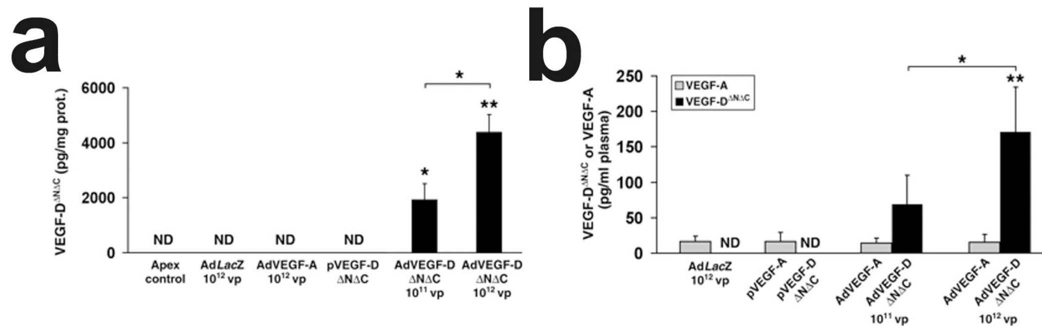


Figure 10. VEGF-D concentrations (pg/mg of total protein) as measured by ELISA in transduced tissues 6 days after the GT (a) and in plasma (pg/ml of plasma) 6 days after the GT (b). From original publication II.

Biodistribution of the adenoviral constructs consists of two entities, the spread of the viral vector shortly after the GT and the spread of the therapeutic protein. Only a few β-gal positive cells were found in the safety tissues analyzed indicating that local gene transfer reduces systemic spread of the virus (Hiltunen et al., 2000b). Growth factors are secreted from the transduced cells, and physical properties of the growth factors govern the distribution of the protein. AdVEGF-A_{165} binds to cell surfaces, whereas VEGF-B_{186} and VEGF-$D^{\Delta N \Delta C}$ are readily soluble. Low levels of VEGF-D protein detected in liver and spleen samples may therefore be due to the high blood content of these tissues, not local protein production in these tissues. Perfused tissue samples would shed more light on this issue.

GROWTH OF BLOOD VESSELS IN MYOCARDIUM AND SKELETAL MUSCLE

VEGFR-2 ligands induce blood vessel growth in skeletal muscle

Capillary size, number and morphology were studied from CD31 stained skeletal muscle sections. Negative control AdLacZ did not alter capillary size or morphology (a). VEGFR-1 ligand AdPIGF induced angiogenesis (b) while AdVEGF-B_{167} (c) and AdVEGF-B_{186} (d) were inefficient. VEGFR-2 ligands AdVEGF-A (e), AdVEGF-$D^{\Delta N \Delta C}$ (f) and AdVEGF-E (g) induced capillary growth. VEGFR-3 ligands AdVEGF-C^{full} (h), AdVEGF-C_{156S} (i) and AdVEGF-D^{full} (j) did not induce blood vessel growth (Figure 11). Several VEGFs bind to more than one receptor. VEGF-A binds to both VEGFR-1 and VEGFR-2 and both full and ΔNΔC isoforms of VEGF-C and VEGF-D bind to VEGFR-2 and VEGFR-3. Growth factors are grouped in the figure based on their main biological responses.

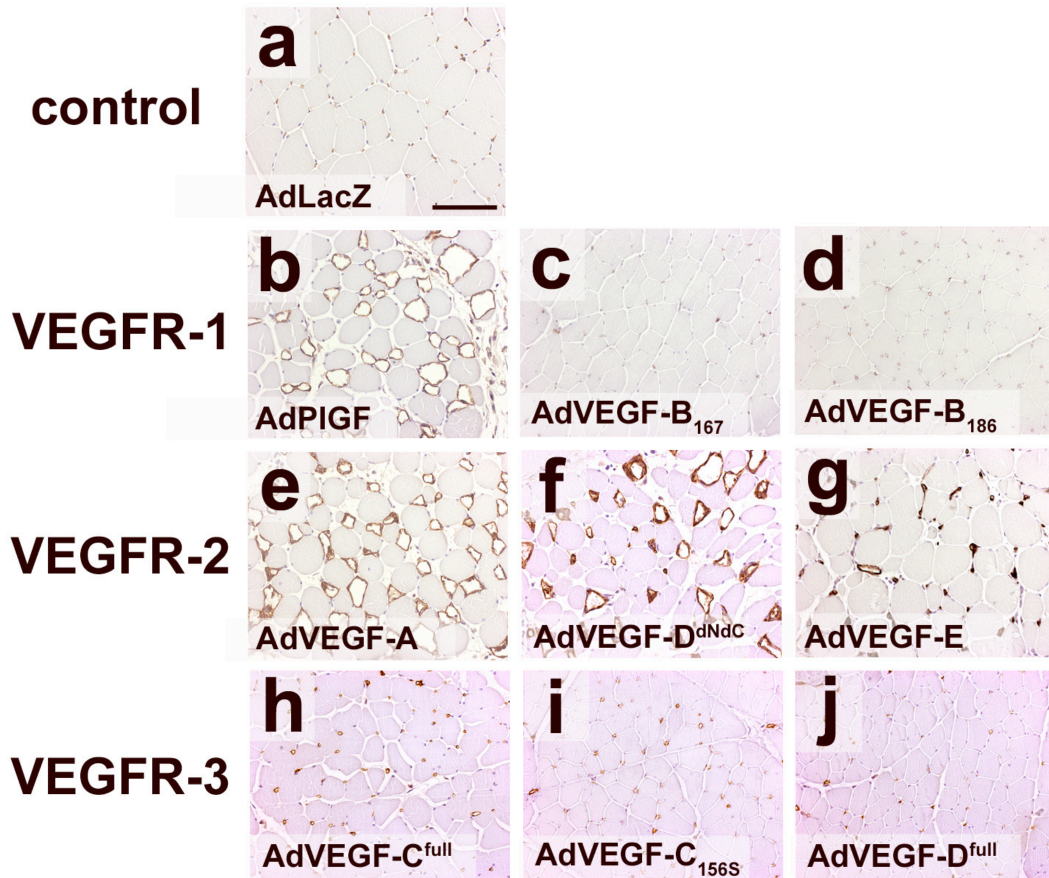


Figure 11. Transduced rabbit skeletal muscles 6 days after the GT. CD31 staining (brown) for endothelial cells, magnification 200X, scale bar 100µm. From original publications I, III and unpublished results.

Quantification of blood vessel growth showed an increase in mean capillary area after the GT of VEGFR-2 ligands and after the AdPlGF GT (figure 12). VEGFR-3 ligands or AdVEGF-B_{167} and AdVEGF-B_{186} did not significantly increase mean capillary area. AdVEGF-$D^{\Delta N\Delta C}$ was the most potent inducer of angiogenesis increasing the mean capillary area 14-fold as compared to AdLacZ (Figure 12a). Capillary number was increased only after AdVEGF-A GT while other growth factors did not significantly increase mean capillary number/mm^2 (Figure 12b).

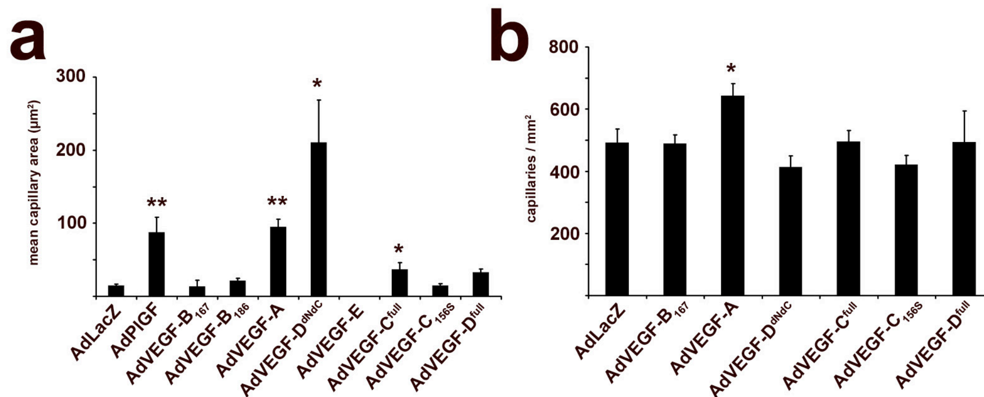


Figure 12. Mean capillary area (μm^2) and capillary density (capillaries/mm^2) in rabbit skeletal muscles six days after the GT. From original publications I and III and unpublished data.

VEGFR-2 is known to mediate angiogenic effects. AdVEGF-A_{165} is a potent inducer of angiogenesis increasing mean capillary size 6-fold as compared to AdLacZ. Surprisingly, AdVEGF-$D^{\Delta N\Delta C}$ was even more potent angiogenic factor. Proteolytic processing increases the affinity of VEGF-D to VEGFR-2 by 17,000 fold (McColl et al., 2007), thus potentiating its angiogenic effects. AdVEGF-C^{full} and AdVEGF-D^{full} failed to induce angiogenesis, indicating that they were not proteolytically processed in this model. The lack of proteolytic processing may be due to species-specific differences as human growth factors were used in rabbit skeletal muscles, or due to non-inflammatory environment lacking proteases such as active plasmin to activate these growth factors (McColl et al., 2003). Although rabbit VEGF-D is 88% identical to human VEGF-D (http://www.ensembl.org), species-specific functions are possible since binding profiles have been shown in mouse with similar gene sequence homology (84%) (Baldwin et al., 2001).

The differences in angiogenic potencies of AdVEGF-A and AdVEGF-$D^{\Delta N\Delta C}$ may be explained by their differential binding to extracellular matrix. VEGF-A_{165} binds to heparin sulphate proteoglycans on cell surfaces and is thus sequestered by the matrix in the vicinity of the transduced cells (Tammela et al., 2005a). AdVEGF-$D^{\Delta N\Delta C}$ on the other hand is freely soluble, and is distributed more widely into the muscle (Achen et al., 1998). AdVEGF-$D^{\Delta N\Delta C}$ may therefore be more readily available for VEGFR-2 on the endothelium.

Capillary number is often used as an end point measurement in angiogenesis studies. Although mean capillary area was significantly increased, only AdVEGF-A significantly increased mean capillary number / myocyte. In previous studies, increase in mean capillary area has led to significant increases in tissue perfusion indicating that increase in capillary size alone is sufficient to increase perfusion in large animal models (Rissanen et al., 2005). Angiogenic mechanisms may vary between species. Increase in capillary density has been observed also in our laboratory in mouse skeletal muscles after adenoviral VEGF-A and VEGF-$D^{\Delta N\Delta C}$ GT (Kholova et al., 2007), while the same vectors only induce capillary enlargement in rabbit and pig skeletal muscles (Rissanen et al., 2003a) and unpublished observations (Lähteenvuo et al. 2006). Expression time may also affect the quality of the angiogenic response observed. Adenoviral GT produces a rapid but transient expression of the therapeutic genes. Capillaries run parallel to myocytes in skeletal muscles, and tight structure of the tissue possibly limits the sprouting of new vessels. Capillaries were large but relatively regular in shape within the muscle bundles after AdVEGF-A GT (Figure 13a). Denser, more disorganized vessel structures were observed in connective tissue surrounding the skeletal muscles six days after the

AdVEGF-A GT supporting this hypothesis (Figure 13b). Flow has also been shown to regulate vessel architecture in AdVEGF-A transduced muscles (Rissanen et al., 2005). In our model arterial flow to transduced muscles was not disturbed, possibly explaining the lack of sprouting angiogenesis.

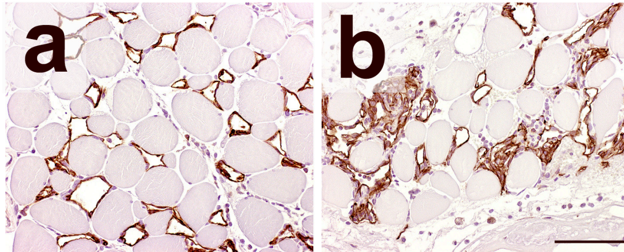


Figure 13. Capillary growth and glomerulus formation in rabbit skeletal muscles, AdVEGF-A transduced muscles six days after the GT. CD31 staining (brown) for ECs, magnification 200X, scale bar 100µm. Lähteenvuo et al., unpublished result.

VEGFR-2 stimulation induces blood vessel growth in ischemic skeletal muscle

PlGF has been proposed to have a specialized function in ischemic tissues (Carmeliet, 2003) and VEGF-B has been shown to induce angiogenesis in ischemic hindlimbs (Silvestre et al., 2003), possibly due to VEGF-B/VEGF-A heterodimer formation (Yoon and Losordo, 2003). The function of VEGFR-1 ligands was therefore further studied in ischemic skeletal muscles. Endogenous angiogenesis was observed in the border of ischemic necrosis in AdLacZ transduced ischemic skeletal muscles (a). The angiogenic responses were in line with our results observed in normoxic skeletal muscles. Both AdVEGF-A (b) and PlGF (c) increased blood vessel growth. AdVEGF-B_{167} (d) and AdVEGF-B_{186} (e) did not induce angiogenesis in the ischemic skeletal muscles (Figure 14).

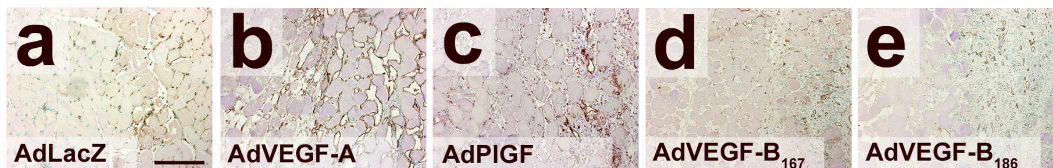


Figure 14. Ischemic rabbit skeletal muscles 6 days after GT. CD31 staining (brown) for endothelial cells, magnification 100X, scale bar 200µm. From original publication III.

VEGF-B is expressed in skeletal muscles (Olofsson et al., 1996a), but is not required for skeletal muscle development as VEGF-B null mice develop normal peripheral musculature (Aase et al., 2001) suggesting a restricted role in the adult. In contrast to previous findings (Silvestre et al., 2003), AdVEGF-B did not increase angiogenesis in the transduced skeletal muscles. This is surprising, as adenoviral gene transfer produces high local protein levels. Differences in angiogenic response may therefore be due to different animal models and differences in end point measurements. However, other effects for VEGF-B such as metabolic and functional effects cannot be ruled out based on this study.

VEGFR-1 and VEGFR-2 ligands induce blood vessel growth in normoxic myocardium

Blood vessel growth in normoxic myocardium was studied 6 days after the GT (Figure 15). Capillaries were normal in AdLacZ transduced muscle samples (a). VEGFR-2 ligands AdVEGF-A (e), AdVEGF-$D^{\Delta N \Delta C}$ (f) and AdVEGF-E (g) induced capillary growth 6 days after the GT. In contrast to results in skeletal muscles, all VEGFR-1 ligands AdPlGF

(b), AdVEGF-B_{167} (c) and AdVEGF-B_{186} (d) induced angiogenesis in the myocardium. VEGFR-3 ligand AdVEGF-C_{156S} induced only weak vessel enlargement (h).

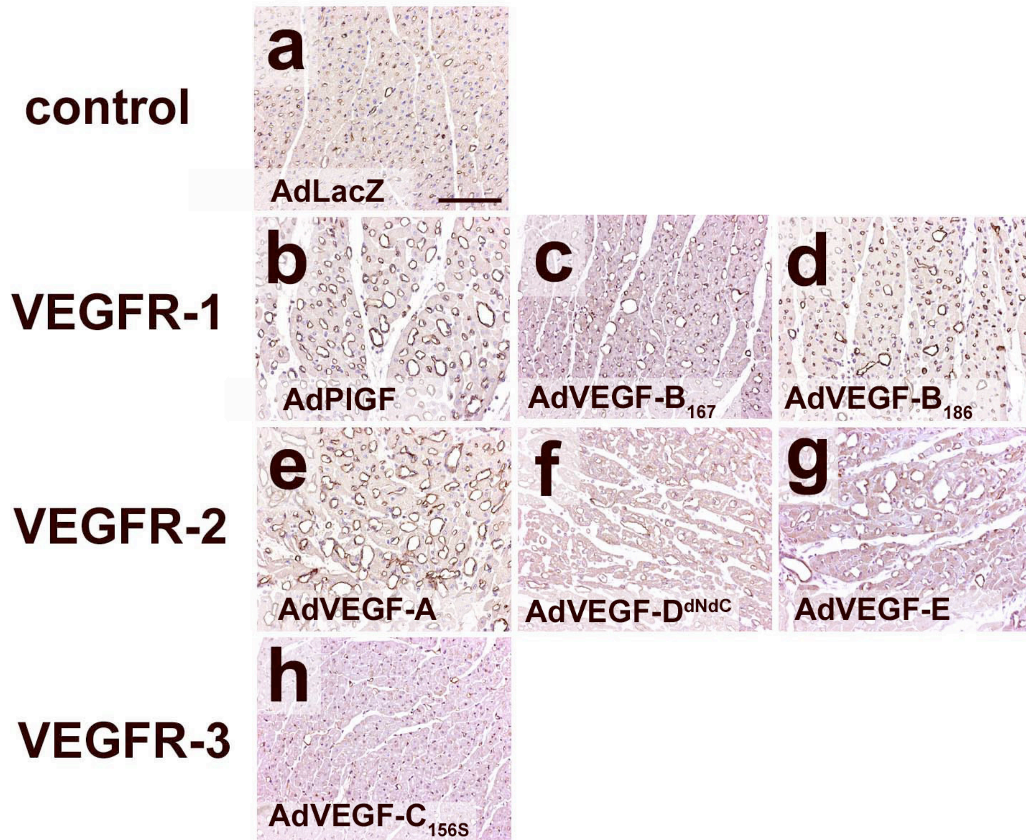


Figure 15. Transduced pig myocardium 6 days after the GT. Pecam-1 staining (brown) for ECs, magnification 200X, scale bar 100μm. From original publication III and unpublished results, figure h Kattainen E et al. unpublished result 2009.

VEGFR-1 and VEGFR-2 ligands induce angiogenesis in ischemic myocardium

Blood vessel growth was studied in the infarction scar edge six days after the GT (Figure 16). In AdLacZ transduced hearts, endogenous angiogenesis was observed both in the outer rim of the fibrotic scar (*) and weak capillary enlargement in the surrounding myocardium (a). AdVEGF-A (b), AdPlGF (c) and both AdVEGF-B_{167} (d) and AdVEGF-B_{186} (e) induced angiogenesis. AdVEGF-B_{186} was the most potent angiogenic factor increasing the total vessel area by 2.9-fold as compared to AdLacZ (Figure 17)

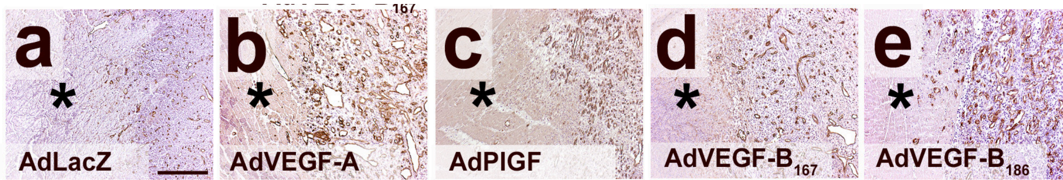


Figure 16. Transduced pig myocardium from the border zone of a myocardial infarction scar 6 days after the GT. Pecam-1 staining (brown) for endothelial cells, magnification 100X, scale bar 200µm. From original publication III.

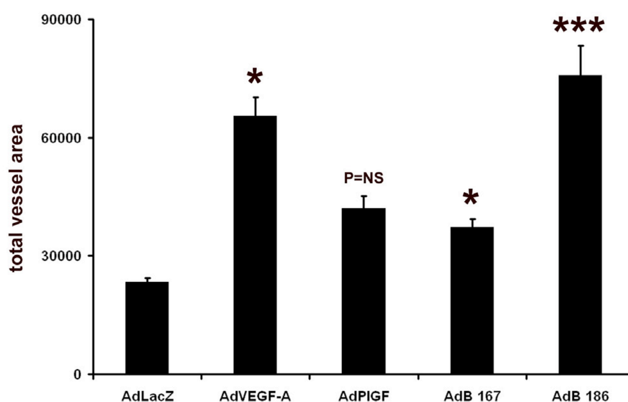


Figure 17. Total vessel area (μm^2) in the infarction border zone 6 days after the GT. From original publication III.

The most potent angiogenic factors were chosen for the studies in ischemic myocardium. While the angiogenic effect of VEGF-B_{186} was weaker than that of AdVEGF-A in normoxic muscle, it was the most efficient growth factor in the infarction edge. This may be due to differences in receptor signaling. VEGFR-1 is up-regulated in the ischemic myocardium (Marti and Risau, 1998). While VEGFR-1 has been shown to be a negative regulator of VEGF-A (Shibuya, 2006), it may be required for the angiogenic function of VEGF-B. If such an endogenous amplification mechanism of VEGF-B exists, it may increase the therapeutic potential of AdVEGF-B_{186} and further target the angiogenic response to ischemic area.

Angiogenic effect persists in the ischemic tissue

The angiogenic effect of AdVEGFA was studied on days 6 and 21 after the GT in normoxic and ischemic myocardium (Figure 18). The angiogenic effect was stronger in the ischemic myocardium (c) although the viral dose was one half of the dose used in the normoxic myocardium (a). Whereas blood vessel size had returned to baseline in the normoxic heart on day 21 (b), in the ischemic hearts angiogenic response persisted on day 21 (d). AdLacZ did not induce blood vessel growth in the normoxic myocardium on day 6 (e) or day 21 (f), and only mild capillary enlargement was seen in the ischemic heart on day 6 (g). The effect had returned almost to baseline by day 21 (h).

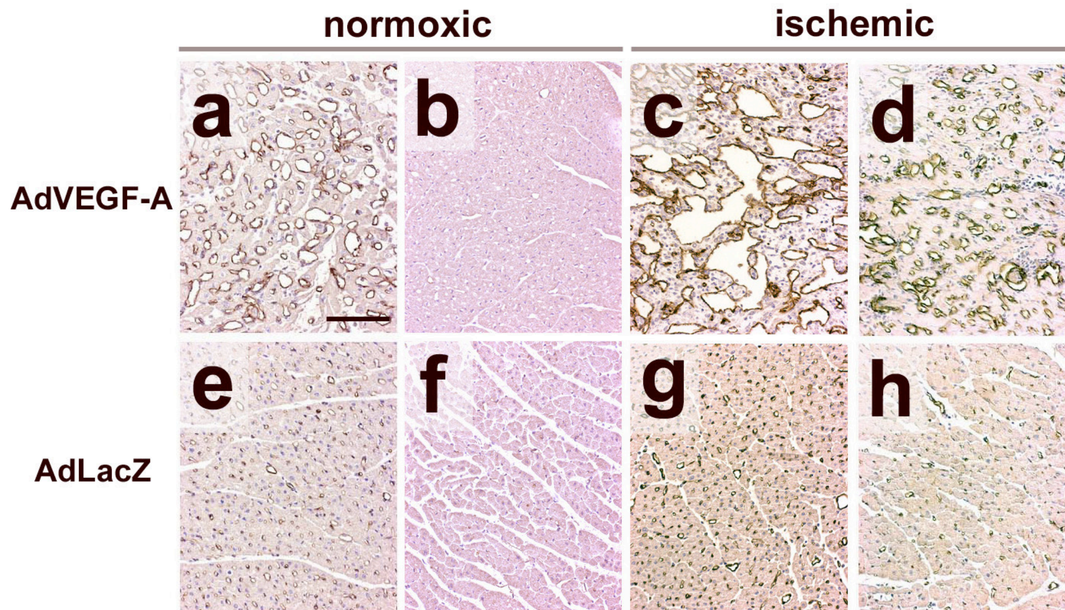


Figure 18. Normoxic (a-b, e-f) and ischemic (c-d, g-h) pig myocardium 6 (a,c,e,g) or 21 (b,d,f,h) days after the GT. Pecam-1 staining (brown) for ECs, magnification 200X, scale bar 100µm. From online data supplement of original publication III and unpublished results.

Angiogenic response in the ischemic myocardium was markedly stronger than in the normoxic myocardium. This is likely due to endogenous growth factor and receptor up-regulation by hypoxia. VEGF-A binds to VEGFR-1 and VEGFR-2. VEGFR-1 has been shown to be directly up-regulated by hypoxia (Gerber et al., 1997; Marti and Risau, 1998), while VEGFR-2 up-regulation has been only demonstrated *in vivo* and occurs likely indirectly due to VEGF-A expression or by post-transcriptional mechanisms (Waltenberger et al., 1996).

Vessels persist in the ischemic tissue due to metabolic need. While in normoxic tissues new vessels or increased perfusion are not needed, in ischemic environment metabolic regulatory mechanisms maintain high perfusion in the enlarged vessels and the vessels do not regress when the growth factor production ceases. Hypoxia and accumulation of metabolites induce relaxation of arterioles, leading to increased blood flow (Guyton and Hall, 2000). This promotes vessel maturation by increasing smooth muscle coverage (Van Gieson et al., 2003).

Angiogenesis is accompanied by arteriogenesis in ischemic myocardium

Ex vivo angiographies were performed six days after the gene transfer to visualize the formation of collateral vessels (Figure 19). Only a few arteries were visible in the infarction border zone in AdLacZ transduced hearts. AdVEGF-A and AdPlGF induced growth of collateral arteries above the infarction scar area. AdVEGF-B_{186} was the most potent inducer of collateral artery growth. A tree-like structure branching from the diagonal branches of LAD was observed in AdVEGF-B_{186} transduced hearts.

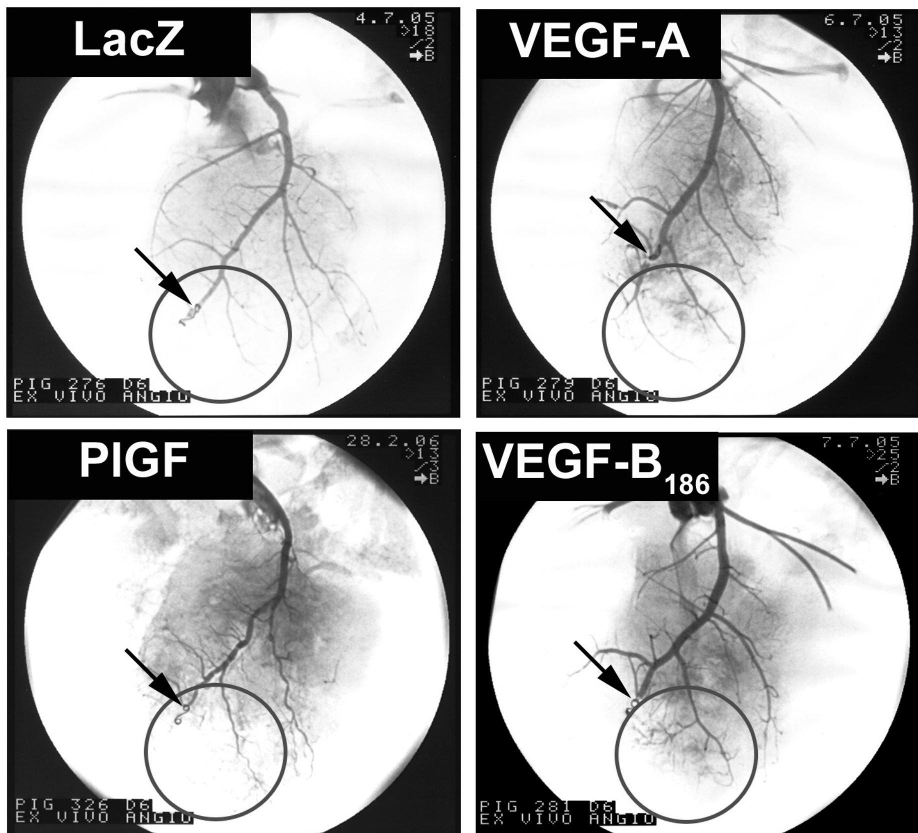


Figure 19. *Ex vivo* angiographies of the transduced pig hearts six days after the GT for visualization of collateral vessels. Infarction scar and border zone are circled, the arrows point to the occlusion site in the LAD. From original publication III.

Collateral arteries are required to supply blood flow to new capillaries in the infarction border zone. Although several studies have demonstrated collateral artery growth after growth factor administration, mechanisms governing arterial growth seem to be different from angiogenic mechanisms. Arteriogenesis is observed in normoxic tissue where no hypoxia-driven growth factor expression is observed (Rockstroh and Brown, 2002). The effect of growth factor GT may therefore be indirect. Blood vessel area increases via growth of the vessel diameter by cell proliferation and vasodilation. This increases the pressure gradient between diagonal branches and capillary bed increasing flow and thus triggering arteriogenesis via increased shear stress. This has previously been demonstrated in rabbit hindlimb ischemia model after AdVEGF-A administration (Rissanen et al., 2005).

Induction of collateral growth indirectly by increasing flow up-stream from the angiogenic capillary bed may not be as straight forward in humans as in young, healthy animals. In the arteries of older patients atherosclerotic changes disturb blood flow and endothelial dysfunction may inhibit endogenous responses. Furthermore, dyslipidemia and hyperglycemia may attenuate vasodilation and recruitment of mononuclear cells essential for collateral growth (LeBlanc et al., 2008).

VEGF-B INDUCES MYOCARDIUM-SPECIFIC ANGIOGENESIS VIA A NOVEL ANGIOGENIC PAHWAY

VEGF-B induced vessel growth is not dependent on nitric oxide production

Nitric oxide synthase inhibitor L-NAME was given p.o. to inhibit NO production. L-NAME blocked the capillary growth induced by AdVEGF-A and AdPlGF but did not inhibit AdVEGF-B_{167} or AdVEGF-B_{186} induced angiogenesis (Figure 20).

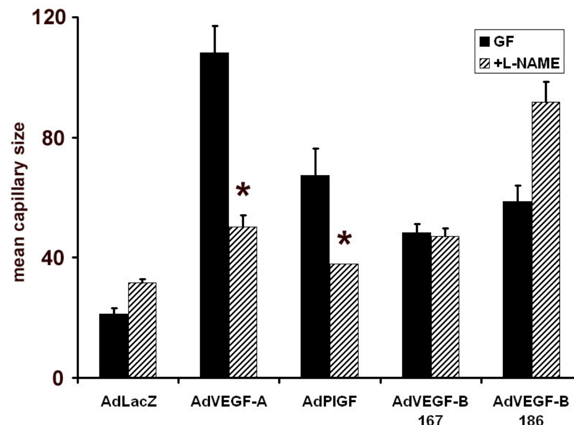


Figure 20. Mean capillary areas (μm^2) in transduced myocardium, GF=growth factor. From the online data supplement of original publication III.

The role of NO is well established for the angiogenic function of VEGFR-2. Angiogenic effects of VEGF-A have been shown to be mediated via NO production (Fukumura et al., 2001) and NO has been shown to be a downstream signaling molecule in the VEGFR-2 signaling pathway. Also VEGFR-1 stimulation with PlGF has been shown to induce angiogenesis via NO production (Bussolati et al., 2001). However, these effects may have been indirect as the authors had previously shown that PlGF induces VEGFR-2 phosphorylation in fibroblasts and the effect of PlGF was inhibited by a VEGFR-2 antibody (Khaliq et al., 1999). Although also anti-VEGF antibody inhibited these effects they were interpreted as indirect VEGFR-2 activation. Such cross-talk between VEGFR-1 and VEGFR-2 has also been suggested by others (Autiero et al., 2003).

NO production is an essential step in angiogenesis. NO induces vasodilation, increases blood flow and NO production also occurs in endogenous angiogenic processes. On the other hand NO increases vascular permeability and induces apoptosis in high concentrations. Lack of NO dependency in blood vessel growth is a new finding and suggests the angiogenic effects of AdVEGF-B_{186} are mediated via a novel angiogenic pathway.

AdPlGF induces angiogenesis via VEGF-A up-regulation but AdVEGF-B_{186} induced angiogenesis is not dependent on a VEGFR-2 ligand

The differences in angiogenic responses of the two VEGFR-1 ligands, AdPlGF and AdVEGF-B were evaluated further. VEGF-A staining of transduced skeletal muscles showed that AdPlGF up-regulated VEGF-A expression while AdVEGF-B did not (Figure 21). Only a few VEGF-A positive cells were observed in the vicinity of the needle track in AdLacZ transduced muscles (a). AdVEGF-A transduced skeletal muscles stained positive for VEGF-A (b). Strong VEGF-A staining was observed also in AdPlGF transduced samples (c), while in AdVEGF-B transduced samples VEGF-A positive cells were only observed in the needle track (d).

In the myocardium, only occasional weakly VEGF-A positive cells were seen in AdLacZ transduced hearts (e). AdVEGF-A transduced tissue was strongly VEGF-A positive (f) and VEGF-A up-regulation was seen in AdPlGF transduced myocardium (g). No VEGF-A expression was seen in AdVEGF-B_{186} transduced myocardium (h).

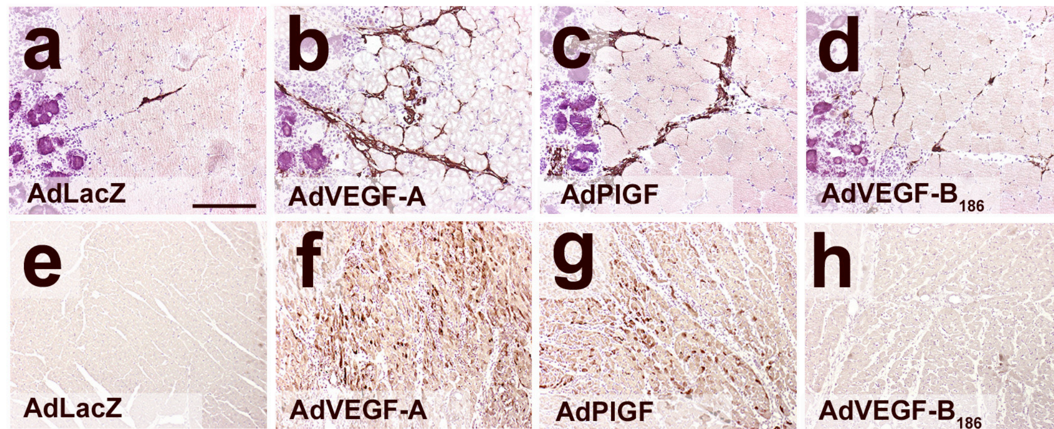


Figure 21. Transduced rabbit skeletal muscles (a-d) and pig myocardium (d-g) six days after GT. VEGF-A staining (brown), magnification 200X, scale bar 100µm. Unpublished results.

VEGF-A staining showed that AdPlGF up-regulates VEGF-A and the amount of VEGF-A staining was comparable to AdVEGF-A transduced samples. Several angiogenic growth factors have been shown to function via VEGF-A up-regulation (Deroanne et al., 1997). Interestingly, PlGF has previously been shown to up-regulate VEGFR-2 (Odorisio et al., 2002) and induce VEGFR-2 autophosphorylation (Autiero et al., 2003) although no direct binding to VEGFR-2 was observed. Previously, PlGF has been shown to induce VEGF-A secretion from human mononuclear cells (Bottomley et al., 2000). A few VEGF-A positive cells were observed in the vicinity of the needle track in AdLacZ and AdVEGF-B transduced muscles. This may be due to endogenous VEGF-A production by the damaged cells in the needle track.

The roles of VEGFR-1 and VEGFR-2 in PlGF-induced angiogenesis were further studied using adenoviruses encoding soluble VEGF-receptors (Figure 22). AdsVEGFR-1 inhibited both AdVEGF-A and AdPlGF induced angiogenesis in skeletal muscles and AdVEGF-B_{186} induced angiogenesis in the myocardium (a and b). Also AdsVEGFR-2 inhibited both AdVEGF-A and AdPlGF induced angiogenesis in the skeletal muscles (a) and AdVEGF-A induced angiogenesis in the myocardium, indicating that AdPlGF induced angiogenesis was dependent on a VEGFR-2 ligand. In contrast, AdVEGF-B_{186} induced angiogenesis in the myocardium was not blocked by AdsVEGFR-2 (b).

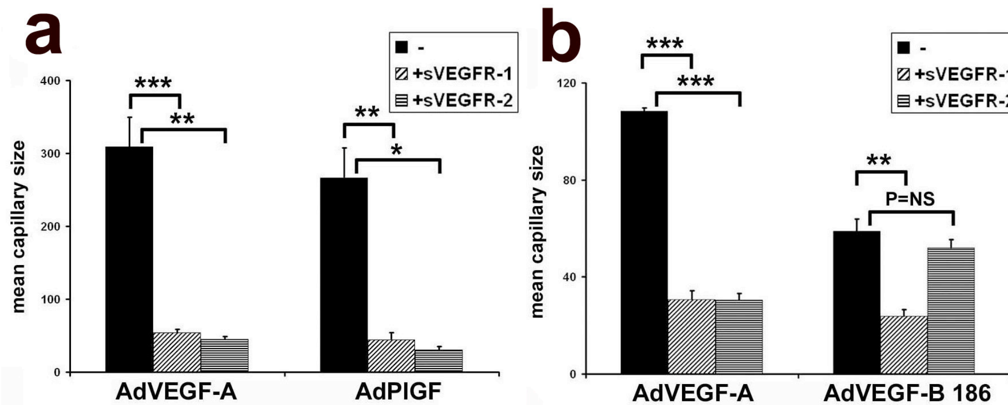


Figure 22. Mean capillary areas (μm^2) in transduced rabbit skeletal muscles (a) and pig myocardium (b). In the legend – indicates growth factor alone. From original publication III.

Over-expression of soluble receptors was used to specify the roles of VEGFR-1 and -2 on the angiogenic response of AdPlGF. Three-fold over-expression of the soluble receptors was used to ensure saturation of the receptor binding site on the growth factor. Both AdsVEGFR-1 and AdsVEGFR-2 inhibited the angiogenic effects of AdPlGF indicating that a VEGFR-2 ligand is required for the angiogenic function of PlGF. Soluble receptors are not optimal tools in receptor function experiments. The soluble receptor may inhibit interactions with other receptors and thus unspecifically inhibit the effect of the growth factor. An optimal approach would be a receptor inhibitor or a blocking antibody. The use of antibodies in large animal models is limited by the amount of antibody needed to reach sufficient local concentrations, and although several VEGFR antibodies are available their specificity to VEGFRs is limited (Kesisis et al., 2007). However, in our setting the limitations are smaller. Since PlGF does not bind to VEGFR-2, inhibitory effect of AdsVEGFR-2 indicates the presence of a VEGFR-2 ligand.

The indirect mode of function was further supported by the notion that while VEGF-A is a potent inducer of vascular permeability in the Miles assay, PlGF homodimer did not induce vascular permeability in 30min while PlGF/VEGF heterodimer did induce a permeability response in higher concentrations (Lähteenvuo et al. unpublished results).

AdVEGF-B induced angiogenesis is dependent on Nrp-1

Several members of the VEGF family bind to neuropilin receptors. In AdLacZ transduced samples a low level of Nrp-1 staining was observed in cardiomyocytes and in ECs. In AdVEGF-A and AdPlGF transduced hearts angiogenic capillaries stained strongly positive for Nrp-1. In AdVEGF-B_{186} transduced samples both angiogenic capillaries and cardiomyocytes in the vicinity of the needle track stained positive for Nrp-1 (Figure 23).

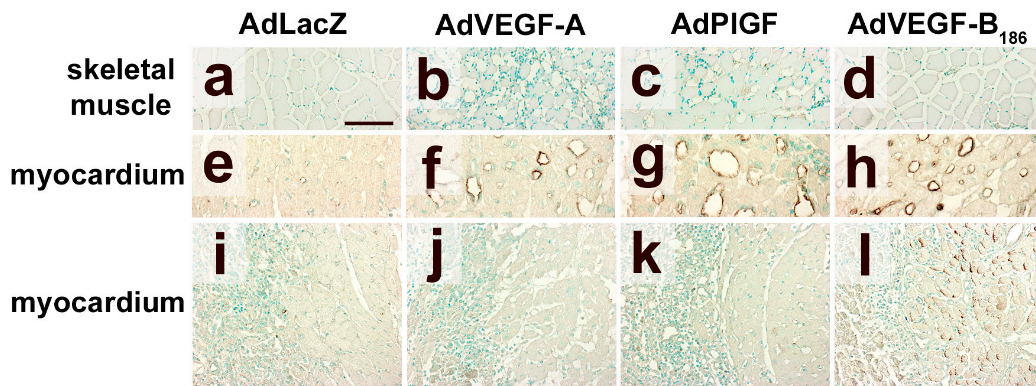


Figure 23. Transduced pig skeletal muscle (a-d) and myocardium (e-l) six days after the GT. Nrp-1 staining (brown), magnification 200X, 100µm. From original publication III.

Immunoprecipitation with Nrp-1 antibody and VEGF-B immunoblotting revealed that VEGF-B was bound to Nrp-1 (Figure 24 a). Some endogenous VEGF-B was also observed in the AdLacZ transduced samples (a). Recently, Nrp-1 was shown to mediate intracellular signaling. Activation of this signaling pathway was confirmed by binding of GIPC protein to Nrp-1 (b) and up-regulation of GAIP (c). Since GAIP was not bound to the signaling complex and was not immunoprecipitated with Nrp-1, the finding was confirmed by immunostaining of GAIP showing redistribution of GAIP in the cardiomyocytes (data not shown).

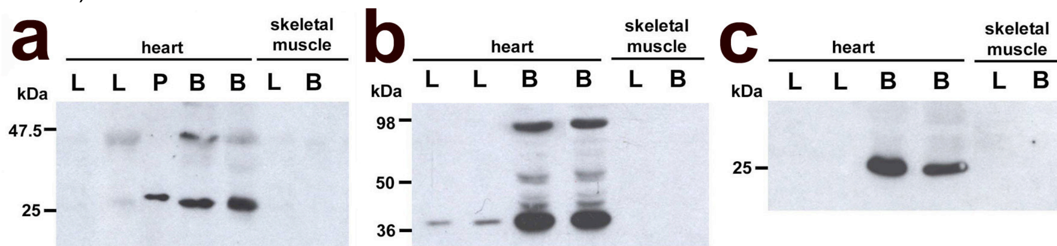


Figure 24. Western blots. L=AdLacZ transduced tissue, B=AdVEGF-B_{186} transduced tissue, P=VEGF-B recombinant protein positive control. A) immunnoprecipitation with Nrp-1 antibody and detection with VEGF-B antibody. B) immunoprecipitation with Nrp-1 antibody and detection with GIPC antibody. C) detection with GAIP antibody.

The role of Nrp-1 in myocardial angiogenesis was studied using an adenoviral construct expressing soluble Nrp-1 (Figure 25). AdsNrp-1 enhances the angiogenic effect of AdVEGF-A (Yamada et al., 2001). Possibly due to endogenous VEGF-A production in the ischemic tissue, AdsNrp-1 enhanced the angiogenic effect in AdLacZ transduced tissues (a vs. c). Also co-administration of AdsVEGFR-1 increased mean vessel size (b). In contrast, the angiogenic effect of VEGF-B_{186} was inhibited by AdsNrp-1 (d vs. e) and by AdsVEGFR-1 (f). Mean vessel sizes in the infarction border zone were quantified to confirm the result.

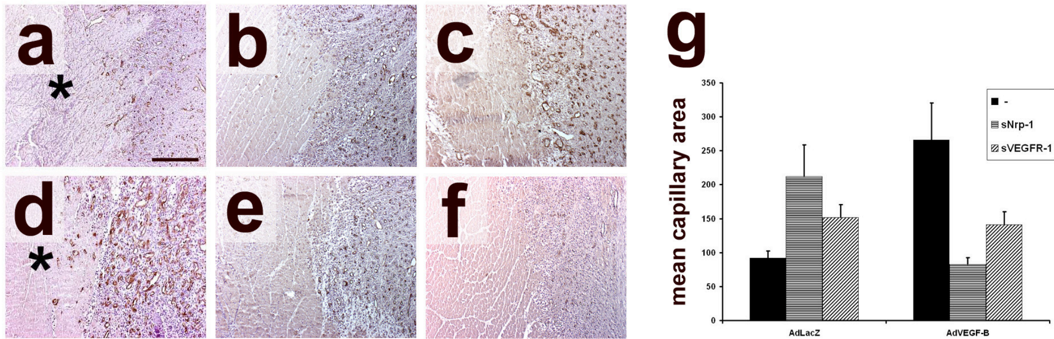


Figure 25. Trancduced pig myocardium 6 days after the GT, infarction border zone. Pecam-1 staining,(brown) for ECs, magnification 100X, scale bar 200μm (a-f) and quantification of mean blood vessel area (μm^2)(g). From original publication III.

Nrp-1 has been shown to be expressed widely in different tissues, and in adults mainly in the arterial endothelium. Here we show that Nrp-1 is also expressed on cardiomyocytes and that Nrp-1 expression is up-regulated after VEGF-B GT. Neuropilins have been thought to function as co-receptors for VEGFRs, but recently an intracellular signaling pathway essential for blood vessel development was published (Wang et al., 2006). Our results indicate that Nrp-1 is essential for VEGF-B mediated effects in the myocardium, but whether it acts independently or whether it modulates VEGFR function remains to be elucidated. Neuropilins have been shown to function in a ligand-dependent manner. Co-administration of AdsNrp-1 increased endogenous angiogenesis in AdLacZ transduced myocardium but blocked the vessel growth induced by AdVEGF-B_{186}.

FUNCTIONAL EFFECTS OF GENE TRANSFER

Angiogenesis increases perfusion in skeletal muscle and myocardium

Perfusion was measured using fluorescent microsphere particles. Angiogenic factors increased perfusion both in the skeletal muscle and myocardium (Figure 26). In skeletal muscles, AdVEGF-A and AdVEGF-$D^{\Delta N \Delta C}$ were the most potent angiogenic factors inducing 4- and 3-fold increases in perfusion ratios, respectively (a), while in the myocardium AdVEGF-A and AdVEGF-B_{186} had the strongest effect on perfusion in the infarction border zone (b).

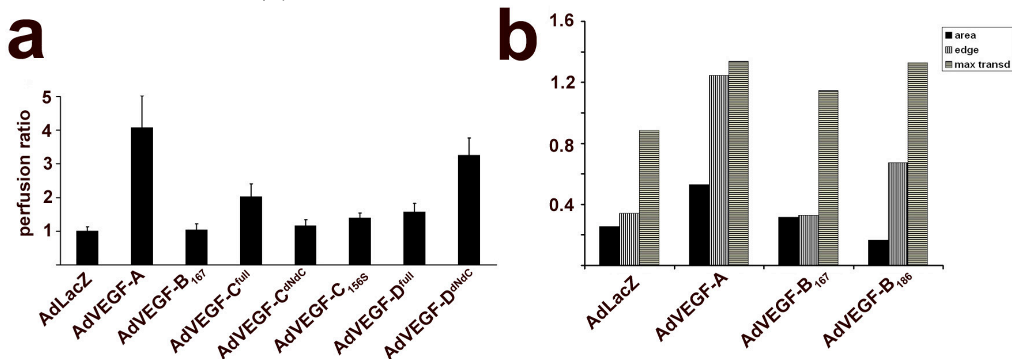


Figure 26. Microsphere perfusion ratios in rabbit skeletal muscle (a) and ischemic pig myocardium (b) as compared to healthy tissue. From original publications I and III and unpublished results.

The microsphere method is the gold standard in perfusion measurement. Microsphere particles are 7µm in diameter and the measurement is based on the microspheres being trapped in the capillary vessels. Mean vessel area in AdVEGF-$D^{\Delta N\Delta C}$ transduced skeletal muscles was 210µm corresponding to diameter of approximately 16µm, too large for the microsphere particles to be trapped in the vessels. In addition, the muscles were perfusion fixed and thus microsphere particles that were not tightly wedged to vessels may have been washed out. The microsphere method may therefore underestimate perfusion ratios. This was confirmed by ultrasound perfusion measurements, where perfusion was increased by 20-30-fold as compared to controls (data not shown).

Angiogenesis increases ejection fraction in ischemic myocardium

Ejection fraction was measured at baseline, after AMI and six days after the gene transfer (Figure 27). Ejection fraction decreased equally in all groups after AMI. In AdLacZ and AdVEGF-B_{167} transduced animals only slight increase in ejection fraction was observed. In contrast, both AdVEGF-A and AdVEGF-B_{186} recovered ejection fraction almost back to the baseline level within six days.

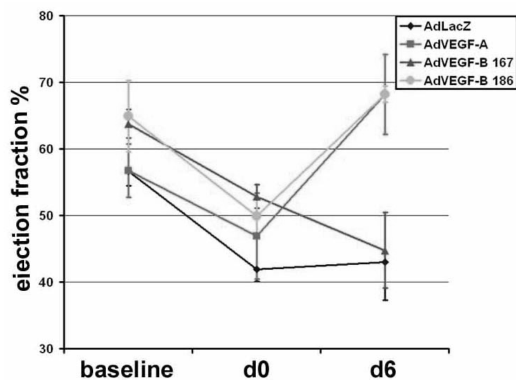


Figure 27. Ejection fraction (%) at baseline, after AMI and 6 days after the GT. From original publication III.

The standard measurement for ejection fraction requires a standardized ultrasound projection of the heart. Pig has very narrow intercostal spaces preventing the use of transthoracic US techniques, and thus measurements were performed from longitudinal projections by using intracardiac ultrasound imaging. GT of the angiogenic factors induced edema in the anterior wall of the left ventricle which may also have affected the EF measurements.

VASCULAR PERMEABILITY

Vascular permeability was measured using modified Miles assay (Figure 28). All angiogenic growth factors increased vascular permeability. AdLacZ GT did not increase leakage of plasma proteins as compared to intact muscles, yielding a permeability ratio of 1.1. Angiogenic factors AdVEGF-A_{165}, AdPlGF, ADVEGF-$D^{\Delta N\Delta C}$ and AdVEGF-E increased vascular permeability in skeletal muscles while VEGFR-3 ligands AdVEGF-C^{full}, AdVEGF-C_{156S} AdVEGF-D^{full} did not increase leakage of plasma proteins.

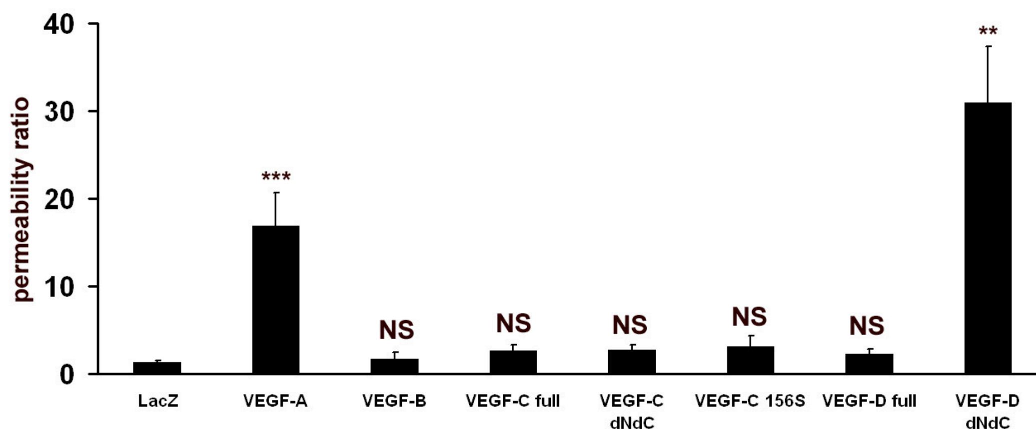


Figure 28. Plasma protein leakage ratios in rabbit skeletal muscles as compared to intact tissue. From original publications I, III and unpublished results.

Blood vessel leakage and tissue edema are among the main safety concerns in angiogenic gene therapy. Several reports have been published about growth factors and growth factor combinations with blood vessel growth without increase in the vascular permeability in mouse models (Luttun et al., 2002; Zheng et al., 2006). However, in our hands all angiogenic growth factors also increased plasma protein leakage. Growth factor expression had lasted for several days at the time of the analysis, and acute and chronic mechanisms of vascular permeability are likely to be activated. EC proliferation was seen in all groups, and detachment of ECs and intercellular leakage are likely inevitable in the process of blood vessel growth.

LYMPHATIC VESSEL GROWTH

VEGFR-3 ligands induce lymphatic vessel growth

Lymphatic vessels were studied using LYVE-1 staining of the transduced tissues (Figure 29). In AdLacZ transduced tissues, lymphatic vessels were observed between the muscle bundles. One or two thin-walled, open lymphatic channels were seen around larger collecting veins (a). VEGFR-1 ligands AdVEGF-A and AdPlGF induced a mild lymphangiogenic response. Both size and number of lymphatic channels were increased in the transduction area. AdVEGF-B_{167} and AdVEGF-B_{186} did not induce lymphatic vessel growth. A simplified grouping according to receptor binding profile is presented.

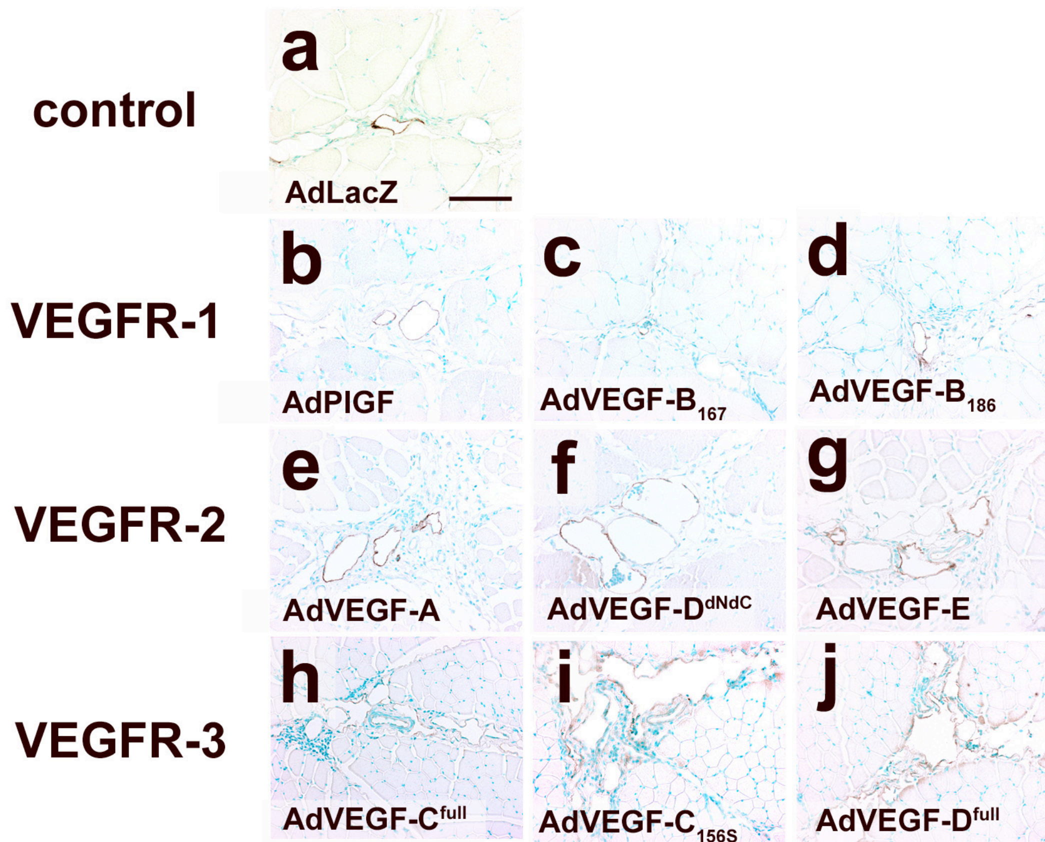


Figure 29. Transduced rabbit skeletal muscles 6 days after the GT. LYVE-1 staining (brown) for lymphatic ECs, magnification 200X, scale bar 100µm. From original publication IV and unpublished results.

AdVEGF-A has been reported to induce lymphangiogenesis (Nagy et al., 2002). Mild lymphangiogenic response studied by LYVE-1 immunostaining was observed in all groups where angiogenesis and increased vascular permeability was observed. Endogenous signals for lymphatic vessel growth are poorly known. Macrophages have been shown to secrete lymphangiogenic factors and to stimulate lymphatic vessel growth in the cornea (Watari et al., 2008). When lymphatic vessel formation was studied in the skin, lymphatic flow was shown to precede the formation of lymphatic channels. It was therefore hypothesized that edema itself was the stimulus for lymphatic vessel growth, not direct receptor stimulation by the growth factors.

Both full and truncated (∆N∆C) forms of VEGF-C and VEGF-D bind to both VEGFR-2 and VEGFR-3, possibly producing a mixed angiogenic and lymphangiogenic response. Receptor-specific effect can be more precisely evaluated by receptor-specific ligands. Previously, no lymphatic vessel growth was observed after VEGFR-2 ligand VEGF-E GT, but only dilation of lymphatic vessels was seen (Wirzenius et al., 2007). In our model, also VEGF-E induced an increase both in the lymphatic vessel size and number suggesting a VEGFR-2 initiated response. The lymphangiogenic response of VEGFR-3 specific ligand VEGF-C_{156S} was comparable to that of VEGF-C and –D full isoforms, suggesting that proteolytic processing does not play a major role and that their functions are mainly mediated via VEGFR-3.

To further assess the role of edema in lymphangiogenesis AdVEGF-A and AdVEGF-D^{full} GTs were done in to the distal end of the rabbit semimembranosus muscle.

AdVEGF-A induced a weak but significant increase in the total lymphatic vessel area / field of view. Lymphatic vessels were organized and had an open lumen. In the AdVEGF-D^{full} GT area lymphatic response was more pronounced and the total lymphatic vessel area was increased by 18-fold as compared to AdLacZ.

Endogenous angiogenesis is accompanied by lymphangiogenesis

Endogenous angiogenesis and lymphangiogenesis were studied in AdLacZ transduced ischemic skeletal muscles (Figure 30). Endogenous angiogenesis was observed in ischemic muscles, and mean capillary area was increased by 4-fold as compared to normal muscles (e and f). Total lymphatic vessel area was increased by 3-fold in ischemic skeletal muscles (b and c). Lymphatic vessel growth was observed in between the muscle bundles in normoxic muscles (a), but both between muscle bundles (arrowheads) and under the muscle fascia (insert) in the border zone of the necrotic muscle (b).

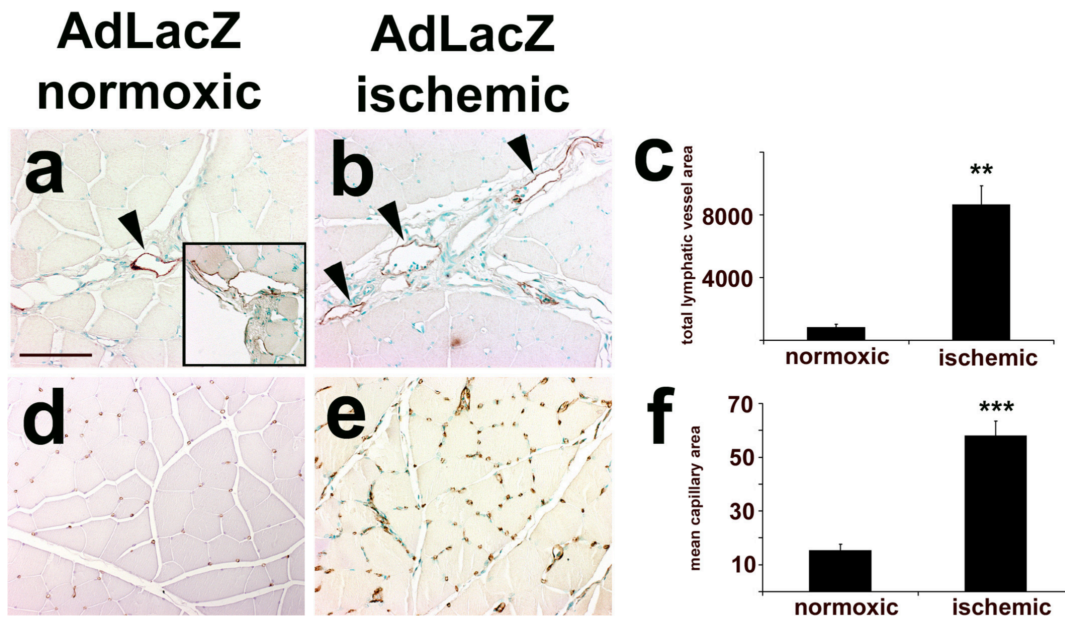


Figure 30. Rabbit skeletal muscles 6 days after the GT, LYVE-1 staining (brown) for lymphatic endothelial cells (top row) and CD31 staining (brown) for ECs (2nd row), magnification 200X, scale bar 100µm. Quantifications for total lymphatic vessel area (µm^2/field of view) (c) and mean capillary area (µm^2) (d). From original publication IV.

Presence of endogenous lymphangiogenesis has been demonstrated in healing myocardial infarction scars (Ishikawa et al., 2007) and in skin wounds (Paavonen et al., 2000). If vascular permeability is an inevitable side-effect of blood vessel growth it is natural that an endogenous response has developed to control the formation of tissue edema.

Lymphatic vessel growth is not dependent on nitric oxide production

Nitric oxide synthase inhibitor was used to study the effect of NO on lymphatic vessel growth (Figure 31). L-NAME did not inhibit the increase in total lymphatic vessel area induced by VEGFR-3 ligand AdVEGF-D^{full}.

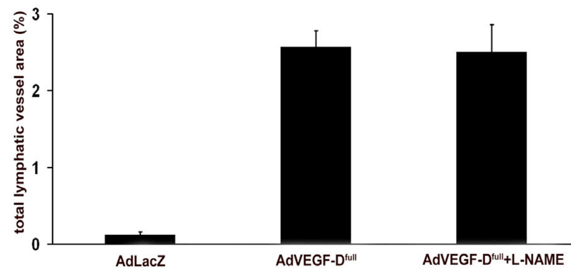


Figure 31. Total lymphatic vessel area (% of total area) (%) in rabbit skeletal muscles 6 days after the GT. From original publication I.

COMBINATION GENE TRARAPY TO REDUCE EDEMA INDUCED BY THERAPEUTIC ANGIOGENESIS

The side-effects of angiogenic AdVEGF-D$^{\Delta N\Delta C}$ GT were treated with co-administration of lymphangiogenic AdVEGF-D^{full} (Figure 32). Mean capillary area was comparable in AdVEGF-D$^{\Delta N\Delta C}$ and combination group (a). Mean lymphatic area was increased in combination group by 4-fold as compared to AdVEGF-D$^{\Delta N\Delta C}$ alone (a). Indeed, the amount of extravasated plasma proteins in the interstitial space had also decreased by 68% in the combination group as compared to AdVEGF-D$^{\Delta N\Delta C}$ alone (b).

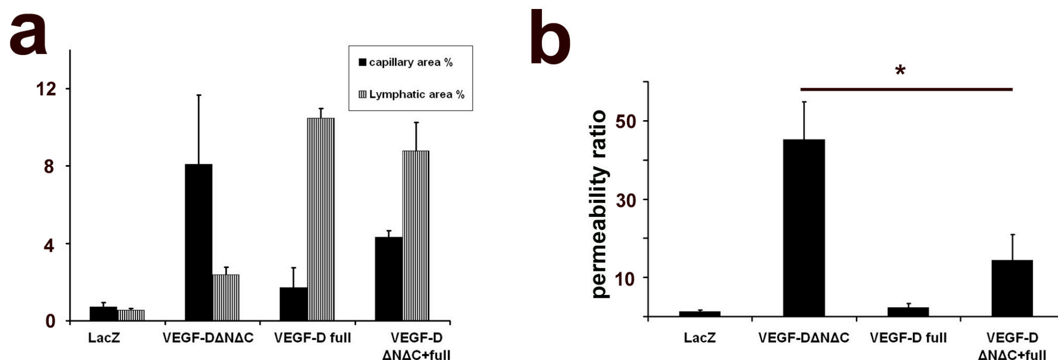


Figure 32. Total blood vessel area and total lymphatic vessel area (% of total area) (a) and permeability ratio (c) in rabbit skeletal muscles 6 days after the GT. From original publication IV.

Co-administration of a lymphangiogenic factor was used to stimulate lymphatic vessel growth and hence to increase clearance of plasma proteins from the interstitial space to alleviate edema. AdVEGF-D$^{\Delta N\Delta C}$ does induce lymphatic vessel growth, but not sufficiently to reduce edema. In combination gene therapy, blood to lymphatic vessel ratio was comparable to that observed in endogenous angiogenesis and lymphatic clearance of plasma proteins was sufficient to alleviate edema.

Growth of blood vessels requires detachment of ECs to allow proliferation and migration. Vascular permeability may therefore be an unavoidable side effect of blood vessel growth. Stimulation of lymphatic vessel growth does not prevent these necessary steps in blood vessel growth but reduces edema by clearing extravasated proteins from the insterstitial space reducing edema.

SAFETY

Blood vessel growth

The major concern in angiogenic gene therapy is unwanted blood vessel growth. Possible effects on blood vessels were assessed from AdVEGF-B_{186} transduced animals. No signs of angiogenesis were observed in any of the control tissues studied (Figure 33). Representative images from liver (a), spleen (b), lung (c), kidney (d) and ovary (e) are presented.

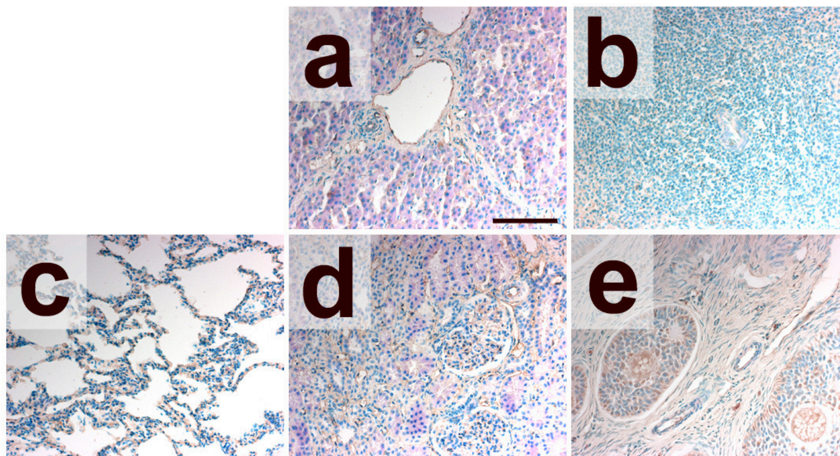


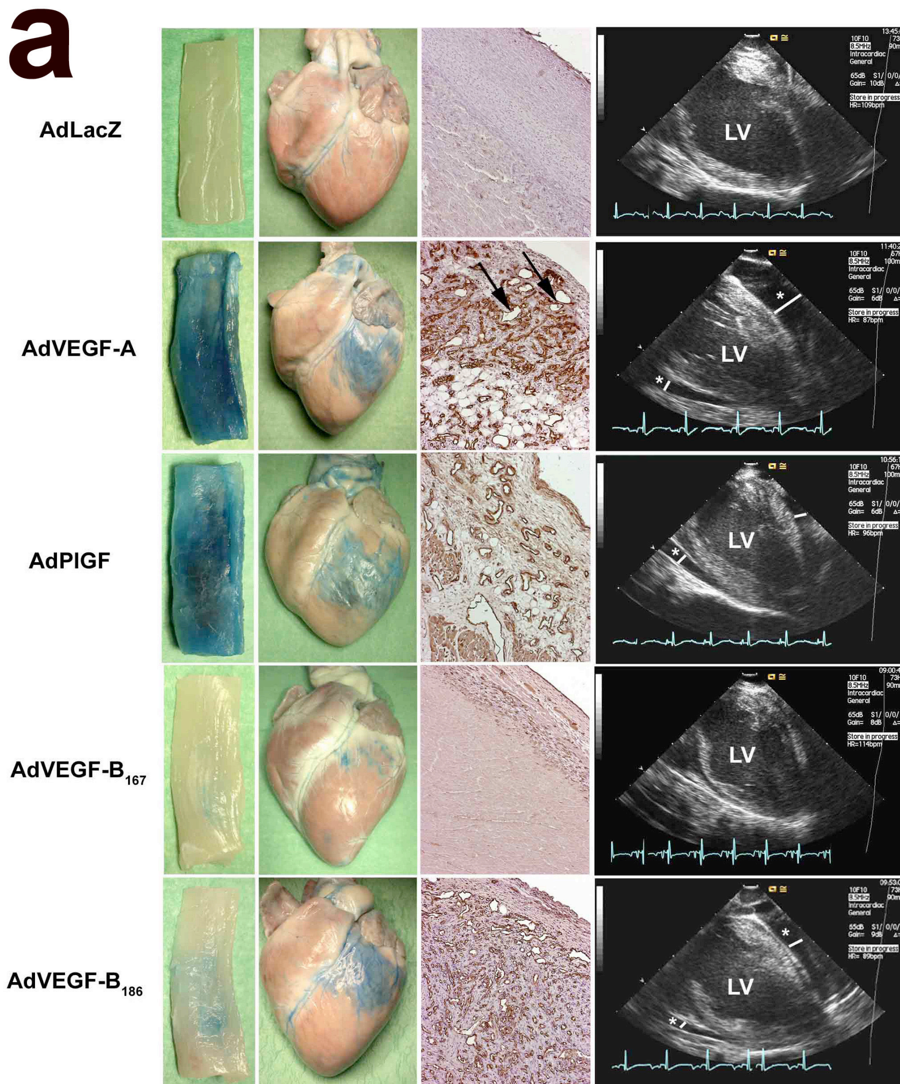
Figure 33. Pecam-1 staining for endothelial cells in safety tissues of a pig 6 days after the intramyocardial GT of VEGF-B_{186}, magnification 200X, scale bar 100µm. Unpublished results.

In VEGF-B null mice all tissues excluding the heart are normal suggesting a tissue-specific role for VEGF-B (Bellomo et al., 2000; Aase et al., 2001). However, since VEGF-B is widely expressed and some tumors have been reported to secrete VEGF-B, systemic effects cannot be excluded in the adult. VEGF-B_{186} is a soluble protein, and is therefore likely transported to systemic circulation by the lymphatic vessels at least to some extent. Lack of blood vessel growth supports the safety of VEGF-B_{186} as a therapeutic protein, but since the metabolic effects of VEGF-B are largely unknown, other systemic effects cannot be excluded.

Vascular permeability

Accumulation of pericardial effusion was studied by ultrasound imaging, and plasma protein leakage was visualized and quantified by i.v. administration of Evans blue dye. To study the effects of blood vessel distribution and morphology, blood vessels in the epicardial surface of the heart were studied (Figure 34).

AdVEGF-A and AdPlGF increased plasma protein extravasation in the skeletal muscles (a, b), while in the myocardium also VEGF-B_{186} increased vascular permeability (a, c). Plasma protein extravasation led to accumulation of pericardial effusion after AdVEGF-A, AdPlGF and to lesser extent after AdVEGF-B_{186} GT. Differences in the amount of effusion may be explained by the pattern of blood vessel growth. AdVEGF-A induced growth of glomerular vessel structures on the epicardial surface, while the angiogenic effect of VEGF-B_{186} was more balanced and wide-spread.



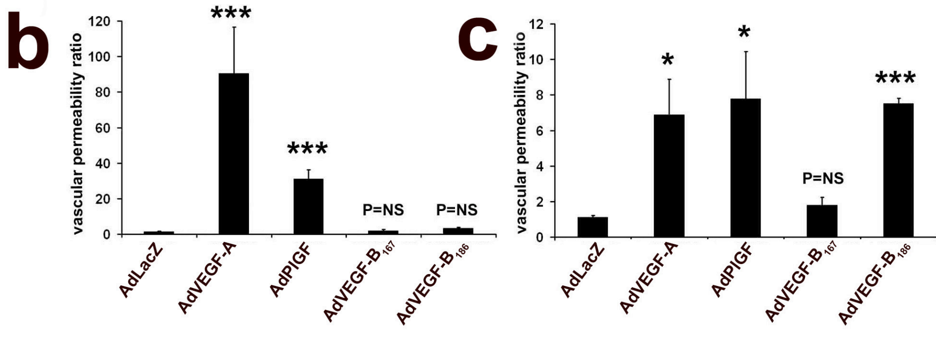


Figure 34. Plasma protein leakage and pericardial effusion. 1st column: photographs of transduced rabbit skeletal muscles. 2nd column: photographs of transduced pig hearts. 3rd column: ultrasound images of the transduced hearts, *=pericardial space. 4th column: Pecam-1 staining, magnification 100X, *=epicardium. Plasma protein extravasation ratios as compared to intact tissue in skeletal muslces (b) and transduced myocardium (c). Unpublished results.

Increased vascular permeability is the main side effect of blood vessel growth. Increased plasma protein leakage was observed after administration of all angiogenic factors. In skeletal muscles plasma protein leakage leads to edema possibly leading to compression of the blood vessels and decreased blood flow. In myocardium excess fluid in the interstitial space may accumulate in the pericardial space restricting heart function. The solubility of the growth factors affected the accumulation of pericardial effusion. VEGF-A secreted from the transduced cells binds to matrix heparin sulphate proteoglycans, and angiogenesis was localized close to transduced cells. As shown by transduction efficiency studies with AdLacZ, the pressure gradient across the myocardial wall pushes the virus towards the epicardium, and the transduction efficiency is highest on the epicardial third of the ventricular wall. Further, blood vessel growth is more aberrant in loose connective tissue compartments. High local concentration of VEGF-A led to formation of leaky glomerular structures. AdVEGF-B_{186} is more soluble, and blood vessel growth was mostly seen within the myocardium and no leaky glomerular structures were observed. Vascular permeability is a dose-limiting side effect, and both the dose and biological properties of the growth factor used and the site of GT have to be chosen carefully to avoid these potentially hazardous side effects.

CONCLUSIONS AND FUTURE PERSPECTIVES

Adenoviral angiogenic gene therapy in large animal models has proved to be an efficient tool to induce blood vessel growth. Angiogenic growth factors increased both tissue area covered by blood vessels and induced growth of angiographically visible collateral arteries. Most importantly, angiogenic gene therapy improved the function of the transduced tissue. Perfusion was increased both in skeletal muscle and myocardium and treatment of ischemic myocardium improved the ejection fraction. Large animal models enabled the use of clinically relevant delivery methods, end point measurements and evaluation of biodistribution and safety in a large mammal.

Angiogenic and lymphangiogenic profiles of the VEGF-family growth factors help to choose a suitable gene for therapeutic applications (Table 8). Quantitative side by side evaluation of blood and lymphatic vessel growth effects in a large animal model sets perspective for experiments performed in different animal models and species. Experiments in tissue environments intended for the clinical use are essential. Local environment, availability of target cells, receptors and other growth factors modulate the therapeutic response. Ischemic tissue was shown to be more responsive to angiogenic growth factors, setting a new dose-response curve for ischemic tissues.

Table 8. Angiogenic and lymphangiogenic responses in normoxic and ischemic skeletal muscle and myocardium. Number of original publication in parentheses, u=unpublished result Lähteenvuo J et al., u*= unpublished result Kattainen E et al., u** unpublished result Lähteenvuo MT et al., ND= not determined.

	ANGIOGENESIS				LYMPHANGIO-GENESIS
	normoxic		ischemic		normoxic
Growth factor	skeletal muscle	myocardium	skeletal muscle	myocardium	skeletal muscle
AdVEGF-A	+++ (I, III)	+++ (II, III)	+++ (III)	+++ (III)	+ (IV)
AdPlGF	+++ (III)	+++ (III)	++ (III)	++ (III)	+ (u)
AdVEGF-B_{167}	- (III)	+ (III)	- (III)	+ (III)	- (I)
AdVEGF-B_{186}	- (III)	+++ (III)	- (III)	+++ (III)	- (u)
AdVEGF-E	+++ (u)	+++ (u)	ND	ND	+ (u)
VEGF-$D^{\Delta N \Delta C}$	+++ (I)	+++ (II)	+++ (u)	+++ (u**)	++ (I, IV)
VEGF-C^{full}	- (I)	ND	ND	ND	+++ (I)
VEGF-D^{full}	- (I)	ND	ND	ND	+++ (I, IV)
VEGF-C_{156S}	- (I)	- (u*)	ND	- (u*)	+++ (I)

Evaluation of the two most potent VEGFR-2 ligands in pig myocardium provided a basis for experiments to treat myocardial ischemia. An optimal vector and dose were established, and the delivery method optimized to recognize and target the area of choice within the myocardial wall. Methods to quantify and monitor vascular permeability in the myocardium are valuable when doses and follow-up methods for clinical trials are chosen.

Evaluation of different VEGFR-1 ligands in skeletal muscle and myocardium revealed a novel angiogenic pathway activated by AdVEGF-B selectively in the heart.

Growth factors have been shown to have different angiogenic and lymphangiogenic profiles in different tissue environments (Byzova et al., 2002), but no tissue-specific growth factors have been reported. Discovery of a tissue-selective function of VEGF-B provides a new promising tool for angiogenic gene therapy. Moreover, VEGF-B may also have beneficial effects on the myocardial metabolism possibly selectively protecting the cardiomyocytes from ischemia and inhibiting apoptosis. The role of this novel pathway in endogenous angiogenesis and arteriogenesis remains to be elucidated. Expression patterns of suggested components of this pathway are all available in the sites of blood vessel growth supporting this hypothesis. VEGFR-1 is diffusely up-regulated in ischemic myocardium (Marti and Risau, 1998). VEGF-B null mice have cardiac defects, and VEGF-B expression during embryogenesis and early postnatal life co-localized with developing coronary vessels and sites of angiogenesis (Bellomo et al., 2000). Further, although not regulated by hypoxia, Nrp-1 is expressed in both myocardium and arterial endothelium.

Metabolic changes observed after AdVEGF-B_{186} GT open a whole new area of VEGF research. Recent reports have supported the concept of VEGF as a metabolic regulator. Kärpänen et al. reported recently that VEGF-B over-expression alters cardiac lipid metabolism, and VEGF-B has been shown to induce expression of fatty acid transporters both *in vitro* and *in vivo* (Carolina Rosenlew and Ulf Eriksson, personal communication).

Efficient induction of lymphatic vessel growth was achieved by adenoviral delivery of VEGFR-3 ligands. Lymphatic vessel area in the transduced muscles was increased, and most importantly, lymphatic vessels were functional and induction of lymphatic vessel growth was able to alleviate edema induced by angiogenic gene therapy. Combination gene therapy with angiogenic growth factors could be used to induce blood vessel growth with less severe side effects. Lymphatic vessel growth is a part of a balanced endogenous healing process. Lymphatic vessels grow in healing wounds (Paavonen et al., 2000), myocardial infarction scars (Ishikawa et al., 2007) and as shown in this study, in ischemic skeletal muscles. Blood/lymphatic vessel ratios calculated from these endogenous growth processes may provide a tool to estimate the increase in lymphatic vessel area required to reduce edema induced by therapeutic angiogenesis.

Experiments in large animal models are the last step before clinical trials. While VEGF-A has been studied in humans for over a decade, growth factor-specific safety information is needed before the less-known members of the VEGF-family are studied in humans. Ligands binding to VEGFR-3 may have side effects connected to lymphatic vessel growth and VEGFR-1 ligands may have yet unknown metabolic functions. VEGFs are known to be involved in both the development and function of the kidneys, and anti-VEGF drugs have been reported to affect kidney function (Chen and Ziyadeh, 2008; Gurevich and Perazella, 2009). Therefore, the effects on kidney function should be addressed.

In conclusion, this study provides new data on both efficacy and safety of VEGFs for clinical angiogenic gene therapy. The optimal therapeutic gene for each application can be chosen based on angiogenic and lymphangiogenic properties of the growth factor. AdVEGF-$D^{\Delta N\Delta}$ was shown to be an efficient angiogenic factor both in skeletal muscle and myocardium. Importantly, AdVEGF-B_{186} induced myocardium- specific angiogenic pathway via Nrp-1 receptor may provide a safer tool for induction of blood vessel growth. The most important side effect of angiogenic gene therapy, increased vascular permeability was assessed, and lymphangiogenic gene therapy to reduce angiogenesis-induced edema was introduced.

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