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A gene transfer approach, based on Adeno-Associated Viral (AAV) vectors, to study the process of vessel maturation and stabilization

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A gene transfer approach, based on Adeno-Associated Viral (AAV) vectors, to study the process of vessel maturation and stabilization

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Zacchigna S, Tasciotti E, Kusmic C, Arsic N, Sorace O, Marini C, Marzullo P, Pardini S, Petroni D, Pattarini L, Moimas S, Giacca M, Sambuceti G. *In vivo imaging shows abnormal function of vascular endothelial growth factor-induced vasculature.* Hum Gene Ther, 2007. 18 (6): 515-24

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ABBREVIATIONS

AAV	Adeno-associated virus
ACE-2	Angiotensin converting enzyme-2
Ang	Angiopoietin
Ad	Adenovirus
α-SMA	α-smooth muscle actin
CAD	Coronary artery disease
CMV	Cytomegalovirus
CsCl	Cesium Chloride
DTPA	Diethylene triamine pentaacetic acid
EC	Endothelial cell
ECM	Extracellular matrix
EPC	Endothelial progenitors cell
ETT	Exercise treadmill test
FGF	Fibroblast growth factor
FGFR	Fibroblast growth factor receptor
bFGF	Basic Fibroblast growth factor
GAG	Glycosaminoglycan
HIF	Hypoxia-inducible transcription factor
HRE	Hypoxia responsive enhancer element
HSC	Hematopoietic stem cell
HSV	Herpes Simplex Virus
ITR	Inverted terminal repeat
i.v.	intravenous
LacZ	β-Galactosidase (marker gene)
MAPK	Mitogen-activated protein kinase
MBF	Muscolar blood flow
NP	Neuropilin
ORF	Opening reading frame
PAD	Peripheral arterial disease
PAH	Pulmonary arterial hypertension
PBS	Phosphate buffered saline

PCR	Polymerase chain reaction
PDGF	Platelet derived growth factor
PDGFR	Platelet derived growth factor receptor
PECAM	Platelet/endothelial adhesion molecule
PET	Positron emission tomography
PlGF	Placental growth factor
PWT	Peak walking time
RGS	Regulator of G-protein signalling
S1P	Sphingosine-1-phosphate
SMC	Smooth muscle cell
SPECT	Single Photon Emission Computed Tomography
TGF	Transforming growth factor
tPA	Tissue plasminogen activator
TRAM	Transverse rectus abdominis musculocutaneous
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor
vgc	Viral genome copies
VP	Viral protein
VPF	Vascular permeability factor
vSMC	Vascular Smooth muscle cells
vWF	Von Willebrand factor

1. ABSTRACT

The main goal of angiogenic gene therapy is the formation of functional new blood vessels adequate to restore blood flow in ischemic tissues. Angiogenesis is a complex process, consisting in the sprouting of new capillaries from pre-existing vessels to form an immature vascular network, which subsequently undergoes functional maturation and remodelling. Many factors are involved in this process and, among them, the VEGF family members are universally recognized as the key players.

During my PhD I exploited gene transfer by vectors based on the Adeno-Associated Virus (AAV) to express several factors involved in the angiogenic process, in an attempt to define the molecular and cellular mechanisms of vessel maturation and stabilization. Most experiments were performed by vector injection in the mouse and rat skeletal muscle, followed by detailed histological, immunohistochemical and functional analysis.

First of all the angiogenic effect driven by two main VEGF isoforms, $VEGF_{165}$ and $VEGF_{121}$ was compared. AAV-$VEGF_{165}$ and AAV-$VEGF_{121}$ appeared equally able to induce endothelial cell proliferation, leading to the formation of new CD31 positive capillaries. However, only the longest $VEGF_{165}$ isoform was capable to recruit α-SMA positive cells around growing capillaries and therefore giving rise to small arteries. The acquisition of a smooth muscle cell layer can be considered as marker of vessel maturation. This was also confirmed by a permeability assay, which showed that $VEGF_{121}$-induced vessels were more permeable compared to those induced by $VEGF_{165}$.

Interestingly, the presence of α-SMA positive vessels was paralleled by the recruitment of CD11b positive mononuclear cells from the bone marrow, cells which were not recruited by $VEGF_{121}$. The presence of these infiltrating cells in close proximity to the newly formed arterioles suggested their possible role in smooth muscle cell recruitment and vessel maturation. Real-time PCR allowed observing that the infiltrating CD11b positive cells expressed a cocktail of cytokines implicated in vessel maturation, such as TGF-β and PDGF-B. As a proof of concept of the paracrine activity of these cells in vessel maturation, we developed an AAV-PDGF-B vector, which, when co-injected with AAV-$VEGF_{121}$, was arteriogenic even in absence of cellular infiltration. Thus, the expression of PDGF-B partially substitutes for the cells observed in the muscles injected by AAV-$VEGF_{165}$ to form arterial vessels. To verify the functionality of the vessels induced by AAV-$VEGF_{165}$ we delivered this vector to different animal models of tissue ischemia: a flap ischemia

model and an *in vivo* chamber for tissue engineering based on an artero-venous loop. In both the models, $VEGF_{165}$ expression induced the formation of α-SMA positive vessels, which turned out to improve flap survival in the flap models, and to promote the formation of new vascularized tissue in the chamber.

Despite the presence of several arteries, other vessels formed by $VEGF_{165}$ were abnormally enlarged and leaky, often forming vascular lacunae. This observation indicated that VEGF gene transfer might not be sufficient for the formation of a fully functional vascular network, and that other factors might be required in order to achieve functional competence of the neovessels. We observed that the combined expression of $VEGF_{165}$ with Angiopoietin-1, which is known to stabilize endothelial and mural cell interactions, resulted in a significant reduction of vessel permeability and improved blood flow, as assessed by positron emission tomography (PET) and single photon emission tomography (SPECT).

These findings reveal that a fine control of the expression of angiogenic factors is needed to achieve the formation of stable and functional vessels. The presence of α-SMA positive cells might be considered as a first step in vessel maturation but further stabilization factors have to take part to the process in order to tighten the cell-cell junctions. Moreover, we showed that a detailed histological and functional analysis ex vivo might not be sufficient to characterized the new vasculature, requiring imaging techniques such as PET or SPECT.

2. INTRODUCTION

2.1 Angiogenesis: a therapeutic goal

Cardiovascular diseases are the most important cause of death in Western countries, and novel therapies are absolutely needed. Several therapeutic advances have been recently achieved for many cardiovascular disorders, although they might not be applicable to all patients. For instance, some patients presenting with myocardial or lower limb ischemia are not suitable candidates for conventional state-of-the-art treatments, such as bypass or angioplastic. Furthermore, in many cases the outcome of these therapies is not completely satisfactory even after a technically successful procedure, and require further intervention.

The devastating effect of tissue ischemia might be overcome by either increasing the cardiac o skeletal muscle survival, or restoring a proper blood supply. During both embryogenesis and adult life, the organism is able to create a new vascular network and to increase it at need at some extent; however, in pathological conditions, this compensatory response might not be sufficient, thus leading to the ischemic death of the tissue. Significant advances have been reached in the last decades in the understanding of the molecular and cellular mechanisms underlying new blood vessel formation, and therapeutic angiogenesis is today considered an actual and feasible therapeutic goal. On the other hand, other pathological conditions take advantage from an increased angiogenesis and in this case inhibiting the angiogenic process might results of therapeutic value; these conditions include cancer, atherosclerosis and diabetic retinopathy (Carmeliet, 2005).

Angiogenesis is an important issue also in plastic and reconstructive surgery, where restoring adequate blood supply is an essential requisite for flap survival. Partial necrosis of skin flaps represents a major clinical problem in patients undergoing reconstructive procedures, with significant morbidity and no effective therapy available. The lack of oxygen and nutrients in the distal part of the flap strongly compromises skin viability, resulting in flap necrosis, which often requires secondary reconstructive interventions (Hallock, 2001).

Another medical field that should benefit from the capacity to induce functional angiogenesis is tissue engineering. Over the recent years, several new materials have been developed and the biggest limitation that they had to face was the limited survival of the new tissue due to lack of a proper blood supply at the time of graft implantation. In vivo, the supply of oxygen and nutrients to the implant naturally occurs by simple diffusion, a

process that can only supply cells in proximity of 100-200 μm from the next capillaries (Morrison, 2009; Rouwkema et al., 2008).

2.2 Structure of blood vessels

Oxygen, metabolites and nutrients are transported throughout the body by the blood flow. In mammals, the vascular network comprises three different vessel types: arteries, capillaries and veins. Arteries are characterized by a thick wall in order to withstand the blood pressure; the arterial wall thickness diminishes as the vessels become smaller, but at the same time the wall-to-lumen ratio becomes greater. Large arteries are muscular conducts with walls comprising an intimal, medial and adventitial layers, divided by the internal and external elastic laminas. The smallest arteries are called arterioles and have a diameter range between 10 and 100 μm. Arterioles, in addition to the endothelial layer, consist of a basement membrane and one or two contractile smooth muscle cell (SMC) layers, capable of regulating the lumen diameter. In the terminal arterioles, the perivascular cells cover the vessel only at intermittent points. After further branching, terminal arterioles finally give rise to capillaries. Capillaries are small vessel, the mean diameter is between 4 and 9 μm, a size that allows the passage of red blood cells. They are particularly suited for the exchange of gases, nutrients, hormones, and cellular metabolites between the circulation and peripheral tissues, because of the slow blood flow, a thin wall and a close physical association with surrounding cells. Capillary endothelial cells (EC) are connected to each other by tight junctions, surrounded by a basement membrane and scattered perivascular SMCs and pericytes. From capillaries blood enters postcapillary venules, which are bigger than capillaries and present more pericytes. Venules drains into the smallest veins and present a continuous and thin SMC layer. Finally blood enters larger collecting veins until it drains into the right side of the heart. Veins have a larger overall diameter, a larger lumen, and a narrowed wall as compared to the corresponding arteries (Figure 2.1).

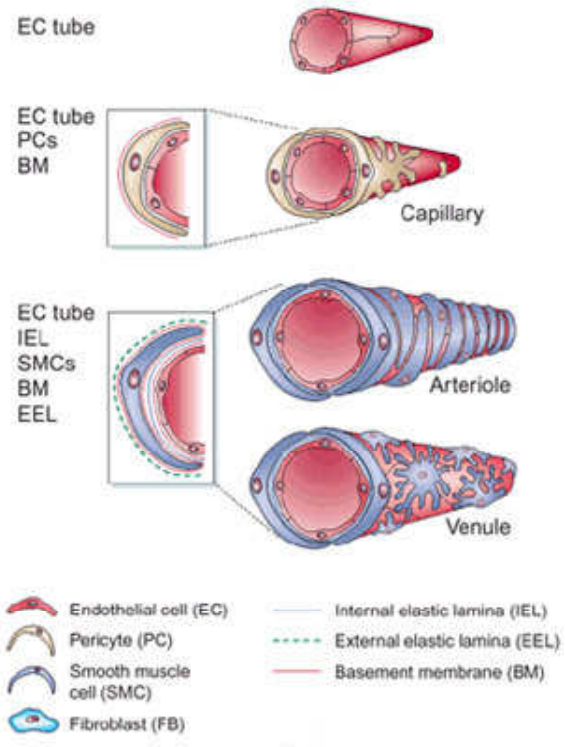


Figure 2.1 Schematic rappresentation of blood vessels structure. The endothelial tube is made by endothelial cells; the capillaries present mural cells and are surrounded by a basement membrane; small arteries in addition to the endothelial layer present one or two layers of smooth muscle cells; bigger arteries are muscular conducts with walls comprising an intimal, medial and adventitial layers, divided by the internal and external lamina; venule are bigger capillaries which present more pericytes and smooth muscle cells. (Figure adapted from Jain 2003)

Due to the functional heterogeneity, it is obvious that ECs, pericytes, SMCs and the basement membrane, are not identical in all blood vessels but have distinct morphology and function depending on their location (Kalluri, 2003).

ECs are present in the inner layer of the vessels and commonly used markers for their identification include CD31 (PECAM-1), CD34, von Willebrand factor (vWF) and Tie-2 (McDonald and Choyke, 2003), arterial endothelial cells express ephrin-B2, whereas eph-B4 (receptor for ephrin-B2), is expressed only in veins (Wang et al., 1998). Pericytes are a heterogeneous population of cells that can differentiate into other mesenchymal cell types, such as SMCs, fibroblasts and osteoblasts (Gerhardt and Betsholtz, 2003). The differentiation into fibroblast-like cells may contribute to collagen deposition in scars in wound healing, in chronic inflammation, and in tumor stroma in cancer (Sundberg et al., 1996). On the other hand, fibroblasts may differentiate into myofibroblasts and

subsequently into pericytes (Tomasek et al., 2002). All pericytes express α-smooth muscle actin (α-SMA), while only the mature ones express desmin (Gerhardt and Betsholtz, 2003; Morikawa et al., 2002). A new marker for pericytes has recently been recognized, the regulator of G-protein signalling 5 (RGS5) (Mitchell et al., 2008). RGS5 is highly expressed in pericyte-covered vessel, whereas its expression is low in veins and capillaries. Other markers for the identification of pericytes are NG-2, platelet-derived growth factor receptor β (PDGFR-β) and aminopeptidase A. Unfortunately some of these markers are not specific for pericytes and none of them recognize all pericytes; their expression is dynamic and varies between organs and developmental stages (Gerhardt and Betsholtz, 2003; Hellstrom et al., 1999).

2.3 Mechanisms of vessel growth

The vascular system, in all vertebrates, is composed of an extensive and complex network, which is spatially organized to provide the finest exchange of gas and metabolites from blood to tissue throughout the body.

Blood vessels can arise from three different mechanisms: vasculogenesis, angiogenesis and arteriogenesis (Isner and Asahara, 1999). Vasculogenesis is the process of *de novo* blood vessel formation that take place during embryonic development. The blood vasculature is the first organ to develop in the embryo and it is formed in the beginning of the third embryonic week, in humans (Risau, 1997; Risau and Flamme, 1995). Blood vessels arise from endothelial progenitor cells (EPC) that are present in the blood islands, together with the hematopoietic stem cells (HSC). Both EPCs and HSCs share a common progenitor, the hemangioblast, characterized by the expression of a few antigenic determinants, including vascular endothelial growth factor receptor-2 (VEGFR-2), Tie-2, Sca-1 and CD34 (Asahara and Kawamoto, 2004). The fusion of multiple blood islands give rise to the yolk sac capillary network, a primitive vascular labyrinth of small capillaries.

In the later phases, this primitive vascular plexus progressively expands by means of vessel sprouting and remodelling in a process called angiogenesis (Carmeliet and Collen, 1999; Conway et al., 2001). The endothelial cells self-organize in order to form nascent endothelial tubes, which become covered by mural cells, pericytes and SMCs. The presence of these additional cell populations provide strength to the vessels and regulate vessel perfusion.

In the adult, angiogenesis takes place in few physiological processes, such as hair growth, follicular growth and the development of the corpus luteum (Carmeliet, 2003), while it

might be induced in pathological conditions, such as ischemia, wound healing, tumors and chronic inflammation (Carmeliet, 2005; Jain, 2003).

The third mechanism that has been proposed to explain blood vessel formation is called arteriogenesis, which is responsible for the growth of collateral arteries from pre-existing arteriolar anastomoses in conditions of chronic ischemia (Herzog et al., 2002). Compromised blood supply to an ischemic region can improve gradually as the pre-existing arteriolar anastomoses enlarge to form collateral arteries that bypass the arterial occlusion (Schaper and Scholz, 2003). This process occurs independently from angiogenic sprouting, and might be considered as an adaptive response in patients with progressive arterial occlusive disease. It is even more important than angiogenesis in supplying blood to ischemic regions, because collaterals provide bulk flow to the tissue, whereas capillaries only provide a limited amount of blood to the immediate cellular milieu (Figure 2.2).

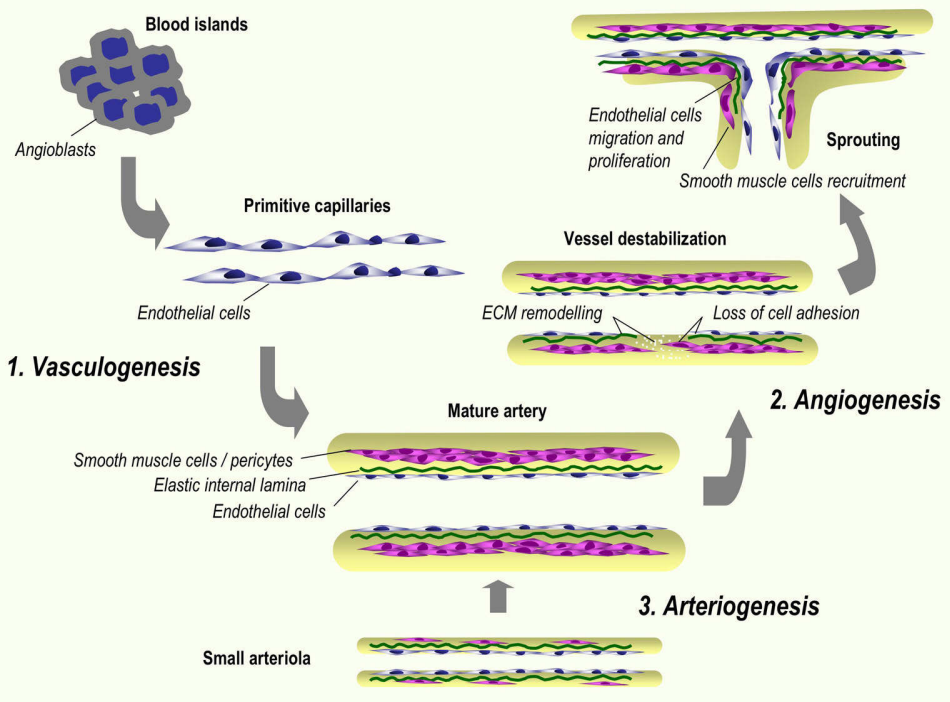


Figure 2.2 Mechanisms of neovascolarization. Blood vessels can arise by three main mechanisms: vasculogenesis, angiogenesis and arteriogenesis. During embryonic development, a population of endothelial cell precursors (angioblasts) start to proliferate within the blood islands, forming a primitive capillary network. The subsequent establishment od cell-to-cell contacts, with the recruitment of pericytes, gives rise to functionally competent vessels. On the contrary, angiogenesis is the main process by which blood vessels can form in adult life, and consists of endothelial sprouting from pre-existing vessels. A transient destabilization of the vessel wall, with extensive extracellular matrix (ECM) degradation and remodeling is required for angiogenesis to start. The latter stages of the process involve the strenghtening of endothelial cell adhesion to ECM components and the enrollment of smooth muscle cells. Arteriogenesis also exclusively occurs in adults, usually as a consequence of a major arterial narrowing, and consists in the remodeling of pre-existing arteriolae to form larger arteries, thus providing a compensatory collateral network during ischemic conditions.

As already mentioned, angiogenesis occurs both in the embryo and in the adult, and it might be induced in pathological conditions. Insufficient availability of oxygen, hypoxia, has been reported to be the most important stimulus for angiogenesis (Carmeliet, 2003; Risau, 1997). The absence of oxygen is also the leading stimulus for angiogenesis during embryonic development (Ikeda et al., 1995). The adult vasculature is normally quiescent but during angiogenesis endothelial cell turnover can be rapid. Endothelial cells proliferate and migrate toward the stimulus, which is often represented by a gradient of Vascular Endothelial Growth Factor (VEGF), the expression of which is induced by the hypoxia inducible factor-1α (HIF-1α) (Pugh and Ratcliffe, 2003; Semenza, 2000). The reduction in oxygen supply leads to the expression of VEGF as well as other factors that cooperate with VEGF in the angiogenic response. Upon VEGF expression, endothelial cells (ECs) proliferate and migrate in order to build an endothelial tube which needs to mature through the acquisition of a periendothelial cell coverage. Therefore, the recruitment of mural cells is a key event for vessel maturation. Pericytes stand as solitary cells with characteristic morphology embedded in the basement membrane of microvessels (Allt and Lawrenson, 2001; Sims, 1986), whereas smooth muscle cells compose a separate layer in the vascular wall. The observation that periendothelial cells are involved after the step of endothelial tube formation (Hungerford et al., 1996) suggests that endothelial cells actively recruit mural precursors of mesenchymal origin and induce their differentiation. Periendothelial cells contribute to the mechanical stability of the microvessels in some organs such as the central nervous system, where they are abundant and cover a major part of the abluminal endothelial surface. However it is more likely that pericytes predominantly influence vessel stability by matrix deposition and by the induction of signals that promote vessel stabilization (Armulik et al., 2005). Indeed several soluble factors which are involved in tightening the junctions between endothelial cells and periendothelial cells, and in modulating the composition of the perivascular extracellular matrix (ECM) are secreted by pericytes (Bazzoni and Dejana, 2004; Dejana, 2004). The ECM have a role for the survival of the quiescent ECs, pericytes and smooth muscle cells providing a necessary contact surface and survival signals (Jain, 2003; Kalluri, 2003). Moreover it acts as a reservoir for growth factors which are bound to ECM and their release is mainly mediated by proteinases (Bergers and Benjamin, 2003). For instance some members of the VEGF family are able to bind to the ECM and the presence of this binding domain essentially controls their biological activity (Carmeliet and Collen, 1999; Ferrara et al., 2003). During the first step of the angiogenetic process, the ECM has to be partially degraded in order to

allow endothelial and mural cell migration whereas in the later steps it has to be produced to give support to the newly formed vessels (Davis and Senger, 2005).

Pericytes make focal contact whit the endothelial cells through specialized junctions; usually a single pericyte contacts several endothelial cells (Armulik et al., 2005) and coordinates neighbouring endothelial cell response. The recruitment of pericytes on the newly formed endothelial tubes is driven by several factors which have been identified by genetic studies in mice. During embryonic development transforming growth factor-β (TGF-β) is the key factor for periendothelial cell recruitment, consistent with the observation that knock-out mice are embryonically lethal (Dickson et al., 1995) due to cardiovascular defects. Moreover TGF-β is also important for the induction of pericyte and SMC differentiation (Carvalho et al., 2004; Hirschi et al., 1998). Angiopoietin-Tie2 signalling pathway is also involved in the reciprocal communications between endothelial cells and pericytes. Angiopoietin-1 is a pericyte-derived paracrine signal for the endothelium, it is mainly produced by perivascular and mural cells. Angiopoietin-1 activate the Tie-2 receptor on endothelial cells, which then produce other factors that recruit mural progenitor cells to the newly formed vessels and promote their differentiation into mural cells (Davis and Yancopoulos, 1999). Instead, Angiopoietin-2 (Ang-2) has been reported to induce vessel destabilization by causing pericyte detachment and loss suggesting an anti-angiogenic role (Zhang et al., 2003). The process of pericyte recruitment is also driven by the PDGF-B PDGFR-β signalling pathway. During angiogenesis endothelial cells secrete PDGF-B, which signals trough PDGFR-β expressed on mural cells, resulting in proliferation and migration of mural cells. PDGFR-β expression on hemangioblasts has been suggested to mediate endothelial cell differentiation during embryonic development (Rolny et al., 2006). Thus the PDGF-B PDGFR-β pathway is critical for both the formation of the vascular plexus and expansion of the pericyte population, and possibly also for pericyte migration along the growing vessel (Bjarnegard et al., 2004). Another factor important for vessel maturation is sphingosine-1-phosphate (S1P), a sphingolipid able to trigger cytoskeletal, adhesiveness, and junctional changes, affecting cell migration, proliferation and survival (Allende and Proia, 2002). S1P signals through Edg1, a G-protein-coupled receptor, and promote N-Cadherin trafficking in endothelial cells, strengthening contacts with mural cells (Paik et al., 2004).

The acquisition of a smooth muscle cell layer surrounding the growing endothelium has been suggested as a hallmark of vessel maturation, even if further steps of stabilization is

required to allow the formation of a functional vasculature (Conway et al., 2001; Jain, 2003).

The last step in the vascular maturation process is the tissue-specific and organ-specific specialization of the vascular wall and network structure (Ruoslahti, 2002). The arterio-venous determination and the endothelial cell differentiation to form organ-specific capillaries were though to mainly depend on mechanical forces such as shear stress, although more recent studies reported indicate the existence of a genetically-determined specialization program (Zhong et al., 2000). One of the key molecules that drive this process is Notch, which commits angioblasts endothelial plexus to either the arterial or venous endothelial lineages (Lanner et al., 2007; Rossant and Howard, 2002). Other molecules essential in the proper branching and interconnections between vessels types are ephrins and their Eph receptors, the former expressed on arterial endothelium and the latter expressed by veins (Adams et al., 1999; Helbling et al., 2000; Thurston et al., 2000a; Wang et al., 1998).

In the whole maturation process endothelial cells, pericytes and the surrounding ECM are reorganized to form stable vessels that are less prone to regression (Benjamin et al., 1998). Indeed, insufficient coverage of newly formed vessels by mural cells leads to excess EC proliferation, permeability, fragility and even regression (Hellstrom et al., 2001).

Several factors involved in the process of vasculogenesis and angiogenesis have been studied and used for therapeutic purposes, with the aim to induce new blood vessel formation in ischemic tissues. Although substantial information has been collected concerning the key molecules that trigger both processes, little is know about the later stages of vessel maturation and stabilization. The definition of the molecular and cellular mechanisms controlling such later stages appear of fundamental importance, as the induction of therapeutic angiogenesis needs to achieve the formation of mature, stable and functional vessels (Figure 2.3).

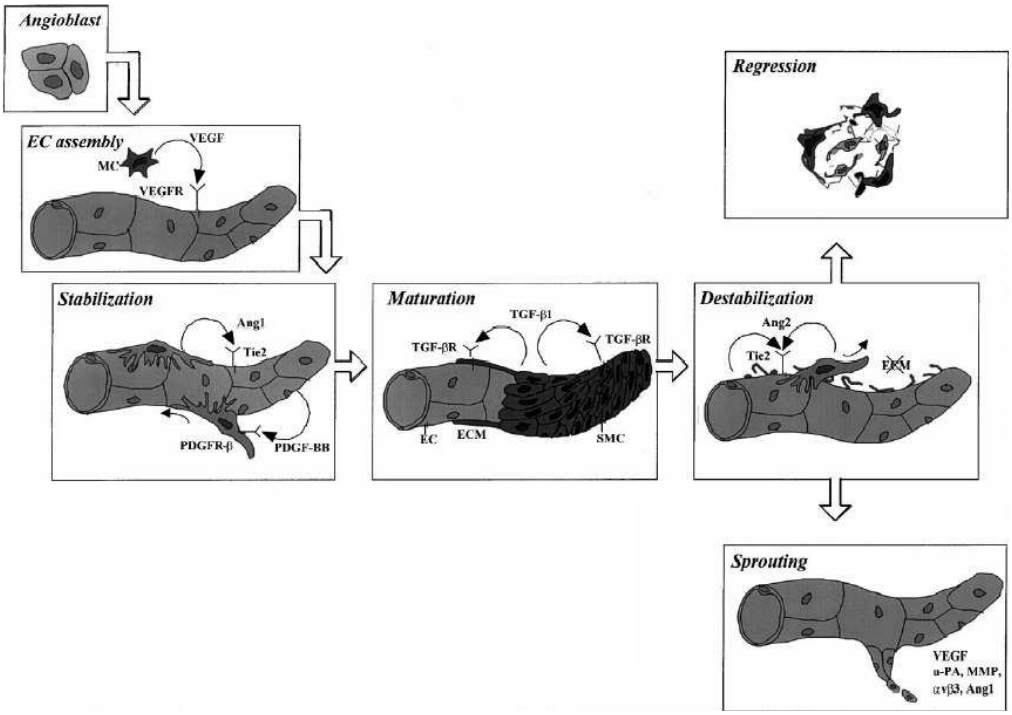


Figure 2.3 The overall process of vessel formation, maturation and stabilization. During embryogenesis a vascular plexus is formed and it expands by means of capillary sprouting induced by VEGF. In later phases, it goes toward stabilization and maturation by recruiting perivascular cells, and by thightening the cell-cell junctions, signals mediated by PDGF-BB, Ang-1 and TGF-β. At this point the vessels might go toward destabilization, mainly mediated by Ang-2 signaling, leading to regression or capillary sprouting, in case of neo-angiogenesis. (Figure adapted from Conway 2001)

2.4 Factors involved in vessel formation, maturation and stabilization

2.4.1 VEGF family members

Vascular endothelial growth factor (VEGF) was discovered twenty years ago as a growth factor that promotes angiogenesis and vascular permeability. Its coding sequence was published by three independent researcher groups in 1989 (Keck et al., 1989; Leung et al., 1989; Plouet et al., 1989), although the very first insights on the existence of this factor actually were obtained few years before by another researcher, who named it 'vascular permeability factor' (VPF) (Senger et al., 1983). Numerous studies have revealed that VEGF is essential for normal embryonic development and that it plays a major role in physiological and pathological angiogenesis in adults. Mice lacking VEGF dye at embryonic day E10.5, not only because of blood vessels defects but also because several organs, such as the heart, kidneys, liver and the neural system, are severely impaired in their development (Carmeliet et al., 1996; Ferrara et al., 1996). After birth, VEGF is not required for organ development and is mainly involved in blood vessel remodelling

(Gerber et al., 1999). Both high and low levels of VEGF are associated to disease conditions, such as diabetic neuropathy, glomerular defects, amyotrophic lateral sclerosis, tumor angiogenesis and ascite formation (Gerber et al., 1999; Kasahara et al., 2000; Maynard et al., 2003) (Figura 2.4).

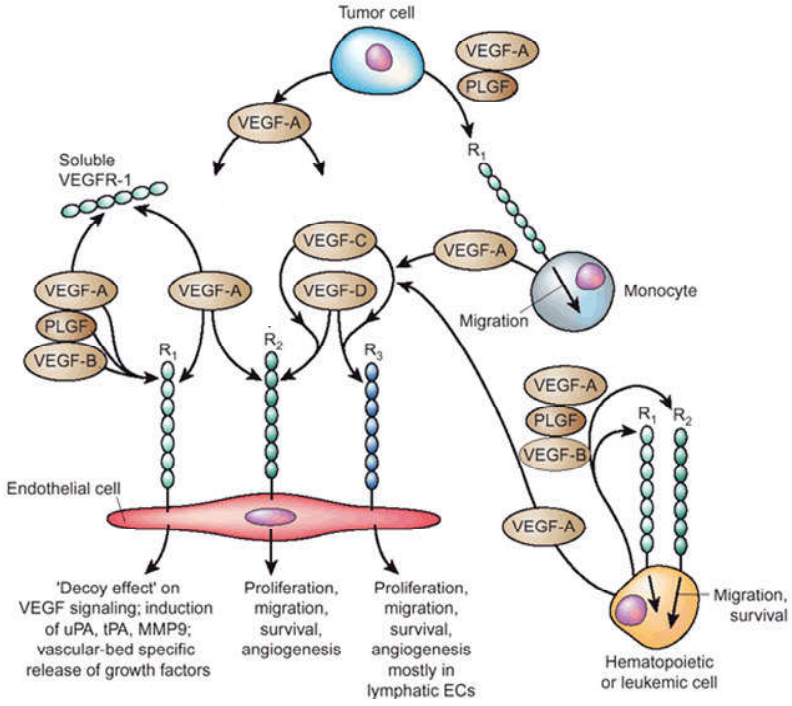


Figure 2.4 VEGF family members and VEGF receptors in different cell types. VEGFR-1 and VEGFR-2 are expressed on the surface of most blood endothelial cells. Instead, VEGFR-3 is largely restricted to lymphatic endothelium. VEGF-A binds both VEGFR-1 and VEGFR-2. In contrast, PlGF and VEGF-B interact only with VEGFR-1. VEGF-C and VEGF-D bind VEGFR-2 and VEGFR-3. There is much evidence that VEGFR-2 is the major mediator in angiogenesis. In contrast, VEGFR-1 seems not able to mediate an effective mitogenic signal and it perform a "decoy" role by sequestering VEGF, thus preventing its interaction with VEGFR-2. However, VEGFR-1 has an established signaling role in mediating monocyte chemotaxis, as well as a survival signal in hematopoietic stem cells (HSC) or leukemic cells. (Figure adapted from Ferrara 2003)

The family of VEGFs modulates a variety of endothelial cell behaviours, commencing with initial embryonic vascular patterning to adult angiogenesis (Ferrara et al., 2003). Indeed, increased formation of capillaries is an expected outcome of short-term VEGF overexpression, given the very well known stimulating properties of VEGF on endothelial cell proliferation and migration (Neufeld et al., 1999). VEGF acts as a prosurvival agent on endothelial cells and it was reported to protect both endothelial and muscular cells from apoptosis (Arsic et al., 2004; Nor and Polverini, 1999; Zachary, 2001).

As endothelial cells are the main target of VEGF, reduction in its expression mostly affects blood vessel: however, the recent evidence that it binds to a variety of cell types such as neurons, skeletal muscles and osteoblasts, explain the observed association between reduced VEGF levels and other diseases. For instance, VEGF have been reported to be critical to prevent motor neuron degeneration, likely acting as a trophic factor on neurons (Oosthuyse et al., 2001). Due to its key role in blood vessel formation, its expression is regulated by several factors such as cytokines, growth factors and hormones but the most important stimulus is the hypoxic conditions. Indeed, VEGF family members expression has been reported to be upregulated in hypoxic conditions, *in vitro* (Shweiki et al., 1992) (Sandner et al., 1997) and *in vivo* (Marti and Risau, 1998) and a HIF-1 binding site has been identified in the VEGF promoter region (Forsythe et al., 1996). Transcriptional regulation of the VEGF-A gene by hypoxia is mediated by binding of the transcription factor HIF-1α to the hypoxia responsive enhancer elements (HREs) (Liu et al., 1995; Minchenko et al., 1994).

The human VEGF-family is composed by five members: VEGF-A, -B, -C, -D and PlGF which differ in their ability to bind to three VEGF receptors (Achen et al., 1998; Joukov et al., 1996; Leung et al., 1989; Maglione et al., 1991; Olofsson et al., 1996; Senger et al., 1983; Yamada et al., 1997)(Senger 1983, Leung 1989, Maglione 1991, Olofsson 1996, Joukov 1996, Yamada 1997, Achen 1998). The VEGF-A gene may give rise to five different isoforms, from 121 to 206 aminoacids, due to alternative splicing (Keck et al., 1989; Leung et al., 1989). These isoforms differ primarily for the presence or absence of a heparin binding domain encoded by exon 6 and 7 (Ferrara et al., 2003). $VEGF_{121}$, made by 121 aminoacids, does not contain these exons and consequently does not bind heparin; however, this form is fully active as an inducer of angiogenesis, and as a blood vessel permeabilizing agent. Since it does not bind heparan-sulfate proteoglycans, $VEGF_{121}$ diffuses freely different from $VEGF_{145}$ and $VEGF_{165}$ stick to the ECM and is released from it slowly (Neufeld et al., 1999; Poltorak et al., 2000).

VEGF-B exist as two splicing variant, VEGF-B_{167} and VEGF-B_{186}, which differ only in their carboxy-terminal domains. VEGF-B_{186} transcript contains the entire exon 6 and encodes a soluble isoform, while VEGF-B_{167} has an alternative exon 6, due to the use of an alternative splice acceptor site, which renders it more sticky to the ECM (Olofsson et al., 1996). VEGF-B is widely expressed in heart, skeletal muscle, and vascular cells and exerts is action mainly through VEGFR-1 (Olofsson et al., 1998; Olofsson et al., 1996). VEGF-C and VEGF-D are closely related, both structurally and functionally. They are both ligands

for VEGFR-3, while VEGF-D is also able to bind to VEGFR-2. They are initially synthesized as disulfide-linked polypeptides containing amino- and carboxy-terminal propeptide extensions. The unprocessed full-length forms preferentially bind VEGFR-3 and have low affinity for VEGFR-2, whereas the fully processed forms have increased affinity for VEGFR-2 (Joukov et al., 1997; Stacker and Achen, 1999). VEGF-C and VEGF-D lack the neuropilin/heparin binding domain found in some VEGF isoforms and appear to be unable to bind neuropilins.

VEGF-C is strongly implicated in the formation of the lymphatic endothelium (lymphangiogenesis). Transgenic mice overexpressing VEGF-C in keratinocytes of the epidermis develop enlarged lymphatic vessels, while mice overexpressing VEGF-A_{164} (homologus of human $VEGF_{165}$) at the same location show only blood-vessel hyperplasia (Jeltsch et al., 1997). VEGF-C also stimulates angiogenesis in the mouse cornea (Cao et al., 1998), however, and also in rabbit models of ischemia in the hindlimb. VEGF-D is mitogenic in endothelial cells and promotes angiogenesis *in vitro* and in several models of angiogenesis *in vivo* (Marconcini et al., 1999). VEGF-D also stimulates lymphangiogenesis in mice when overexpressed in skin keratinocytes and tumors (Stacker et al., 2001), and it induces the survival and migration of lymphatic endothelial cells.

Human PlGF exist in four isoforms, PlGF-1 to PlGF-4, with PlGF-1 and -2 believed to be the major isoforms. PlGF-2 is able to bind heparin and neuropilin-1, a VEGF co-receptor, through an exon 6 encoded heparin-binding domain (Maglione et al., 1993a; Maglione et al., 1993b; Migdal et al., 1998), whereas PlGF-1 lacks exon 6 and is thus unable to bind heparin (Maglione et al., 1991). Mice lacking PlGF are viable and develop normally, and they have reduced angiogenesis only in pathological situations, such as ischemia. PlGF-deficient mice also have delayed collateral artery growth following blockage of an artery, indicating that PlGF stimulates collateral vessel growth (Carmeliet et al., 2001). Finally, PlGF stimulates monocyte chemotaxis through VEGFR-1, and there is increasing evidence that the biological effects of PlGF are mediated, at least in part, the by mobilization of bone-marrow-derived haematopoietic progenitors (Hattori et al., 2002).

All VEGF family members form disulphide-bond active dimmers. Homodimers and heterodimers are formed as VEGF-A, PlGF and VEGF-B, thus further increasing the diversity of their biological effects (Joukov et al., 1997; Olofsson et al., 1996; Stacker and Achen, 1999). VEGF dimers are able to interact with VEGF receptors, which in turn undergo dimerization upon ligand binding. Heterodimer formation is also possible

betweenthe VEGF receptors, including VEGFR1-VEGFR2 and VEGFR2-VEGFR3 heterodimers (Dixelius et al., 2003; Huang et al., 2001).

The VEGF family members exert their functions through three tyrosine kinase receptors: VEGFR-1 (Flt-1), VEGFR-2 (Flk-1) and VEGFR-3 (Flt-4), whose relative affinity and distribution mediate their biological effect (Carmeliet and Collen, 1999; Neufeld et al., 1999).

These receptors have seven immunoglobulin-like domains in the extracellular portion, a transmembrane region and a consensus tyrosine kinase domain in their intracellular part (Fournier et al., 1997; Shibuya et al., 1999; Terman et al., 1991).

VEGFR-1 exists as either a transmembrane receptor or as a soluble form (a decoy receptor trapping excess VEGF). Null mice for the VEGFR-1 gene die in utero before day E10 as a result of excessive angioblast proliferation and endothelial cells organization failure (Fong et al., 1995). Its expression is not restricted to endothelial cells thus its activation does not induce an important angiogenic response. Consistently, PlGF and VEGF-B, which bind to VEGFR-1, do not lead to strong angiogenic responses, while they are mainly involved in monocyte chemotaxis (Barleon et al., 1996; Waltenberger et al., 1994). This receptor is also expressed in the myocardium and it has just been demonstrated to regulate VEGF-B induced angiogenic effect (Lahteenvuo et al., 2009). PlGF-1, appears to be weakly angiogenic when acting alone through VEGFR-1, but the formation of a VEGF-A-PlGF heterodimer was reported to be able to bind to VEGFR-2, inducing a mitogenic effect on endothelial cells, thereby stimualting angiogenesis *in vivo* (Autiero et al., 2003).

VEGFR-2 mediates most of the effects of VEGFs on blood vessels, such as endothelial proliferation, angiogenesis, endothelial cell survival and vascular permeability (Ferrara et al., 2003; Gille et al., 2001). Indeed, its deficiency was reported to be embryonically lethal: VEGFR-2 knock out mice die in utero between day 8.5 and 9.5, and manifest a lack of vasculogenesis (Shalaby et al., 1995). VEGFR-2 expression is restricted to endothelial cells and the binding of an agonist, such as VEGF-A, results in the activation of a well known intracellular mitogenic signalling cascade (Petrova et al., 1999), involving MAPK and PI3K pathways, eventually leading to endothelial cell migration, proliferation and protection from apoptosis (Gerber et al., 1998a; Gerber et al., 1998b).

Hypoxia is a key regulator for VEGF expression and it was suggested to be an important regulator also for both VEGFR-1 and -2 expression. *In vitro* studied showed that VEGFR-1 expression is upregulated by hypoxia by a HIF-1α dependent mechanism (Gerber et al., 1997), whereas the same is not true for VEGFR-2.

VEGFR-3 is mainly involved in lymphatic vessel growth, as its activation leads to growth, migration and survival of lymphatic endothelial cells (Makinen et al., 2001; Veikkola et al., 2001). Studies in null mice showed that its inactivation causes lymphedema due to a lack of cutaneous lymphatics (Karkkainen et al., 2000; Karkkainen et al., 2001). In embryos its expression is not restricted to lymphatic endothelial cells, but it extends to blood vessels; in contrast, in the adult its expression become restricted to the lymphatic endothelium (Kaipainen et al., 1995; Valtola et al., 1999). VEGFR-3 is not a receptor for VEGF-A or -B, while it binds to VEGF-C and -D (Karkkainen et al., 2002).

Beside canonical VEGF receptor, endothelial cells express two co-receptors, which bind to $VEGF_{165}$ but not $VEGF_{121}$: neuropilin-1 and neuropilin-2 (Soker et al., 1996).

Neuropilin-1 (NP-1) and neuropilin-2 (NP-2) are transmembrane glycoproteins with large extracellular domains and a very short cytoplasmatic tail (Gu et al., 2002). Neuropilins are receptors for class-3 semaphorins, originally discovered as neuronal guidance cues, providing repulsive signals to growing axons (Neufeld et al., 2002). The heart, glomeruli and osteoblasts express both NP-1 and NP-2. In the adult vasculature, NP-1 is expressed mainly by arterial endothelium, whereas NP-2 is only expressed by venous and lymphatic endothelium (Herzog et al., 2001). Both neuropilins are commonly over-expressed in regions of physiological (wound-healing) and pathological (tumour) angiogenesis, but the signal transduction pathways, neuropilin-mediated gene expression and the definitive role of neuropilins in angiogenesis are not fully characterized. Genetic studies in mice demonstrated that NP-1 plays a critical role in embryonic vascular development, NP-1 knockout mice have defects in yolk sac, neuronal vascularization, and impaired development of large vessels and heart (Kawasaki et al., 1999). On the other hand, overexpression of NP-1 in transgenic mice resulted in the formation of excess capillaries, haemorrhages, and profound alterations of nervous system development, leading to embryonic lethality (Kitsukawa et al., 1995).

The fact that $VEGF_{121}$ does not bind neuropilins suggested that the exon-7-encoded basic sequences are required for such interaction (Soker et al., 1996). Neuropilins were found to form complexes with VEGFR-1 and VEGFR-2. In particular, the association of NP-1 to VEGFR-1 inhibits the binding of VEGF to the receptor (Fuh et al., 2000), whereas its association to VEGFR-2 enhances both the binding and the biological activities of $VEGF_{165}$ (Soker 1998). It has been proposed that NP-1 presents $VEGF_{165}$ to VEGFR-2 in a manner that enhances the potency of VEGFR-2-mediated signal transduction (Soker et

al.,1998; Yamada et al., 2001), it thus serves as a $VEGF_{165}$ co-receptor that increases the ability of the growth factor to activate its main receptor.

2.4.2 Platelet-derived growth factors

Platelet-derived growth factor (PDGF) was originally identified in platelets and in serum. It was found in plasma as a heterodimer composed by PDGF-A and –B; the latter was cloned from human endothelial cells (Collins et al., 1985).

The PDGF family members were discovered for their role as mitogens for many cell types of mesenchymal and neuro-ectodermal origin (Heldin and Westermark, 1999)(Figure 2.5).

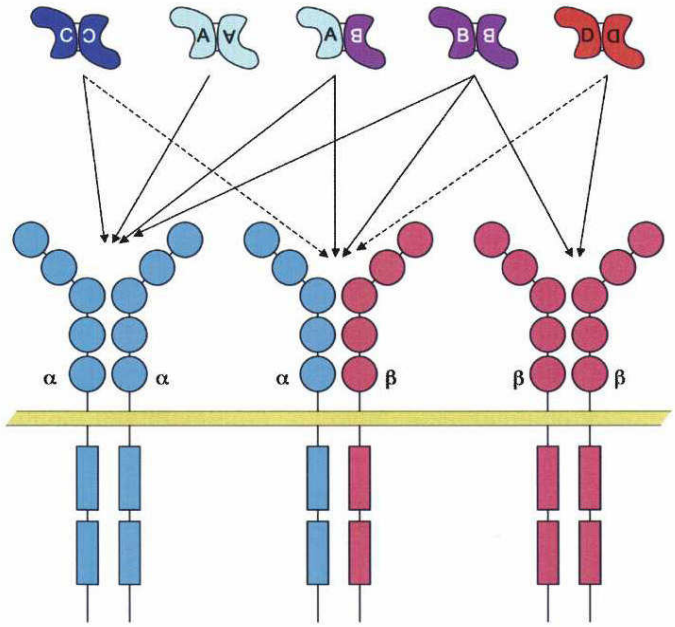


Figure 2.5 PDGF family members and PDGF receptors. The PDGF family is composed by 4 members which form homodimers or hetherodimers, PDGF-C and –D are not able to form heterodimers. They signals through the PDGFRs, which also form homodimers and heterodimers. Each chain of the PDGF dimer interacts with one receptor subunit. Indeed the active receptor configuration is determined by the ligand dimer configuration. (Figure adapted from Andrae 2008)

Insight in the role of PDGFs came from studies on transgenic and knockout animal models. PDGFs have important functions during the embryogenesis, in particular for the development of the kidneys, blood vessels, lungs, and central nervous system. In these organs, several connective tissue-like cell types are dependent on PDGFs, including mesangial cells, pericytes, alveolar fibroblasts, and glial cells. The significant role of PDGF in the formation of connective tissue is still valid during wound healing in the adult.

In the adult, PDGFs overactivity correlated to several serious diseases, such as vascular disorders, fibrosis and cancer. In atherosclerotic lesions, PDGF-A and -B are abundantly

expressed, as are PDGFR-α and -β on SMCs of the vessel wall. The high proliferative rate of vascular SMCs characterizes pulmonary arterial hypertension (PAH), a severe condition that leads to heart failure and death. Pulmonary artery vSMC hyperplasia is a hallmark of the disease, and up-regulated expression of PDGFs and PDGFRs has been correlated with PAH in various experimental animal models (Balasubramaniam et al., 2003; Humbert et al., 1998) and in humans (Schermuly et al., 2005). The role of PDGFs in the development of theses diseases is connected to the pro-mitogenic action on the different cell types such as smooth muscle cells and myofibroblast . PDGF expression can be induced in cells that compose the normal arterial wall as well as in inflammatory cells that infiltrate the artery in response to pathological stimuli.

Focusing on blood vessel development and angiogenesis, studies on knock out animal models helped in understanding the role of either isoform and cognate receptors.

These studies showed that PDGF-A and PDGFR-α are required during embryogenesis, with essential roles in numerous contexts, including central nervous system, neural crest and organ development (Soriano, 1994) (Bostrom et al., 1996; Fruttiger et al., 1999; Karlsson et al., 1999; Karlsson et al., 2000; Soriano, 1997). Indeed, PDGF-A deficiency is lethal but mice dye at various developmental times, spanning from E10 to postnatal day 60. PDGFR-α -/- also die, but always at embryonic stages (Soriano, 1997). Moreover, PDGF-B -/- mice die perinatally and display several anatomical and histological abnormalities (Leveen et al., 1994), including abnormal kidney glomeruli, heart and blood vessel dilatation, anaemia, thrombocytopenia, and haemorrhages. Similarly, PDGFR-β null mutants die at late gestation from wide spread microvascular bleeding (Leveen et al., 1994; Soriano, 1994), further confirming the role of this pathway on vessel formation.

In particular, PDGF-B/PDGFR-β signaling during embryonic development seems to essentially promote the proliferation of vSMC/pericyte progenitors during their recruitment to new vessels (Hellstrom et al., 2001; Hellstrom et al., 1999; Lindahl et al., 1997). In the PDGFR-β null mice vSMCs and pericytes were the cell types more severely affected (Lindahl and Betsholtz, 1998; Lindahl et al., 1997).

Pericyte deficiency, as seen in mice lacking PDGF-B and its cognate receptor PDGFR-β, promotes a range of microvascular changes such as endothelial hyperplasia, vessel dilatation, tortuosity and leakage (Leveen et al., 1994; Soriano, 1994). The role of PDGF-B for pericyte recruitment and proper vessel formation was also studied by exploiting a PDGF-receptor inhibitor, which affected pericyte viability, leading to extensive pericyte loss (Wilkinson-Berka et al., 2004).

During development PDGFR-β is expressed rather specifically on vSMC/pericytes surrounding the blood vessels, whereas PDGF-B is mainly produced by endothelial cells (Lindahl et al., 1997). Tissue-restricted PDGF-B knockout mice have confirmed that the endothelium is the most important source of PDGF-B in vSMC/pericyete recruitment . The strongest expression of PDGF-B during angiogenic sprouting occurs from the specialized endothelial cell situated at the tip of the sprout (tip cells) (Gerhardt and Betsholtz, 2003). Thus, PDGF-B and PDGFR-β expression occurs in different but closely located cell types in the blood vessels. PDGF-B therefore likely acts as a short-range paracrine factor, its action appears to be facilitated by the presence of a retention motif, which helps to stick the secreted PDGF-B to proteins or proteoglycans on the endothelial cell surface or in the periendothelial matrix, thereby promoting its recognition by neighbouring receptor-carrying cells (pericytes). Retention of PDGF-B in microvessels is essential for proper recruitment and organization of pericyets and for renal and retinal function in adult mice (Lindblom et al., 2003). In these models, absence of PDGF-B and PDGFR-β induces a reduction in the number of pericyte and partial dissociation of pericytes from the abluminal endothelial surface (Abramsson et al., 2007). Pericytes normally extend dendritic processes that associate intimately with the abluminal endothelial surface (Allt and Lawrenson, 2001). Normal blood microvessels are lined by pericytes, which contribute to microvessel development and stability through mechanisms that are poorly understood. Pericyte deficiency has been implicated in the pathogenesis of microvascular abnormalities associated with diabetes and tumors.

Pericytes have been heralded as potential target for vascular destruction. Pericyte recruitment to coat nascent vessels is essential for the stabilization and further establishment of the vascular network, and, conversely, vessels lacking adequate pericyte coverage were reported to be more prone to regression.

2.4.3 Angiopoietins

Besides endothelial cell sprouting, the formation of a full blown and functional vasculature requires the outward remodeling of the newly formed vessels, with the acquisition of a smooth muscle cell coat and the deposition of extracellular matrix.

Studies performed over the last decade showed that Angiopoietins are key factors in these later stages of blood vessel formation (Davis et al., 1996; Thurston et al., 2000b).

Angiopoietin-1 (Ang-1) and -2 (Ang-2) are the two well characterized members of the Angiopoietin family, and they act through the Tie-1 and Tie-2 tyrosine kinase receptors.

Ang-1 has been implicated in the remodelling, maturation, stabilization and survival of blood vessels via triggering Tie-2 receptor activation in endothelial cells (Asahara et al., 1998; Hawighorst et al., 2002). Studies performed on the Ang-1 knockout mouse, showed that the absence of Ang-1 does not allow a proper development of the vascular system, leading to embryonic death before birth. Interestingly, a similar phenotype was previously observed in Tie-2 knock out mice, suggesting the essential role of the Ang-1/Tie-2 signaling for proper vessel development (Sato et al., 1995; Suri et al., 1996).

Ang-2, which was identified by a homology screening (Maisonpierre et al., 1997), acts a natural antagonist of Ang-1 by binding but not activating the Tie-2 receptor, which results in apoptosis of endothelial cells and disruption of angiogenesis (Lobov et al., 2002; Maisonpierre et al., 1997; Nor and Polverini, 1999). Ang-2 overexpression, in transgenic mice, was able to interfere with blood vessel formation in the mouse embryo leading to blood vessel destruction. Studies performed Ang-2-deficient mice revealed that Ang-2 is dispensable for embryonic vascular development but is required for subsequent angiogenic remodelling. Moreover, mice lacking Ang-2 exhibited major lymphatic vessel defects. It was suggested that Ang-2 might act as a Tie-2 agonist for lymphatic vessel development and as an antagonist of the same receptor in angiogenesis (Gale et al., 2002; Veikkola and Alitalo, 2002) (Figure 2.6).

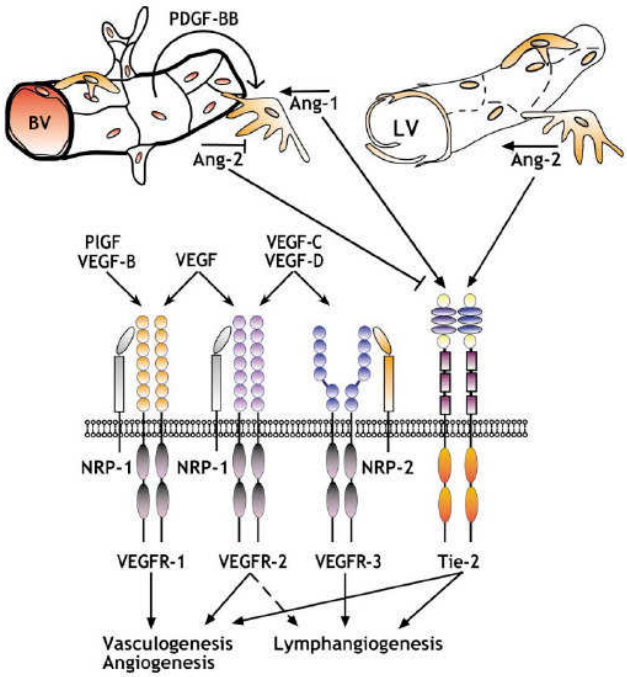


Figure 2.6 Angiopoietins in angiogenesis. During angiogenesis Ang-1 is involved in periendothelial cell recruitment (yellow) and vessel stabilization, while Ang-2 destablilizes these interactions in blood vessels (BV), allowing endothelial cells to respond to the angiogenic stimuli, such as VEGF. During lymphangiogenesis Ang-2 is able to recruit smooth muscle cells, required for the stability and the proper functions of the lymphatic vessels (LV). Angiopoietins mediate their signals through the Tie-2 receptor. (Figure adapted from Veikkola 2002)

In the adult, Ang-1 is essential for proper vessel maturation and stabilization, by reinforcing endothelial cell connections, as well as by recruiting periendothelial cells (Thurston et al., 1999). Ang-1 mitigates vessel leakiness promoted by inflammation in the adult vasculature (Thurston et al., 2000b)confirming the notion that it is able to counteract vascular permeability. In combination with VEGF overexpression, Ang-1 counteracts the vascular permeability induced by VEGF, thus leading to a more mature vasculature (Arsic et al., 2003). In a model of wound healing, it was reported that Ang-1 overexpression is able to induce the formation of larger vessels whit a reduced leakiness (Suri et al., 1998).

Beside Ang-1 and Ang-2, over the last years new members of the Angiopoietin family have been identified: angiopoietin-like factors (Kim et al., 1999; Oike et al., 2003; Valenzuela et al., 1999). These factors contain both a N-terminal coiled-coil domain and a C-terminal fibrinogen-like domain, similar to the other family members, but they are not able to signal through the canonical angiopoietin receptors, Tie-1 and Tie-2. Angiopoietin-like factors therefore were initially thought unable to induce angiogenesis; surprisingly

recent evidence indicate that they participate in new blood vessel formation (Oike et al., 2004).

2.5 Cardiovascular gene therapy

2.5.1 Gene therapy for the induction of neo-angiogenesis (new blood vessel formation)

As previously described, new blood vessel formation is a multistep process sustained by the sequential and coordinated activity of several factors. Therapeutic angiogenesis aims not only at increasing vessel number in ischemic tissues but also at achieving a proper functional maturation.

Despite quite exciting results obtained in experimental animal models, the results of the first clinical trials have been rather disappointing. A better understanding of the mechanisms that regulate the angiogenic process is therefore mandatory to improve the clinical translatability of the approaches developed so far. This effort will eventually be useful for the development of both pro-angiogenic therapies and anti-angiogenic therapies. Similarly, molecular therapies aimed at inducing vessel maturation and stabilization are gaining increasing interest in both therapeutic angiogenesis and anti-angiogenic cancer therapies.

Gene therapy is the transfer of nucleic acids to somatic cells of an individual with a resulting therapeutic effect (Yla-Herttuala and Alitalo, 2003). The final result of gene therapy is dependent from many variables, such as the efficiency of gene delivery to the target tissue, the choice of the proper therapeutic gene and of the proper delivery method. Beside its direct and obvious therapeutic goal, *in vivo* gene transfer might well be considered a potent tool to study the role of specific factors *in vivo* in physiological and pathological conditions, in order to unveil novel possible molecular targets.

Choosing the right vector for gene therapy application is one of the biggest challenges faced by gene therapists (Baker, 2004), together with the choice of the genes of interest. An ideal vector for gene therapy should combine the following features: high efficient transduction, large cloning capacity, long-term expression restricted to the target tissue, no cytotoxicity and no induction of inflammatory or immune responses (Gaffney et al., 2007).

Many clinical trials in the cardiovascular filed used naked plasmid DNA because of its easy production and safety, but the low gene transfer efficiency, resulting in very low levels of the therapeutic protein at the target site, did not allow to reach satisfactory results in humans (Hedman et al., 2003; Makinen et al., 2002). In order to improve the gene transfer efficiency, viral vector have been developed, which allow high levels of transgene

expression *in vivo*. Adenoviral (Ad) and Adeno-Associated Viral (AAV) vectors are the two classes of viral vectors mainly used in the cardiovascular field. They both showed high transduction efficiency in blood vessels, heart and skeletal muscles, the major target tissues in cardiovascular gene therapy.

The most important features of vectors derived from AAV are the ability to mediate prolonged transgene expression in the target tissue and the absence of pathogenicity (Favre et al., 2001). Moreover, AAVs can transduce cells at high multiplicity of infection, allowing the expression of more than one factor by the same cell (Arsic et al., 2003). The features of these viral vectors will be discussed in details in the following paragraph.

Adenoviral vectors are characterized by a high cloning capacity and they could be easily produced at high titers, however they are affected by two limitations: they evoke a strong inflammatory response, which might be reduced with the development of new gutless vectors, and their expression in the target tissue is short and limited to 15 days. This later feature might be of interest for gene therapy approaches for acute pathologies but would not be feasible for situation that require a prolonged expression of the transgene. Moreover, beside the strong inflammatory response, the death of a patient enrolled in a clinical trial has been raising doubts on their safety (Raper et al., 2003).

Another class of viral vectors of interest for the cardiovascular gene therapy are Retroviruses, which have been highly used in the past (Nabel et al., 1990), but recent evidences concerning their safety limited their application (Hacein-Bey-Abina et al., 2003). These vectors are able to transduce dividing cells, such as hematopoietic cells, due to their ability in inserting their genome into the host chromosomes, while are not able to transduce nondividing cells. The ability to integrate in the host genome, beside allowing prolonged expression, might account for random insertional events that should lead to carcinogenesis. However Lentivirus, which are a subgroup of retroviruses, are able to transduce both dividing and quiescent cells, and have been recently introduced into cardiovascular gene therapy field. Gene therapy vectors derived from Lentivirus were reported to be able to transduce both neonatal and adult rat cardiomyocytes *in vitro* and *in vivo* (Sakoda et al., 2003; Zhao et al., 2002). Recently, a lentiviral vector for angiotensin converting enzyme-2 (ACE-2) was reported to be efficient in protecting cardiomyocytes during myocardial infarction, in a rat model (Der Sarkissian et al., 2008).

In Table 1 a summary of the vectors used in cardiovascular gene therapy is reported.

Table 1: Vector used in cardiovascular gene therapy. (Adapted from (Rissanen and Yla-Herttuala, 2007))

Vector	Advantages	Disadvantages
Naked plasmid DNA	Easy to produce safe	Very low transduction efficiency Transient expression
Adenovirus	High transduction efficiency Relatively high transgene capacity Easy to produce in high titers Transduces quiescent cells Tropism for multiple cells	Inflammation with high doses Transient expression
Adeno-associated virus (AAV-1, -2, 5, 6, 8, 9)	Long-term gene expression Moderate immune response Transduces quiescent cells High tropism for skeletal muscle (AAV-1, -6) and myocardium (AAV-8 and -9) Wild type does not cause disease in humans	Limited transgene capacity Difficult to produce in large quantities
Lentivirus	Long-term gene expression Transduces quiescent cells Relatively high transgene capacity Low immune response	Non-specific integration Low transduction efficiency Limited tropism Difficult to produce in large quantities
Retrovirus	Long-term gene expression Relatively easy to produce Low immune response	Non-specific integration Transduces only dividing cells Low transfection efficiency Limited tropism Difficult to produce in large quantities

In recent years, much effort has been spent to achieve tight regulation of transgene expression by the use of tissue specific or inducible promoters, which allow a spatial and temporal regulation of the expression. In inducible vectors, the expression of the transgene is regulated by drugs such as tetracycline, doxycycline or rapamycine, which bind to the transactivator allowing or inhibiting the transcription of the gene (Chenuaud et al., 2004; Chtarto et al., 2003; Gossen and Bujard, 1992; Gossen et al., 1995). In these systems, two transgenes have to be expressed at the same time: the transactivator, which is usually under the control of a constitutive promoter, and the gene of interest, the expression of which is induced (TetON system) or abolished (TetOFF system) by the administration of the drug (i.e. doxycycline).

Inducible AAVs have allowed to obtain encouraging results in preclinical animal models, although recent evidence indicate an important immune response toward the viral components, such as transactivators, thus limiting the clinical applicability of this approach (Favre et al., 2002; Favre et al., 2001).

2.5.2 Adeno-associated virus biology and rAAV vectors

AAVs are small viruses (25nm) belonging to the *Parvoviridae* family, their genome is single stranded DNA and they need the presence of an helper virus, such as Ad or Herpes simplex virus (HSV), to facilitate productive infection and replication. In the absence of a helper virus, AAV integrates into a specific region of human chromosome 19, termed AAV1S, thus establishing a latent infection (Kotin et al., 1990) (Figure 2.7).

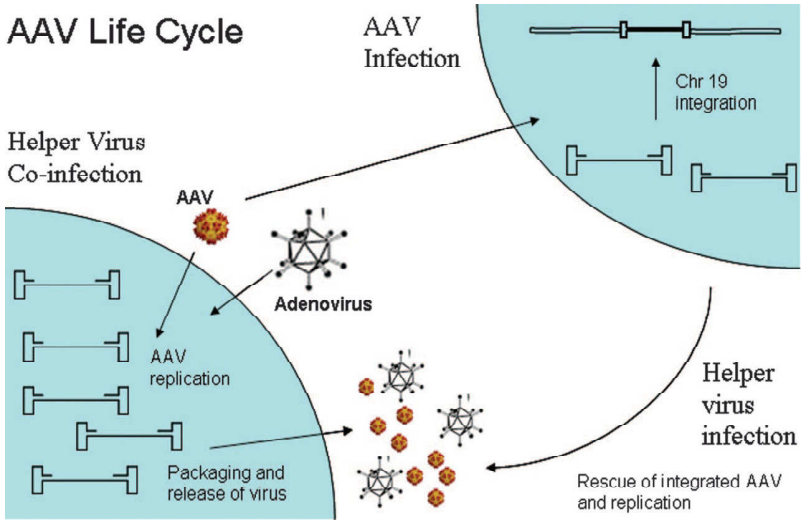


Figure 2.7 AAV life cycle. AAV needs the presence of an helper virus, such as adenovirus, to undergo productive infection which is characterized by genome replication, viral gene expression, and virion production. In the absence of adenovirus, AAV can establish latency by integrating into the host genome, chromosome 19. (Figure adapted from Daya 2008).

The DNA genome of 4.7 kb consists of two large opening reading frames (ORFs) flanked by inverted terminal repeats (ITR), which are required for genome replication and packaging. The two ITRs are 145 bp long, show complementarities within the first 125 bp and form a T-shape hairpin at both ends of the genome. In the current model of AAV replication, the ITR is the origin of replication and acts as a primer for second strand synthesis by DNA polymerase. The left ORF contains the Rep genes, which produces four Rep proteins, Rep78, Rep68, Rep52 and Rep40, which regulate AAV gene expression in the presence or absence of helper virus and are required for DNA replication (Pereira et al., 1997). The right ORF contains the Cap gene, which produces three viral capsid proteins (VP1, VP2 and VP3) by alternative splicing. The AAV-2 capsid is composed by 60 viral proteins arranged into a icosahedral structure and the capsid proteins are present on the surface in a 1:1:10 ratio (VP1, VP2 and VP3 respectively) (Daya and Berns, 2008).

AAV vectors share many features with the wild type virus. In order to obtain a good viral vector for gene therapy, most of the viral sequences are removed, except for the ITRs, which are essential for packaging. The absence of the Rep protein, which is required for site-specific integration, is the reason why recombinant AAV genomes persist primarily as extrachromosomal elements (Afione et al., 1996; Schnepp et al., 2005).

The increasing popularity of rAAV vectors relies on their efficient and long-term persistent infection of a series of tissues and organs in vivo (Xiao et al., 1996). For some still unexplored reasons, however, transduction occurs essentially only in cells of muscular origin (including skeletal and SMCs and cardiomyocytes), in neuronal cells (both in the central and peripheral nervous system and in the retina), in retinal pigmental cells, and, at a lesser extent, in hepatocytes. The reasons for this selective tropism are most likely unrelated to viral penetration into the cells, since AAV particles are able to efficiently bind and enter a large number of cells, due to the usage of widely expressed molecules as receptors, including heparan sulphate proteoglycans, FGFR-1 and αVβ5 integrin (Bartlett et al., 2000). After infection, however, different and unknown cellular factors influence the outcome of viral transduction that limit the *in vivo* efficiency. A peculiar molecular event limiting the infection efficiency is the synthesis of the complementary strand of the viral genome, which converts the single-stranded AAV DNA into its transcriptionally active, double-stranded form (Ferrari et al., 1996). Intriguingly, this event often takes a long time to occur: even in highly permissive tissues, the maximum levels of transgene expression is detected only after several weeks, being preceded by a lag period during which some still not completely understood molecular events take place (Zentilin et al., 2001).

Recombinant AAV vectors have been based mostly on serotype 2 but up to 12 different serotypes have been discovered up to date (Schmidt et al., 2008). They differ for the capsid proteins, which determine their tropism. However some tissues remain refractory to AAV transdcution by the available serotypes. Of interest for cardiovascular gene therapy, AAV-1 and AAV-6 were reported to efficiently transduce skeletal muscles, while AAV-8 and AAV-9 showed a strong transduction of the heart and of all the muscles (Palomeque et al., 2007). In particular, AAV-9 was shown to transduce myocardium much more efficiently (5- to 10-fold) than AAV-8, resulting in over 80% cardiomyocyte transduction (Bish et al., 2008; Inagaki et al., 2006).

A second administration of a different serotype-based AAV into immunocompetent animals appears to be fully efficient. Therefore, cross-administration of AAV-1, AAV-2 and AAV-5 is a very promising approach for skeletal muscle gene transfer, as it allows to

overcome the risk of low gene transfer efficiency due by pre-existing immunity in the host organism due to an initial virus exposure (Riviere et al., 2006). Indeed, studies performed on human blood samples highlighted the presence of antibodies against AAV capsid proteins (Chirmule et al., 1999; Manno et al., 2006), suggesting that in a clinical setting the transduction efficiency might depend on previous exposure to the wild-type virus. In this respect, immunosuppressive therapy might be admnistered in combination with AAV; this treatment was shown to overcome the presence of an immune response against the capsid proteins, at least in animal models (Mingozzi et al., 2007; Wang et al., 2007).

2.5.3 Production of rAAV vectors

Since the AAV genome cloned into a plasmid is still infectious and able to produce viral particles, any exogenous gene (less than 4.5 kb in length) can theoretically be placed within the two 145 bp ITRs to obtain a circular backbone suitable for vector production.

Unlike other delivery systems that have evolved into several generations, the original composition of the AAV vector plasmid (a transgene expression cassette flanked by the two ITRs) is essentially the same as in the current version. The traditional method for rAAV production is based on co-transfection of the vector plasmid together with a second plasmid, supplementing the rep and cap gene functions, into helper-infected cells (usually HeLa or 293 cells). The identification of the adenoviral genes essential to provide helper activity to AAV replication in packaging cells led to the development of new protocols that allow for vector manufacturing in a setting totally free of helper virus, based on plasmids containing part of the adenoviral genome. These plasmids are completely non-infectious, carrying only the subset of adenoviral genes that is essential for rAAV production. Recently, both the AAV and adenoviral genes were assembled in one plasmid, thus reducing the number of plasmids required to transfect for recombinant particles production (Grimm et al., 1998). In this way, it is now possible to obtain rAAV preparations free of contaminating helper virus and unwanted adenoviral protein at a yield even higher than that achieved by using infectious helper virus.

In Figure 2.8 a schematic diagram of the rAAV production method is reported.

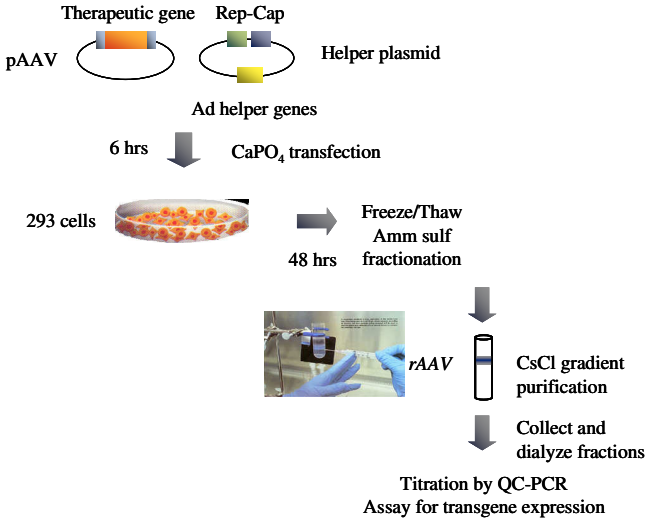


Figure 2.8 Production of recombinant AAV vectors. After co-transfection of the AAV vector plasmid (carrying the cDNA of the therapeutic gene), together with the helper plasmid (supplementing the rep and cap gene functions, as well as adenoviral genes with helper activity), HEK293 cell lysate is obtained by repeated freezing and thawing, and subsequently fractionated by $(NH_4)_2SO_4$ precipitation. rAAV particles are then purified by CsCl density gradient ultracentrifugation and dialyzed. Viral preparation stocks are finally titrated and assessed for transgene expression.

2.5.4 Cardiovascular gene therapy: pre-clinical studies and clinical trials

The concept of therapeutic angiogenesis originated from studies investigating tumor angiogenesis factors in the 1970's (Folkman, 1971). However only after sequencing the cDNA of FGF-1 and -2 and VEGF in 1986-89, it was possible to produced purified growth factors and move to *in vivo* studies.

The use of recombinant proteins was the first approch to achieve therapeutic angiogenesis but it soon revealed the limitations imposed by their short half life. For instance, after an intradermic injection of VEGF, its biological activity is reduced already after 60 minutes (Dafni et al., 2002).

As mentioned before, naked plasmid DNA provided promising results in animal models (Shyu et al., 1998; Tsurumi et al., 1996; Vincent et al., 2000) and in initiale phase I clinical trials, but the larger randomized clinical trials have not supported its use because of its low transduction efficiency (Hedman et al., 2003; Makinen et al., 2002).

The introduction of viral vectors have allowed the development of several successful preclinical studies. For instance, some studies performed with adenovirus expressing VEGF were successful in inducing capillary sprouting and the formation of a vascular network confirming the key role of VEGF in the agiogenetic process and suggesting its use

in clinical trials (Isner et al., 1996; Magovern et al., 1997; Mesri et al., 1995; Pettersson et al., 2000; Poliakova et al., 1999).

The promising results obtained in these studies led to the translation to the clinic with the development of clinical trials. The first one for therapeutic angiogenesis started in 1994 (Isner et al., 1996), in this trial patients affected by critical hindlimb ischemia were enrolled and treated with naked plasmid DNA for VEGF. The overall effect was then evaluated by angiography and nuclear magnetic resonance, which revealed the formation of new collaterals and a significant improvement in perfusion in the VEGF-treated group (Baumgartner et al., 1998). The only major side effect was the occurrence of a remarkable edema of the leg, probably related to the potent permeabilizing effect of VEGF (Baumgartner et al., 2000). After this first trial additional studies were performed and extended also to patients affected by myocardial ischemia (Fortuin et al., 2003; Losordo et al., 2002; Losordo et al., 1998).

Several patients have been enrolled so far in clinical studies for therapeutic angiogenesis but the results were not always successful. Patient with either critical limb ischemia or CAD have been enrolled in many clinical trials which are still ongoing and a summary is presented in Table 2 (Rissanen and Yla-Herttuala, 2007).

Table 2: Phase II/III randomized controlled gene therapy trials for therapeutic angiogenesis

Trial	Therapeutic agent	Disease target	N° of patients	Primary endpoint	Results	References
VEGF peripheral vascular disease trial	Adenovirus VEGF165 Plasmid/liposome VEGF165	PAD	54	Increased vascularity in angiography at 3 months	Positive	(Makinen et al., 2002)
RAVE trial	Adenovirus VEGF121	PAD	105	PWT at 12 weeks	Negative	(Rajagopalan et al., 2001)
Groningen trial	Naked VEGF165 plasmid	PAD	54	Decrease in amputation rate	Negative	(Kusumanto et al., 2006)
KAT trial	Adenovitus VEGF165 Plasmid/liposome VEGF165	CAD	103	Improved myocardial perfusion at 6 months	Positive (Adenovirus group only)	(Hedman et al., 2003)
REVASC trial	Adenovirus VEGF121	CAD	67	Time to 1 mm ST-segment depression on ETT at 26 weeks	Positive	(Stewart et al., 2002)
Euroinject one trial	Naked VEGF165 Plasmid	CAD	74	Improved myocardial perfusion at 3 months	Negative	(Kastrup, 2003)
AGENT trial	Adenovirus FGF-4	CAD	79	ETT at 4 weeks	Positive (only one group)	(Grines et al., 2002)
AGENT-2	Adenovirus FGF-4	CAD	52	SPECT at 8 weeks	Positive	(Grines et al., 2003)
VIVA trial	rVEGF protein	CAD	178	ETT at 4 weeks	Negative	(Henry et al., 2003)
FIRST trial	rFGF-2 protein	CAD	337	ETT at 12 weeks	Negative	(Simons et al., 2002)
TRAFFIC trial	rFGF protein	PAD	190	ETT at 12 weeks	Positive	(Lederman et al., 2002)

Despite good results obtained in the animal models and in the phase I studies the overall results from the clinical studies are quite negative, many have to be stopped due to the presence of side effects or had negative outcome (Simari and O'Brien, 2002; Yla-Herttuala et al., 2004).

The variable success of trials conducted to date might depend on a few recurrent reasons, including choice and formulation of growth factors, short exposure, route of administration and selection of patients.

The negative results observed in the clinical studies might be due the complexity of the angiogenetic process suggesting that one factor is not able to recapitulate the complete process properly. Moreover they pointed out that some potent angiogenetic factor may also potentially lead to tissue edema and death (Isner, 2001; Lee et al., 2000).

Therefore, simultaneous induction of angiogenesis and arteriogenesis would be an optimal clinical outcome. In animal models the combination of two or more genes for the induction of therapeutic angiogensis have already begun leading to successful result. Synergistic effect on blood vessel formation of $VEGF_{165}$ isoform delivered in AAV vector together with angiopoietin-1 (Ang-1), which is required for vessel maturation, was observed by Arsic (Arsic et al., 2003). Combinations of VEGF with other angiogenic growth factors, such as FGF-2 and platelet-derived growth factor-BB (PDGF-BB), were studied (Cao et al., 2003). Indeed, the majority of current approaches for therapeutic neovascularization aim at delivering highly effective angiogenic factors to the ischemic regions of limb or heart to simulate vessel sprouting and remodeling of the newly formed capillaries (Cao et al., 2005).

In this respect, rAAV vectors are a promising candidate due to fact they infect cells at high multiplicity, allowing the simultaneous delivery of several combinations of genes in the same tissue, and to their ability to ensure prolonged expression of the therapeutic genes in absence of inflammatory response.

3. AIMS OF THE STUDY

The main goal of angiogenic gene therapy is the formation of functional new blood vessels adequate to restore blood flow in ischemic tissues. Angiogenesis is a complex process, consisting in the sprouting of new capillaries from pre-existing vessels to form an immature vascular network, which subsequently undergoes functional maturation and remodelling. Many factors are involved in this process and, among them, the VEGF family members are universally recognized as the key players.

The experiment reported in this thesis were aimed at defining the molecular and cellular mechanisms of vessel maturation and stabilization by a gene transfer approach based on Adeno-Associated Viral (AAV) vectors.

The work might be divided in three main chapters: i) define the angiogenic properties of the two VEGF isoforms $VEGF_{121}$ and $VEGF_{165}$, which share many structural features except the ability in NP-1 binding; ii) study the effect of $VEGF_{165}$ overexpression in two models of ischemia, that might be of clinical interest in the plastic and reconstructive surgery field; iii) unravel the role of Angiopoietin-1 in the process of new vessel formation induced by VEGF by the co-injections of two rAAVs.

Most experiments were performed by vector injection in the mouse or rat skeletal muscle, followed by detailed histological, immunohistochemical and functional analysis. Moreover, the availability of imaging techniques, such as PET and SPECT, allowed to obtain results at the functional level and not restricted to ex vivo analysis.

4. MATERIALS AND METHODS

4.1 Recombinant AAV vector preparation

The rAAV vectors used in this study were produced by the AAV Vector Unit at ICGEB Trieste (http://www.icgeb.org/avu-core-facility.html).

Infectious AAV-2 vector particles were generated in 293 cells, cultured in 150-mm-diameter Petri dishes, by co-transfecting each plate with 15 µg of each vector plasmid together with 45 µg of the packaging/helper plasmid, pDG, expressing AAV and adenovirus helper functions. Viral stocks were obtained by CsCl gradient centrifugation and rAAV titers were determined by measuring the copy number of viral genomes in pooled, dialyzed gradient fractions using real-time PCR.

The vectors used in this study express the various cDNAs under investigation ($VEGF_{165}$, $VEGF_{121}$, PDGF-B) or the LacZ gene under the control of the constitutive CMV immediate early promoter.

All the viral stocks used in this study had a titer $\geq 1x10^{12}$ viral genome particles/ml. The proper expression of all transgenes was tested *in vitro* by western blotting using specific antibodies after transduction of 293T cells and *in vivo* by real-time PCR, quantification of the transgene mRNAs in transduced tissues.

4.2 Animal treatment

Animal care and treatment were conducted in conformity with institutional guidelines in compliance with national and international laws and policies (EEC Council Directive 86/ 609, OJL 358, December 12th 1987). CD1 mice and Wistar rats were obtained from Harlan and maintained under controlled environmental conditions.

Animals were injected in the tibialis anterior (40 µl of vector preparation for mice, 120 µl for rats) or heart (20 µl of vector preparation for mice, 40 µl for rats). For PET imaging, in which a larger area was transduced, 400 µl of vector preparation were injected, as described below. In the TRAM flap model 160 µl of vector preparation were injected, 4 injections were performed in the region where each perforator artery arises from the rectus sheet. In the AV-loop model, animals were injected in the pectineus muscular flap (30 µl of vector preparation).

4.3 Cell culture

CD11b+ cells were isolated from total bone marrow (extracted by tibiae and femurs of CD1 mice) using CD11b magnetic cell separation system beads (Miltenyi Biotec, Germany), and cultured in RPMI 1640 supplemented with 10% high quality fetal bovine serum (GIBCO).

Coronary artery smooth muscle cells were purchased from Clonetics and cultured in their own medium, also provided by the manufacturer.

4.4 Migration assay

Migration assays were performed with the use of 8-μm (for SMCs) pore size transwell permeable supports with a polycarbonate membrane (Costar, Corning Incorporated). 1 x 10^5 cells were seeded in serum-free medium in the upper chamber, while chemoattractants were placed in the lower chamber. After 16 hours incubation, migrated cells on the lower surface of the membrane were fixed in methanol and stained with Giemsa. Each assay was carried out in triplicate, by counting 8 fields per membrane at 400x magnification. Results are expressed as the mean number of migrated cells ± standard deviation.

4.5 Proliferation assay

SMC proliferation was estimated by the MMT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] colorimetric assay. CD11b+ cells were cultured and conditioned media were added to SMCs, plated in a 96 multiwell plate. At the end of the incubation time, culture media were removed and 100 μl of MTT (5 mg/ml) (Roche Molecular Biochemicals) was added to each well for 4 hours. The precipitated MTT formazan was dissolved in 100 μl SDS 10%, 10 μM HCl, and the absorbance read the day after at 570 nm with an ELISA Reader (BioRad). Cell number was determined from absorbance values, by using a pre-established calibration curve.

4.6 Histology

For histological evaluation, tissue samples were either snap frozen or fixed in 4% formaldehyde and embedded in paraffin. In the AV-loop model, tissue chamber was exposed and the vessels were ligated proximally and distally before chamber removal. The sample was then fixed in 4% formaldehyde and embedded in paraffin.

Histological sections were either stained with Hematoxylin and Eosin and Azan trichromic staining.

The area of new tissue was measured by the use of ImageJ software and expressed as a percentage relative to the total section area.

Immunohistochemistry was performed on paraffin-embedded sections, with the use of the following antibodies: mouse monoclonal against α-SMA (clone 1A4, Sigma) and goat polyclonal against CD31 (Santa-Cruz Biotechnology). Protocols were according to the Vectastain Elite ABC kit (universal or goat) from Vector Laboratories. After treatment, slides were rinsed in PBS and signals were developed using 3,3'-diaminobenzidine as a substrate for the peroxidase chromogenic reaction (Lab Vision Corporation).

Immunofluorescence, was performed either on paraffin-embedded sections or on frozen sections (5-μm thick) which were fixed in cold acetone (-20°C) and blocked for 30 min with 5% which goat or 5% horse serum in PBS, depending on the secondary antibody. The following primary antibodies were used: anti CD11b (clone M1/70), anti CD31 (clone 390) and CD45 (all from Pharmingen BD), anti NP-1 (R&D Systems), Cy3-coniugated anti-α-SMA (clone 1A4) (Sigma).

The secondary antibodies used were Alexa Fluor 488– and AlexaFluor 555–conjugated goat antirat, Alexa Fluor 488–conjugated donkey antirabbit, and Alexa Fluor 594–conjugated donkey antimouse (all from Molecular Probes). Nuclei were counterstained with DAPI. Relative areas occupied by differently stained cells were quantified by the use of ImageJ software.

For the detection of the β-galactosidase activity, 2-4 mm fresh tissue biopsies were fixed in 4% paraformaldehyde (or 0.5 glutheraldeyde in case of carotid arteries) and then stained in X-gal solution (1mg/ml X-gal, 5mM K3Fe(CN)6, 5mM $K_4Fe(CN)_6$, 2mM $MgCl_2$). Samples were then rinsed in water and counterstained with nuclear fast red.

4.7 Real-time PCR

Total RNA from purified CD11b+ cells or AAV-injected tibialis anterior muscle samples was extracted using TRIzol reagent (Invitrogen) according to manufacturer instructions, and reverse transcribed using hexameric random primers. The cDNA was then used as a template for real-time PCR amplification to detect the expression levels of the three murine VEGF receptors (VEGFR-1, VEGFR-2 and NP-1) as well as of TGF-β, PDGF-B, CXCR4, Ang-1, ephrin-B2, SDF-1 and hVEGF; the housekeeping genes GAPDH and r18s were also amplified and used to normalize the results. All the amplifications were performed on a 7000 ABI Prism Instrument (Applied Biosystems), using pre-developed assays (Applied Biosystems).

4.8 In vivo perfusion with fluorescent microspheres

To quantify the vascular volume of treated and untreated muscles, 300 µl of orange fluorescent microspheres (FluoSpheres, Molecular Probe) were injected i.v. into living mice. After one minute (to ensure a proper distribution of the microspheres throughout the body), animals were sacrified and both tibialis anterior muscles immediately harvested. Recovery of microspheres and extraction of fluorescent dye was performed according to manufacturer's instruction. Briefly, weighted muscles were digested in 2M ethanolic KOH, 0.5% Tween 80 (v/v) (Sigma) at 60˚C with periodic shaking. Homogenates were centrifuged in a swinging rotor and the pellet resuspended in 0.25% Tween 80. After an additional centrifugation and washing, microspheres were dissolved in 2-etoxyethyl acetate (Sigma). Debris were removed by centrifugation and fluorescence in the supernatant was measured with the VersaFluor Fluorometer System (BioRad). Values were normalized and expressed as ratios between fluorescence of treated and untreated controlateral muscle for each animal (n=6 per group).

To obtain a 3-D reconstruction of the vascular network, mice were subjected to in vivo perfusion with a 1:6 dilution of the same fluorescent microsphere solution (FluoSpheres, Molecular Probe). Afetr anesthesia, the chest was opened and the vasculature perfused with PBS for 3 minutes, followed by 20 ml of FluoSpheres (1:6 dilution of the stock), via a blunt 13 G needle inserted through the left ventricle into the ascending aorta. The treated muscles were removed and immediately frozen for cryosectioning. Thick (50-100 µm) sections were analyzed by confocal microscopy to obtain a Z-stack for the 3-D reconstruction of the vascular network.

4.9 Vessel permeability

Analysis of vessel permeability was performed by a modification of the Miles' assay (Miles and Miles, 1952) to rodent muscles. Mice or rats (n=6 per group) were injected in the jugular vein with 250 µl or 2.5 ml of 0.5% Evans blue (Sigma), respectively, and sacrificed after 30 minutes. The tibialis anterior muscles were removed and weighted. The dye was extracted by incubation in 2% formamide at 55˚C and quantified spectrophotometrically at 610 nm. Absorbance values were converted, according to a standard curve, in Evan's blue content and expressed as a ratio between treated and control muscles from the same animal.

4.10 PET and SPECT Imaging

The PET and SPECT experiments on rats were performed at Institute of Clinical Physiology, CNR, Pisa (Italy), in collaboration with G. Sambuceti.

In the rat model, after mild general anesthesia, the animals were injected with 400 µl of AAV vector (containing $4x10^{11}$ particles of each vector) in their right leg and with a similar volume of PBS in their left leg. Different muscles were transduced, defining 8 injection sites per leg: 2x50 µl into the tibialis anterior, 3x50 µl into the gastrocnemius, 3x50 µl into the rectus femoris. All the injections were performed transcutaneously, using a syringe with a 29 G needle. The animals were divided into 4 experimental groups according to the treatment they received: AAV-LacZ, AAV-VEGF, AAV-Ang1, or AAV-VEGF + AAV-Ang1 (n=21 for each group).

A clinical PET scanner was used to detect an index of muscle blood flow, by injecting the rats i.v. with 3.7 Mbq of ^{13}N-ammonia (n=21 per group). All the animals were tested both under resting conditions and after 15 minutes of muscle activity induced by electrical stimulation at 180 spikes/min.

For the quantitative evaluation of intravascular volume and permeability of the differently treated muscles, ^{99m}Tc-diethylenetriaminepenta acetic acid (DTPA) was systemically injected into the jugular vein, and data elaborated according to a published protocol (Peters et al., 1987).

4.11 Assessment of artero-venous shunting

To evaluate the presence and quantify the entity of artero-venous shunts in the differently treated animals, a bolus of ^{99m}Tc macroaggregate, which do not normally transverse the capillary bed, was injected into the abdominal aorta. The detection of radioactive counts in the lungs provided an accurate quantification of the fraction of the abdominal aortic blood flow shunted to the venous circulation.

4.12 TRAM flap model

A total of 60 adult male Wistar rats weighting 250-300 g were employed for this study and housed under controlled environmental conditions. After general anesthesia with Avertin 2% (10ml/Kg), abdominal hair was removed and skin washed with sterile water. A flap measuring 5 x 8 cm was outlined on the skin, extending distally from the xiphoid process and bilaterally from the midline.

The entire left part of the flap was raised on a plane between the panniculus carnosus and the abdominal fascia, but on the right side the medial portion of the flap was left attached to the anterior rectus sheet. In this way, the rectus abdominis represents the muscular component of the flap, providing the blood supply to the cutaneous component through the perforator arteries. In this model 160 µl of either AAV-$VEGF_{165}$ or placebo were injected in the same region where each perforator artery arises from the rectus abdominis (4 injection, 40 µl each one).

Each of the 6 experimental groups described in Figure 5.16 was initially composed of 4 rats. In control groups, AAV-LacZ or saline was administered (in every control group, 2 animals received AAV-LacZ and the other 2 received saline). Twelve additional rats were subsequently employed to confirm the results obtained in the TRAM model by injecting the animals 14 days prior to surgery.

The treated and control flaps were evaluated both grossly and histologically at day 7 following surgery. Digital images of the flaps were taken by a Nikon E995 camera and analyzed by UTHSCSA Image Tool software. The area of the necrotic zone was measured and expressed as a percentage relative to the total flap surface area. Immediately after animal sacrifice, the flaps were harvested following the scars, and tissue biopsies were fixed overnight in 10% buffered formalin for histological evaluation.

4.13 Construction of the AV -loop and generation of the collagen tissue chamber

A microvascular loop was generated between the femoral artery and vein using a vein graft harvested from the controlateral leg. End-to-side anastomoses were performed with 11/0 nylon sutures both on recipient artery and vein. After perfusion, the AV-loop was embedded into a sandwich composed of 2.4x1.4 cm portion of a commercially available bi-layered dermal substitute template (inner layer: matrix of bovine collagen-GAG; outer layer: silicon; INTEGRA^{TM}). The template was secured on three sides by multiple 9/0 silk sutures as a sandwich including the loop.

4.14 Laser Doppler perfusion imaging

In the AV-loop model, the blood flow was assessed by laser doppler analysis (Laser Doppler Perfusion Imager, Perimed, Sweden) either at day 0, before and after the application of the scaffold, and at day 30. The laser Doppler source was mounted onto a movable rack exactly 20 cm above the rat limbs, the animals being under anaesthesia and

restrained on the operation table. The laser beam (780 nm), reflected from moving RBCs in nutritional capillaries, arterioles and venules, was detected and processed to provide a computerized, color-coded image. Using image analysis software (Laser Doppler Perfusion Imager, Perimed), mean flux values representing perfusion were calculated.

4.15 Statistical analysis

One-way ANOVA and Benferroni/Dunn's post-hoc test was used to compare multiple groups. Pair-wise comparison between groups was performed using the Student's t test. $P<0.05$ was considered statistically significant.

5. RESULTS

5.1 Different angiogenic properties of the two VEGF isoforms $VEGF_{121}$ and $VEGF_{165}$

5.1.1 Efficient transduction of muscle tissues by AAV vectors

The tropism of AAV vectors for muscle tissues was assessed by injecting an AAV-LacZ (serotype 2) in different muscle compartments of mice and rats, and evaluating transgene expression at different time points after transduction. In particular 40 µl of a viral preparation (viral titer ~ 10^{12} viral genome copies (vgc)/ml) were injected into the tibialis anterior muscle of mice, while 3 injections of 40 µl were performed in the same muscle of rats. To transduce the heart, a volume of 20 or 40 µl was injected intramyocardially in the mouse and rat, respectively. All the transduced tissues were analyzed for β-galactosidase expression by X-gal staining at 15 days, 1 month and 3 months after treatment. Transgene expression was found to be already high at two weeks after vector delivery and to persist, almost invariably, afterwards. A representative picture of a section of a rat skeletal muscle and a rat myocardium 1 month after AAV-LacZ injection is shown in Figure 5.1.

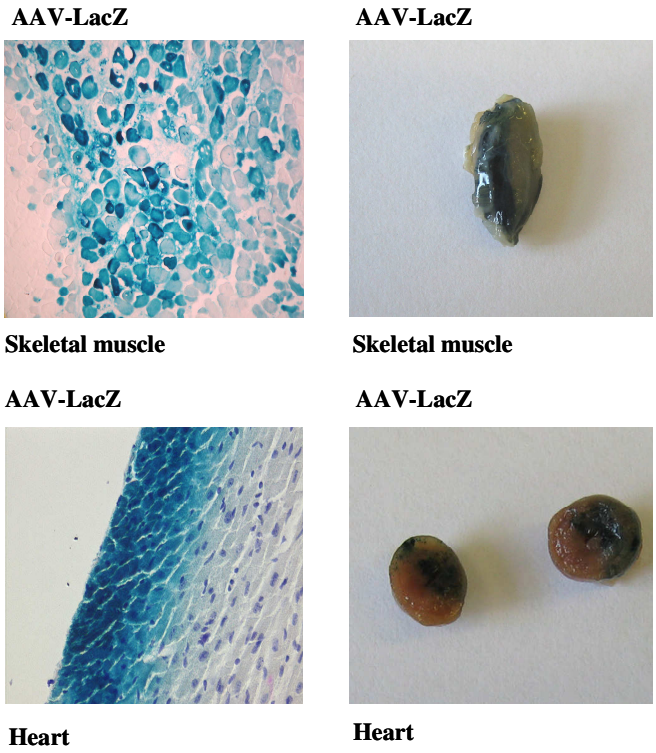


Figure 5.1 AAV-LacZ transduction of skeletal muscle and myocardium in rodents. Comparable amounts of AAV-LacZ were delivered to the different tissues of mice and rats to assess trangene expression by X-Gal staining after 1 month

5.1.2 VEGF $_{165}$ induces a potent angiogenic effect in vivo

The $VEGF_{165}$ isoform is one of the most studied growth factor for therapeutic angiogenesis and we have previously demonstrated the powerful angiogenetic response observed by AAV-$VEGF_{165}$ transduction in skeletal muscle in rats (Arsic et al., 2003).

To confirm the biological effect of $VEGF_{165}$ expression an AAV-$VEGF_{165}$ vector was injected in the right tibialis anterior muscle of normoperfused CD1 mice. Each animal received an equal amount of AAV-LacZ or PBS in the same muscle of the controlateral leg, as an internal control (Figure 5.2)

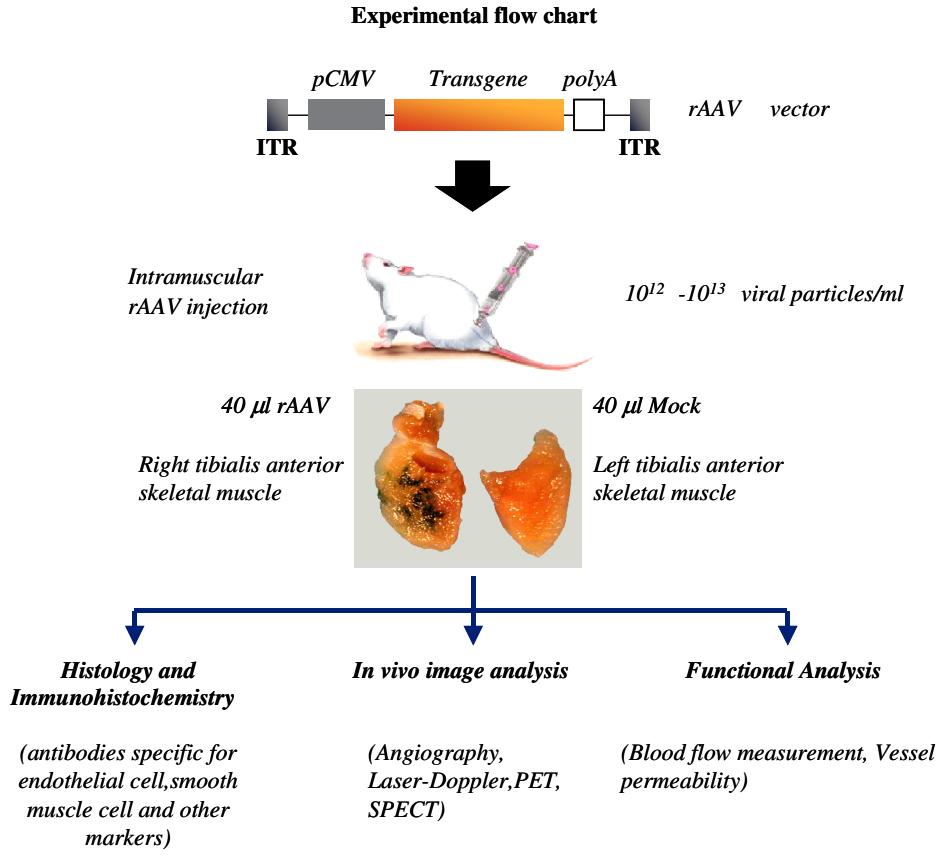


Figure 5.2 Experimental flow chart. This scheme represents the general experimental flowchart of the studies reported in this thesis. Animal were injected with rAAV in their right leg while received the same volume of PBS in their left leg, as an internal control. They underwent *in vivo* image analysis and functional analysis, while after sacrifice they were subjected to histological analysis.

Histological sections were examined 1 month after transduction. A striking angiogenic effect was detected, consisting of an impressive number of new blood vessels as compared to control muscle. In order to identify the phenotype of the newly formed vascular structures, we detected the presence of endothelial cells by an immunofluorescence

staining against the endothelial marker CD31 (PECAM-1). The number of CD31-positive cells was greatly increased in muscles transduced with AAV-VEGF$_{165}$. The quantification of the area occupied by the CD31 positive cells revealed a huge increase compared to control muscle (Figura 5.3).

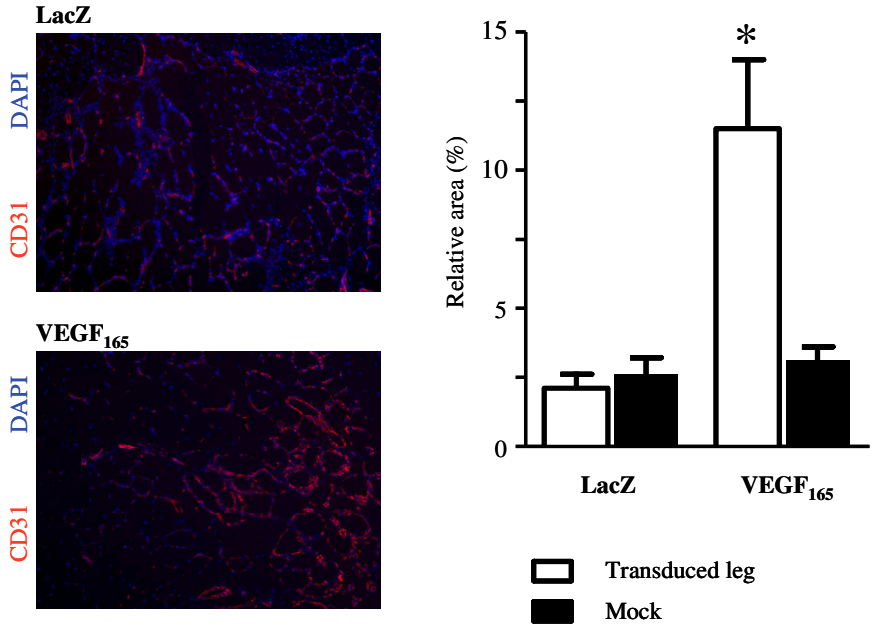


Figure 5.3 The long term expression of VEGF$_{165}$ into the skeletal muscle induced the formation of a great number of capillaries. The prolonged expression of VEGF$_{165}$ into the normoperfused muscle resulted in endothelial cell activation and formation of new capillaries, assessed by CD31 staining. Histograms on the right show the quantification of the relative area occupied by cellular endothelial cells (CD31). *Statistical significance over control P<0.01

To recognize the presence of pericytes, indicative of vessel maturation, we performed an immunostaining against the pericyte marker α-SMA. We detected a striking increase in the number of α-SMA positive vessels in the muscles transduced with AAV-VEGF$_{165}$. Quantification of this effect revealed a ~ 6 fold increase in the number of α-SMA positive vessels as compared to control (Figure 5.4). The α-SMA positive vessels essentially are small arterioles, having a diameter between 20 and 120 μm.

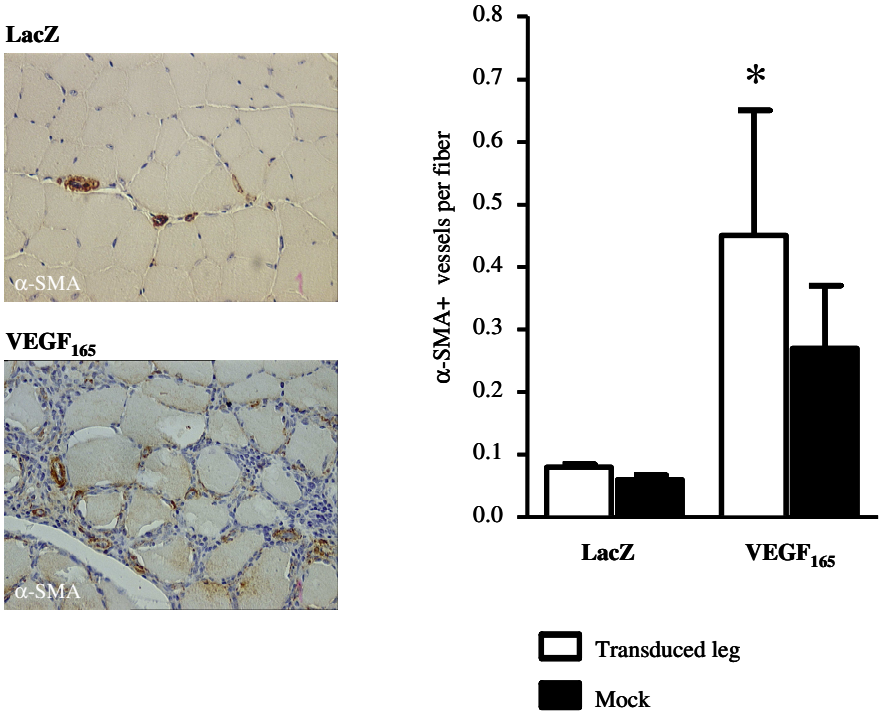


Figure 5.4 The long term expression of $VEGF_{165}$ into the skeletal muscle induced the formation of a great numebr of arteries. The prolonged expression of $VEGF_{165}$ into the normoperfused muscle resulted in 2-fold increase in vessels positive for α-smooth muscle actin having a diameter of 20-120 μm. This resulted in an overall 6-fold increase in the number of arteriolae in the treated muscle compared to control. *Statistical significance over control (P<0.01).

The strong angiogenic effect observed upon $VEGF_{165}$ expression in normoperfused animals confirmed that this VEGF isoform is able to induce both capillary sprouting and maturation of the newly formed vessels, as highlighted by the presence of several α-SMA positive vessels.

5.1.3 $VEGF_{121}$ is angiogenic but not arteriogenic in vivo

The current literature suggests that other VEGF isoforms might be able to induce angiogenesis. We wanted to assess whether $VEGF_{121}$ shares the same angiogenic properties of the longer $VEGF_{165}$. Therefore, an AAV vector expressing $VEGF_{121}$ was injected into the right tibialis anterior muscle of normoperfused CD1 mice. Again, mice received an equal amount of AAV-LacZ or PBS in the controlateral leg.

In order to perform a comparison among both VEGF isoforms, we sacrificed the animals 1 month after injection, as we did for mice injected with $VEGF_{165,}$ and simultaneously quantified the effects of the two VEGF isoforms in terms of both capillary and arterial

vessel formation. A CD31/α-SMA double-immunofluorescence staining was performed on muscle sections from mice injected with either AAV-VEGF$_{165}$ or AAV-VEGF$_{121}$ and the relative area occupied by nuclei (DAPI staining), endothelial cells (CD31) and arterial smooth muscle cells (α-SMA) was quantified by means of the ImageJ software. The results of the quantification are reported in Figure 5.5 together with representative images. As expected, the shorter VEGF isoform induced a massive capillary sprouting comparable with the effect observed in muscle injected with AAV-VEGF$_{165}$. Quite surprisingly, however, it was not able to induce the formation of small arterial vessels, as very few α-SMA positive vessels were detected in the AAV-VEGF$_{121}$ injected muscles.

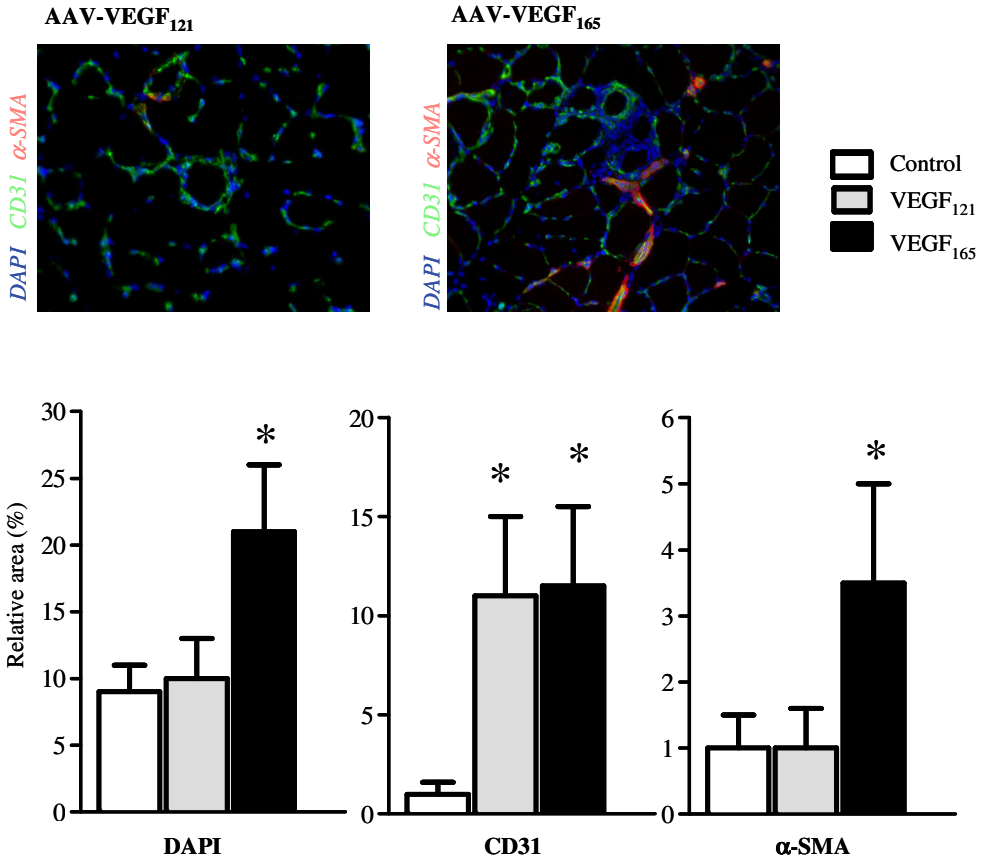


Figure 5. 5 Comparison between VEGF$_{121}$- and VEGF$_{165}$-induced angiogenesis. While both VEGF isoforms induce endothelial cell proliferation to a similar extent (CD31 staining in green), onlyVEGF$_{165}$ expression results in a significant increase in the total number of cells (DAPI nuclear staining in blue) paralleled by the formation of new arteries (α-SMA staining in red). Histograms on the bottom show the quantification of the relative area occupied by cellular nuclei (DAPI), endothelial cells (CD31), or SMCs (α-SMA) in 20 independent sections for each treatment. *Statistical significance over control $P < 0.05$

To verify whether the new vessels observed by histological examination were functional and connected to the circulation, we quantified the blood volume of the differentially treated muscles. To this purpose, animals were injected with either AAV-$VEGF_{121}$ or AAV-$VEGF_{165}$ and, after 1 month, they were in vivo perfused with a bolus of fluorescent microspheres. By extracting and measuring fluorescent microspheres from the muscles we could obtain a quantitative measurement of the perfusion of the treated muscle relative to the controlateral untreated one, as well as a 3D-reconstruction of the vascular network.

The results of the quantification are presented in Figure 5.6, showing that the treatment with the long isoform $VEGF_{165}$ resulted in a marked increase in the blood volume (~ 6-fold), while the treatment with $VEGF_{121}$ resulted in a milder increase in the blood volume (~ 4-fold), in agreement with the observation that the vessels induced by the short VEGF isoform are mainly capillaries. Moreover we observed that the vascular network induced by $VEGF_{165}$ was formed by vessels that appeared abnormally large and poorly organized (Figure 5.6).

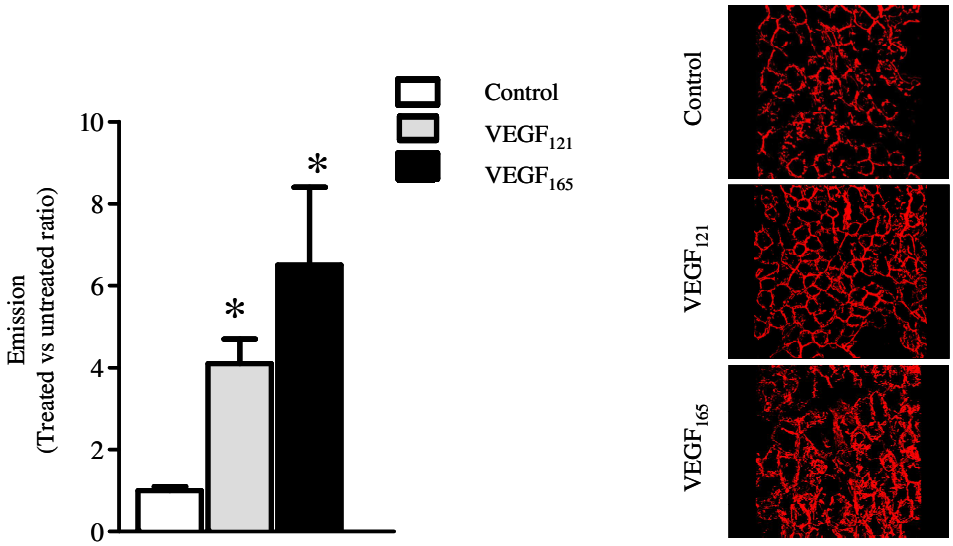


Figure 5.6 ***In vivo*** **perfusion with fluorescent microspheres.** Fluorescent microspheres injected i.v. into the animal were recovered from both the right (treated) and the left (untreated) tibialis anterior muscle, and quantified by fluorimetric analysis. Shown are means and standard deviation of the ratios between the two measurements, together with a 3D-reconstruction of the vascular network in each muscle. *Statistical significance over control $P < 0.05$

The presence of an α-SMA layer is a hallmark of maturation and the differences observed between the two VEGF isoforms suggested that $VEGF_{121}$ might drive the formation of an immature vasculature. As it is widely accepted that vascular leakiness is a peculiar feature

of newly formed, still immature vessels, we injected a new set of animals (n=5 per group) with AAV-$VEGF_{121}$ and AAV-$VEGF_{165}$ to assess vascular permeability. To this purpose, we performed a modified Miles' assay, by systemically injecting a bolus of Evan's blue and assessing dye extravasation into muscle tissue. The results of the Miles' assay are presented in Figure 5.7. As expected, AAV-$VEGF_{121}$ exerted a potent permeabilizing activity (~7 fold increase in vascular permeability relative to the mock-treated controlateral muscle). In contrast, AAV-$VEGF_{165}$ displayed a lower permeabilizing effect although $VEGF_{165}$ induced vessels were still permeable (~ 6-fold increase in vascular permeability relative to the mock-treated controlateral muscle).

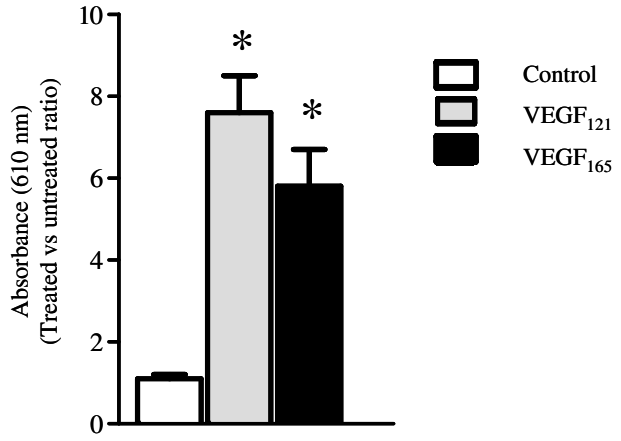


Figure 5.7 Miles' assay for vascular permeability assessment. At 1 month after treatment a bolus of Evan's blue was injected into the jugular vein and both tibialis anterior muscles were harvested 30 minutes later. The histogram reports spectrophotometric quantification of the dye infiltrated in each muscle, expressed as a ratio between treated and untreated legs. *Statistical significance over control $P < 0.05$

This result confirmed that $VEGF_{165}$ induces the formation of a vascular network which is relatively more mature as compared to the one formed in response to $VEGF_{121}$, also consistent with the results of the tissue perfusion assay based on the injection of fluorescent microspheres.

How could this difference be explained? Both isoforms stimulated the proliferation of endothelial cells at a similar extent; however, a specific increase in the number of α-SMA-positive arterial vessels was specifically evident in muscles expressing $VEGF_{165}$. Another major difference, appreciable at histological examination, was that $VEGF_{165}$ but not $VEGF_{121}$, induce a massive infiltration of muscle tissues by mononuclear cells, as assessed

by DAPI nuclear staining (see Figure 5.5). The different angiogenic phenotype induced by $VEGF_{121}$ and $VEGF_{165}$ suggested a functional relationship between the recruitment of these cells and the formation of arterial vessels.

In addition, the notion that the two isoforms mainly differ in term of NP-1-binding ability, suggested that NP-1 might be involved in the recruitment of these cells as well as in arteriogenesis.

Collectively, these observations suggested us the hypothesis that the cells recruited by $VEGF_{165}$ might be recruited through the NP-1 receptor and have role in the process of vessel maturation, perhaps by a paracrine effect. To better characterize the infiltrating cells, we performed an immunofluorsecence staining for the myeloid marker CD11b, and observed that CD11b+ cells were detected only in the AAV-$VEGF_{165}$ injected muscle, in close association with α-SMA positive vessels (Figure 5.8).

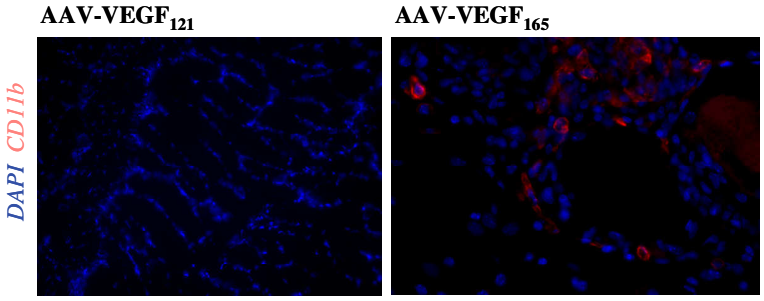


Figure 5.8 $VEGF_{165}$ recruits CD11b+ cells from the bone marrow. An immunofluorescence staining for the myeloid marker CD11b showed that $VEGF_{165}$ is able to recruite CD11b+ cells from the bone marrow, whereas $VEGF_{121}$ did not show any chemotactic activity toward bone marrow cells.

Beside CD11b, these cells also expressed abundant levels of the pan-leukocytic marker CD45, as shown in Figure 5.9 by immunofluorescence staining of sections from muscles injected with AAV-VEGF$_{165}$. Finally, we further confirmed by double immunostaining that the vast majority of the infiltrating CD11b+ cells also expressed NP-1 (Figure 5.9).

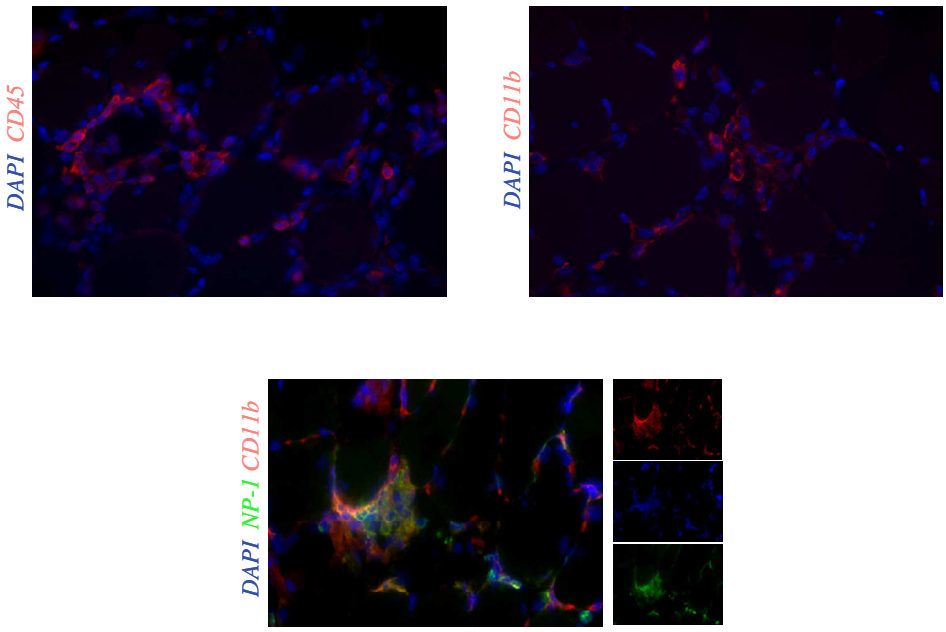


Figure 5.9 Characterization of the cellular infiltrates recruited by VEGF$_{165}$. As already described, VEGF$_{165}$ is able to recruite CD11b+ cells (here shown in the right upper panel), these cells also scored positive for the panleucocytic marker CD45 (upper panel left). A double immunofluorescence staining for CD11b and NP-1 confirmed that the vast majority of the CD11b+ cells express also NP-1(lower panel).

In order to confirm these results, we purified CD11b+ cells from total bone marrow by means of magnetic beads. The CD11b+ cells were subsequently used for RNA extraction, followed by retrotranscription and real-time PCR. As reported in Figure 5.10, the isolated CD11b+ expressed NP-1, VEGFR-1 and VEGFR-2, all receptors needed to respond to VEGF$_{165}$.

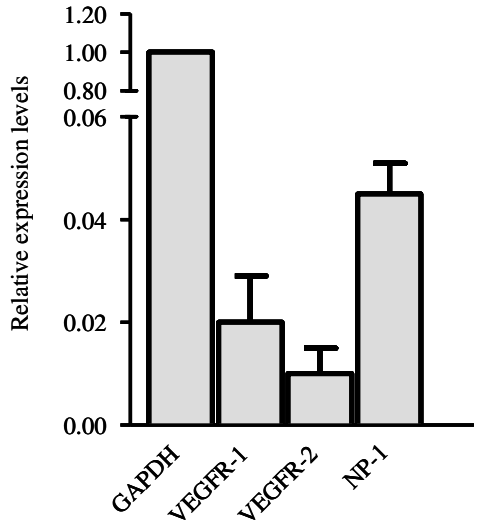


Figure 5.10 Realtime quantification of VEGFR1, VEGFR2 and NP-1 in CD11b+ cells. CD11b+ cells were sorted from bone marrow to detect the expression levels of the free receptors VEGFR1, VEGFR2 and NP-1. As expected, the CD11b+ cells express NP-1 as well as the two VEGF receptors. Alla data are normalized taking the GADPH housekeeping gene as a reference.

5.1.4 Bone marrow CD11b+ cells stimulate smooth muscle cell proliferation and migration

The intriguing observation that $VEGF_{121}$, is neither able to recruit BM CD11b+ cells nor it forms arteries *in vivo*, suggested us that these cells might play an important role during the later phases of the angiogenic process, possibly by promoting smooth muscle cell recruitment and the subsequent formation of arterial vessels. As a first step to verify this hypothesis, we fractionated total BM according to CD11b expression with the use of magnetic beads, and both the positive and the negative fractions were tested for their ability to attract primary arterial smooth muscle cells (SMCs). As shown in Figure 5.11, the CD11b+ fraction exhibited a marked, dose-dependent chemotactic activity toward SMCs.

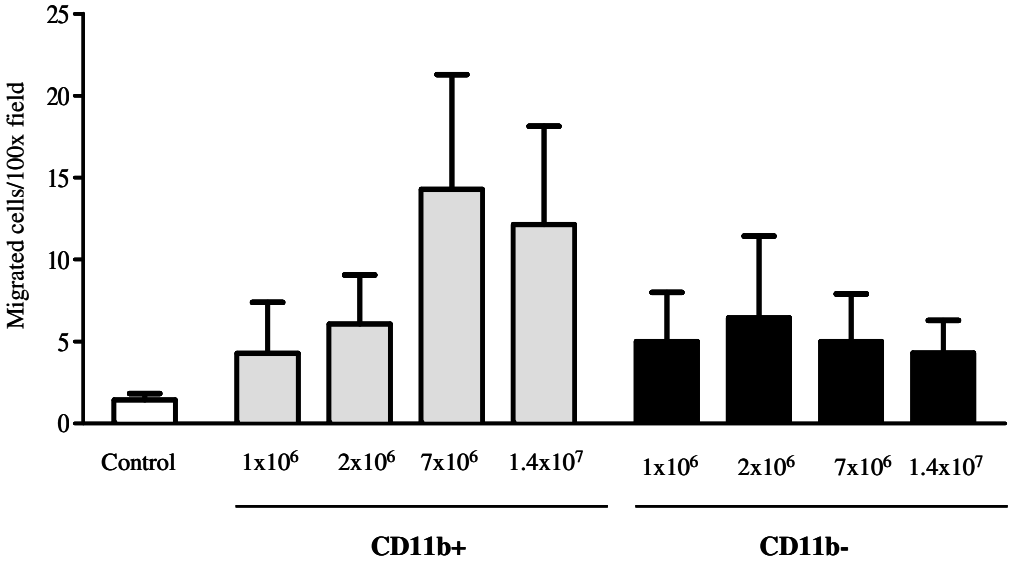


Figure 5.11 Smooth muscle cell recruitment by CD11b+ cells. After bone marrow fractionation according to CD11b expression, an increasing number of cells from the two fractions was used as chemoattractant for primaty smooth muscle cells. Only CD11b+ cells were found to be able to attract SMCs in a dose-dependent manner.

Next we investigated the ability of CD11b+ cells to influence SMC proliferation. We found that the CD11b+ cell-conditioned medium significantly stimulated the proliferation of SMCs (Figure 5.12). This result indicates that unstimulated CD11b+ cells secrete factors able to drive mitogenic signals in SMCs.

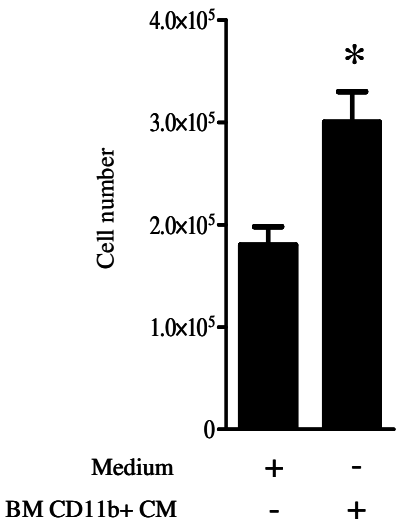


Figure 5.12 Bone marrow CD11b+ cells stimulate smooth muscle cell proliferation. SMCs were exposed to medium conditioned by CD11b+ cells, and cell proliferation was measured by MTT assay. SMCs responded to mitogens secreted by not-stimulated CD11b+ cells. Shown are means ± SD of 3 experiments. *Statistical significance over unstimulated cells ($P<0.05$).

Overall, these experiments indicate that BM CD11b+ cells secrete soluble factors able to trigger both the proliferation and the migration of SMCs, thus supporting a possible paracrine action of these cells in the recruitment of SMCs during neo-vascularization *in vivo*. We therefore wanted to obtain additional insights into the molecular mechanisms that mediate the recruitment of SMCs by myeloid CD11b+ cells. Animals injected with AAV-$VEGF_{121}$ or AAV-$VEGF_{165}$ were sacrificed at 1 month after vector injection (n=6 per group) to determine the expression levels of a panel of molecules previously shown to play a role in the process of new blood vessel formation and maturation. As shown in Figure 5.13, muscles injected with AAV-$VEGF_{165}$ exhibited a specific up-regulation of TGF-β, PDGF-B, and of the CXCR4 chemokine receptor as compared to muscles injected with AAV-$VEGF_{121}$. We then analyzed the gene expression levels of the same target genes in primary BM CD11b+ cells and interestingly we observed that most of these transcripts were also abundantly expressed in these primary cells (Figure 5.13, panel B). In contrast, no significant variations could be detected in the expression levels of Ang-1, Ephrin-B2 and SDF-1 (a CXCR4 ligand). The striking differences detected in the former group of genes cannot be attributed to different levels of expression of $VEGF_{165}$ versus $VEGF_{121}$, as shown by the specific quantification of the respective human mRNAs expressed from the transduced vectors (Figure 5.13, panel C).

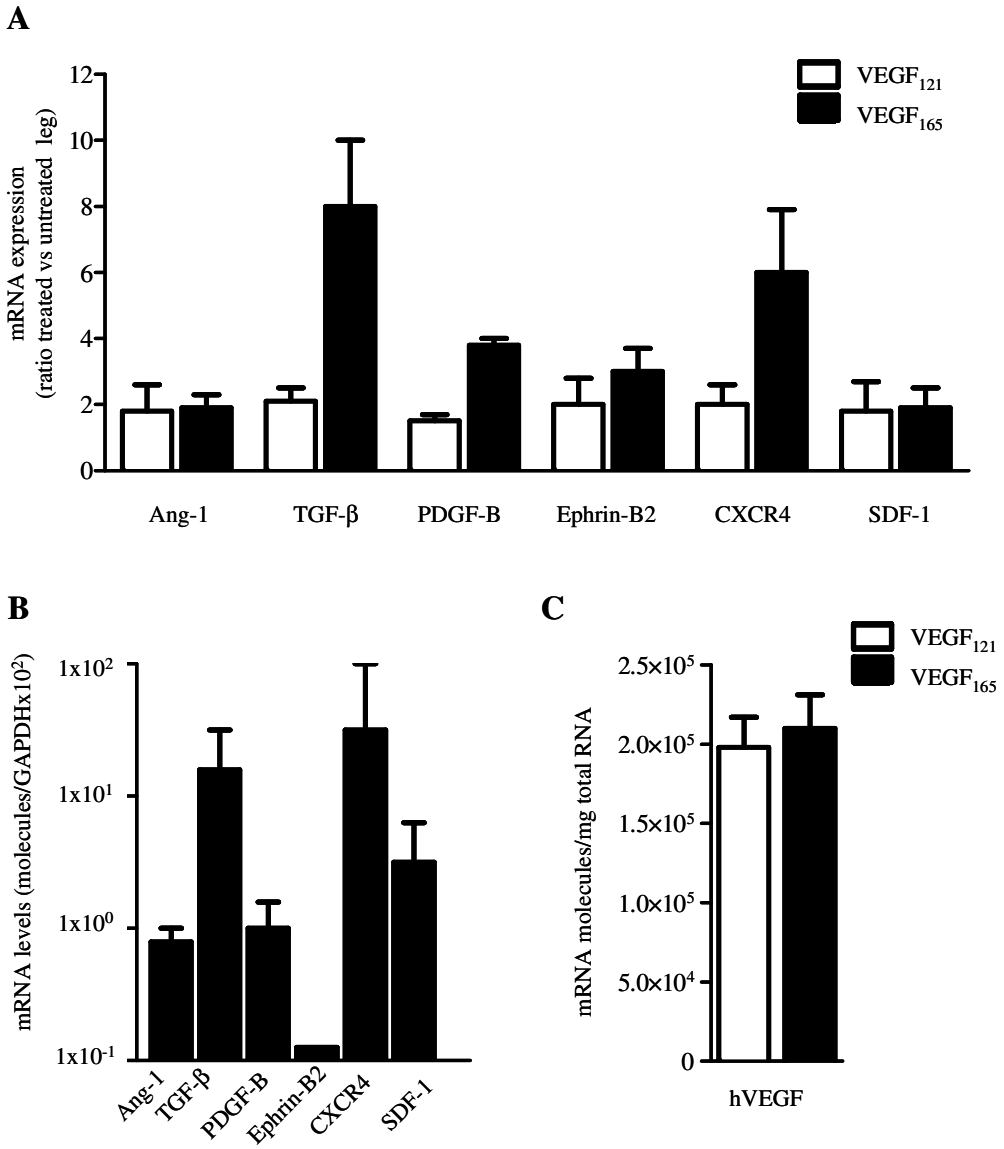


Figure 5.13 Bone marrow derived CD11b+ cells express factors involved in vessel maturation. (A) The expression levels of a panel of candidate genes were determined by real-time PCR in muscles injected with AAV-$VEGF_{121}$ or AAV-$VEGF_{165}$. Data are presented as a ratio between the VEGF-expressing and the mock-injected contralateral muscles. (B) The expression levels of the same target genes were also analyzed in primary CD11b+ cells. (C) The expression levels of $VEGF_{121}$ and $VEGF_{165}$ were analyzed in the injecetd muscles 1 month after transduction using a human VEGF-specific assay. Similar expression levels were deteceted for both isoforms, indicating that both vectors were equally effective in vivo. Alla data are normalized taking the GADPH housekeeping gene as a reference.

5.1.5 PDGF-B cooperates with $VEGF_{121}$ to promote arterial formation in vivo

Our model predicts that BM-derived CD11b+ cells promote the activation of SMCs in a paracrine fashion. Indeed, expression of cytokines that attract SMCs, including PDFG-B and TFG-β, was detected in BM CD11b+ cells. In particular, several studies both during development and in the adult life have disclosed an essential role for PDGF-B in the development of multilayered vessels through the interaction of this factor with the PDGFR-β present in the progenitors of vascular smooth muscle cells/pericytes (Bjarnegard et al., 2004; Lindahl et al., 1997). On the basis of these considerations and on the observed effect of $VEGF_{121}$ on endothelial cells, we hypothesized that the simultaneous expression of $VEGF_{121}$ and PDGF-B should result in the formation of new arteries. To test this hypothesis, we developed an AAV-vector expressing PDGF-B (AAV-PDGF) and injected mice with this vector either alone or together with AAV-$VEGF_{121}$ (n=6 per group). We observed that AAV-PDGF alone had no significant effect on the number of either endothelial cells or α-SMA-positive cells, nor it increased the total number of cells in the injected areas. In contrast, the simultaneous injection of AAV-PDFG and AAV-$VEGF_{121}$ was clearly arteriogenic, in the absence of cellular infiltration.

Figure 5.14 summarizes these differences, by showing a representative CD31/α-SMA double-immunofluorescence on a muscle section for each of the two isoforms, as well as the quantification of the relative area occupied by cellular nuclei (DAPI staining), endothelial cells (CD31) and arterial smooth muscle cells (α-SMA) at 1 month after transduction.

Overall, these results confirmed that the expression of PDGF-B might partially substitute for the recruited CD11b+ cells and provides a proof-of-principle of the validity of our model.

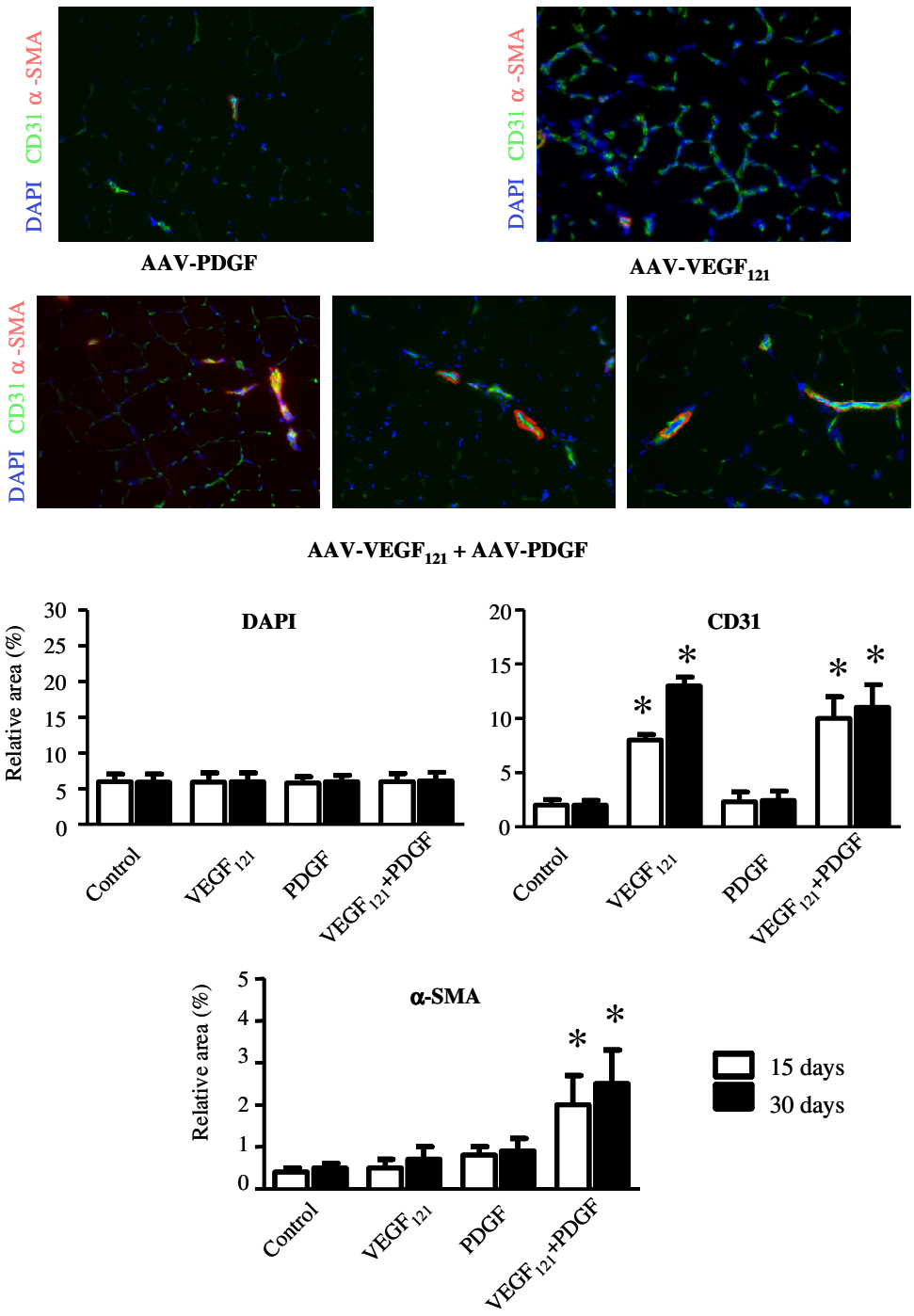


Figure 5.14 PDGF-B cooperates with $VEGF_{121}$ to promote arterial formation *in vivo*. Animal were injected with AAV-PDGF alone or in combination with $AAVVEGF_{121}$. While PDGF-B cDNA delivery alone had no remarkable effect on either capillary (green) or artery (red) formation, its co-expression with $VEGF_{121}$ determined the formation of a remarkable number of new α-SMA-positive arterial vessels, in the absence of cellular infiltration (upper panel). Quantification of the relative areas occupied by cellular nuclei (DAPI), endothelial cells (CD31) or smooth muscle cells (α-SMA) are presented in the lower panel. Data are presented as means ± SD. * Statistical significance over control ($P<0.05$).

5.2 Animal models of ischemia

The process of neoangiogenesis would be essential in ischemic conditions, such as lower limb ischemia or myocardial infarction, as well as in reconstructive surgery, where neovessel formation might improve tissue grafting and viability. The results we obtained in the normoperfused mice injected with AAV-$VEGF_{165}$ suggested that, in comparison with $VEGF_{121}$, the longer isoform is able to induce the formation of α-SMA positive vessels which turned out to be less leaky and to increase tissue perfusion. We therefore decided to test whether the newly formed vasculature is functional and might improve tissue survival and regeneration in ischemic animal models of reconstructive surgery and tissue engineering.

5.2.1 The musculocutaneous flap model

$VEGF_{165}$ expression reduces TRAM flap necrosis

Considering the strong angiogenic effect exerted over time by the AAV-$VEGF_{165}$ vector, we wanted to assess its therapeutic potential to improve skin flap survival.

First, we developed a transverse rectus abdominis musculo-cutaneous (TRAM) flap model, which involves the dermis, the epidermis and the muscular layer of the rat (Figure 5.15).

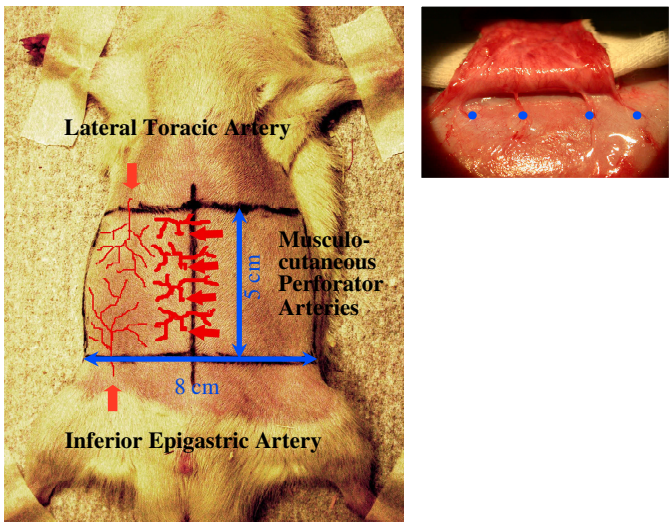


Figura 5.15 Schematic representation of themusculocutaneous TRAP flaps and its vascular components, with the indication of the vector injection sites. The surgical models of skin flap used in this study is based on a rectangular skin paddle measuring 5 x 8 cm, drawn on the abdomen of the animals. The predictable vascular system of the flap is composed of the lateral thoracic arteries, the inferior epigastric arteries and the musculo-cutaneous perforator arteries arising from the rectus abdominis (usually four vessels on each side). The TRAM flap was raised on a plane between the panniculus carnosus and the abdominal fascia, with the rectus abdominis as the only source of blood supply through the perforator arteries. In this model, the vector was administered by intramuscular injection in the region where each perforator artery arises from the rectus sheet (inset in the upper right part).

In order to assess the optimal timing for AAV vector delivery, we performed a first set of experiments in adult male Wistar rats (weight ~ 300gr). After TRAM flap elevation, AAV-$VEGF_{165}$ or AAV-LacZ was injected intramuscularly in the same region where each perforator artery arises from the rectus abdominis.

According to the vector injected (either AAV-LacZ or AAV-$VEGF_{165}$) and to the time of vector administration (0, 7 or 14 days before surgery) animal were divided in 6 groups (n=4 per group).

All animals were sacrified 7 days after surgery to assess flap necrosis. The overall results of this set of experiments are reported in Figure 5.16. The flaps treated with AAV-LacZ predictably underwent necrosis in their distal portion, similar to the untreated flaps, in which the extent of the necrotic area reached 24.4 ± 2.3% of the total flap surface (data not shown; calculated on a total of 20 animals). As shown in Figure 5.16, the treatment with AAV-$VEGF_{165}$ reduced the area of necrosis, the reduction of flap necrosis in the VEGF-treated animals vs LacZ controls was statistically not significant when the vector was injected during surgery, while it was 38.1% and 50.0% ($P<0.05$ in both cases) when AAV-$VEGF_{165}$ was injected at 7 or 14 days before surgery, respectively.

Taken together, these results indicated that the injection of AAV-$VEGF_{165}$ before surgery significantly increases flap survival.

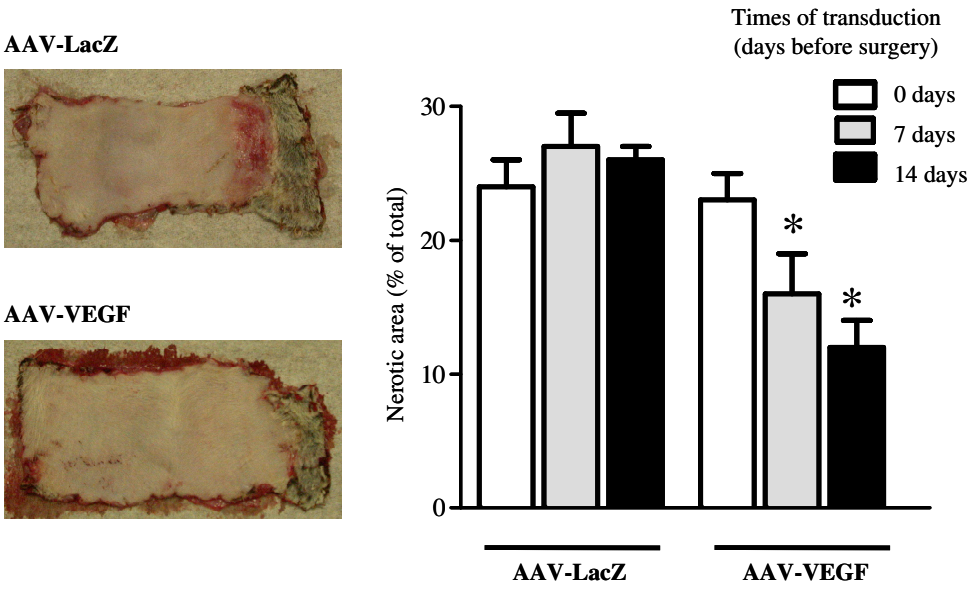


Figure 5.16 Effect of VEGF on skin flap survival at different times of administration. At postoperative day 7, the regions of survival and necrosis were clearly defined in all the flaps: the surviving skin appeared pink and tender, whereas the distal necrotic portion was black and rigid. The pictures show two selected TRAM flaps, treated with AAV-LacZ and AAV-VEGF, 14 days before flap elevation. The treatment with AAV-VEGF resulted in a significant improvement in tissue viability. The histograms represent the mean values ± SD of the necrotic area relative to the total flap area. * Statistical significance over control P<0.05

Increased flap survival is due to VEGF-induced angiogenesis

We then assessed whether the pro-survival effect of AAV-$VEGF_{165}$ might be due to an increased angiogenesis in the flap. Therefore, we performed histological and immunohistochemical evaluation of the injected tissues. A larger set of animals were treated with either AAV-LacZ or AAV-$VEGF_{165}$ (n=12 per group) 14 days before surgery and were sacrificed 7 days after surgery, followed by the measurement of the necrotic area. As already observed in the previous experiment, the expression of $VEGF_{165}$ induced a reduction in the flap necrotic area, the percentage of the necrotic area was reduced from 22.8±2.7% to 8.9±2.8% (>50% reduction; P<0.01) (Figure 5.17).

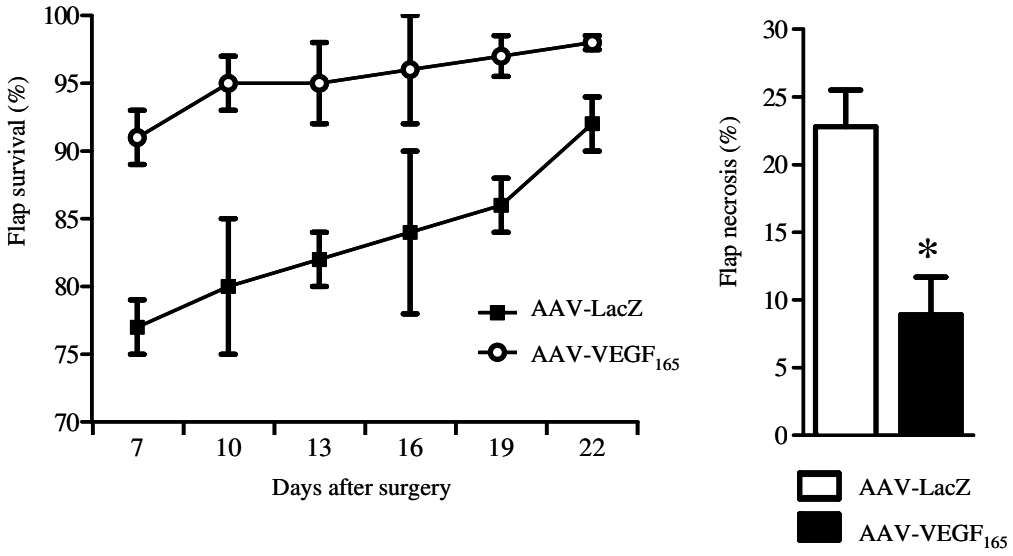


Figure 5.17 Pretreatment with AAV-VEGF significantly improves TRAM flap survival and healing. The healing process was monitored throughout time after surgery up to 22 days, by measuring the extent of flap survival. The means of survived flaps area are shown at each time points (expressed as a percentage of the total flap area). The histogram shows the extent of percentage flap necrosis in rat treated with AAV-LacZ or AAV-VEGF at day 14 before flap elevation. Measurements were performed 7 days after surgery. * Statistical significance over control $P<0.01$

In order to determine whether the improved tissue viability was actually due to a sustained angiogenic response induced by $VEGF_{165}$, representative sections from flap biopsies were stained with Hematoxylin and Eosin. In all flaps we were able to identify three zones (indicated as a, b, c in Figure 5.18), according to their distance from the vascular pedicle: a survived zone (a), from which we took sample A, corresponding to the injection site; an intermediate zone (b), from which we took sample B at ~ 3 cm from sample A, a region corresponding to the border between viable and necrotic skin; and a necrotic zone (c). Histological analysis revealed marked differences between AAV-$VEGF_{165}$ and AAV-LacZ-treated animals (Figure 5.18).

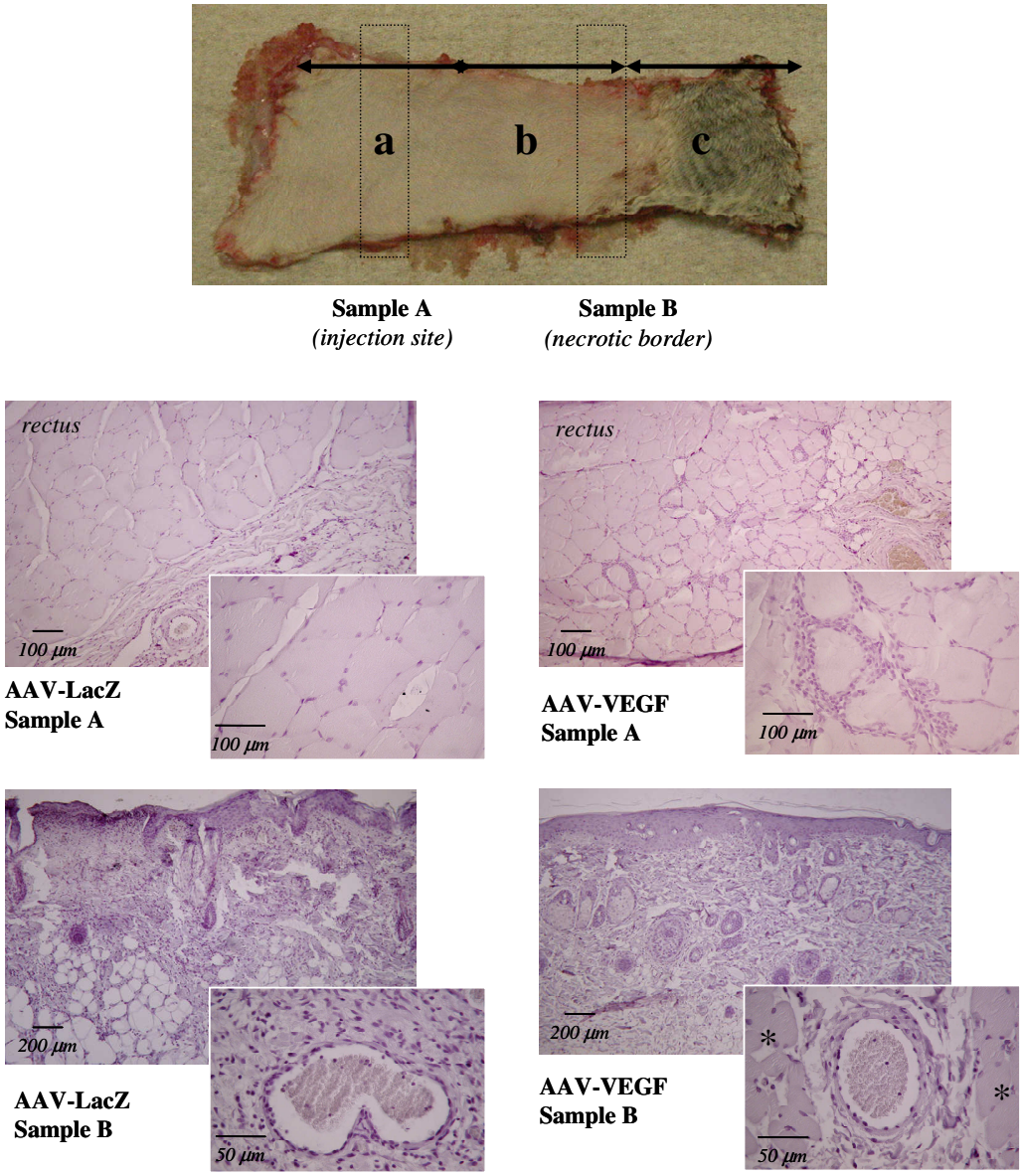


Figure 5.18 Histological sampling and assessment of TRAM flap viability. Each flap presented three distinguishable zones, according to their distance from the vascular pedicle: a survived zone (a), an intermediate zone (b), and a necrotic zone (c). To histologically examine flap viability, we harvested the skin and the muscular sheet from the injection site (sample A), as well as a more distal cutaneous sample (sample B, at ~ 3 cm from sample A) from the intermediate zone. Histological analysis were performed on sections of samples A and B from AAV-VEGF-treated and control animals. At the injection site (sample A), a massive cellular infiltration appeared as a consequence of AAV-VEGF treatment (upper panels). More notably, sample B of VEGF-treated flaps showed an intact and viable epithelial layer with conserved tissue architecture, whereas, in control flaps, the epithelium was thin and discontinuous, with massive inflammation and adipose substitution. Myonecrosis was detected only in LacZ-treated flaps, as indicated by the disappearance of the panniculus carnosus (shown by asterisks in the VEGF sample).

In the area of injection (sample A) the animals transduced with AAV-$VEGF_{165}$ showed massive cellular infiltration, consistent with the data obtained in the normoperfused skeletal muscle. In the border region (sample B), a remarkable difference was also observed in terms of tissue viability. In the control animals, the epithelial layer was thin and immature, with evidence of acute inflammation, adipose substitution and myonecrosis (as shown by the almost complete disappearance of the panniculus carnosus); the infiltrating inflammatory cells, mostly monocytes and neutrophils, were dispersed throughout the skin layers, concomitant with a severe disruption of the tissue architecture. In contrast, the morphology was preserved in the AAV-$VEGF_{165}$-treated flaps, as revealed by the presence of an intact epithelial layer and of skin appendages, with only mild and focal inflammation, and poor accumulation of adipose tissue. In the same groups of animals we also assessed the effect of vector transduction on the extent of new blood vessel formation. Therefore, sections were stained with either an anti CD31 antibody in order to detect endothelial cells or an anti- α-SMA to detect smooth muscle cells. A massive formation of new capillaries was detected (Figure 5.19) both in the proximity of the injection site (Figure 5.19, panel A) and in the skin of the more distal intermediate zone (Figure 5.19, panel B). The effect was most apparent deep to the panniculus carnosus layer, as shown by the insets, resulting in a 2-fold increase in the number of capillaries detectable in the dermal sheet ($P<0.05$) (Figure 5.19). As shown in Figures 5.20, an increase in the number of arterioles was also detected at the level of the injection site (sample A) and, more evidently, in the dermis of the intermediate zone (sample B) in the AAV-$VEGF_{165}$-treated animals (a 3-fold increase was detected in sample B; $P<0.05$).

The overall results in the TRAM flap model confirmed that $VEGF_{165}$ is able to induce the formation of a vessel network composed mainly by small arteries and this angiogenic response results in a beneficial effect on flap survival.

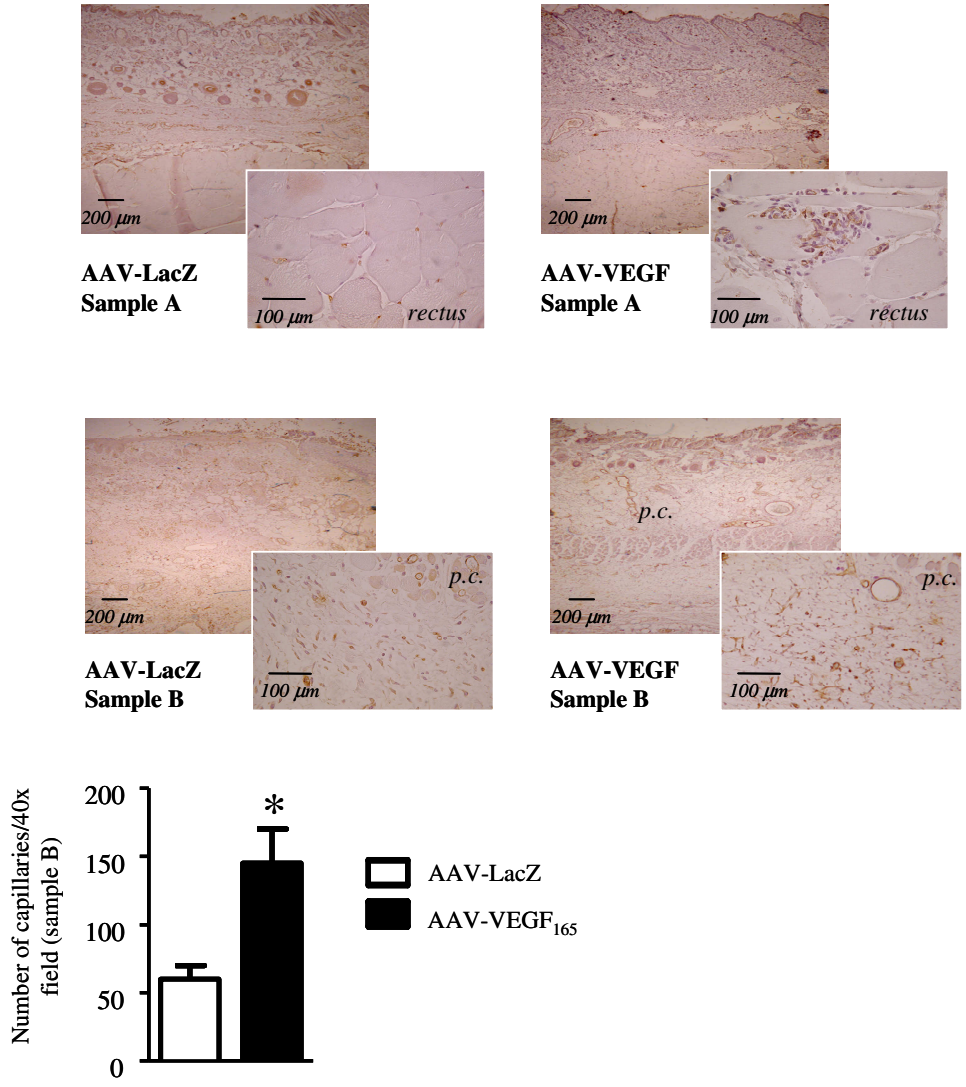
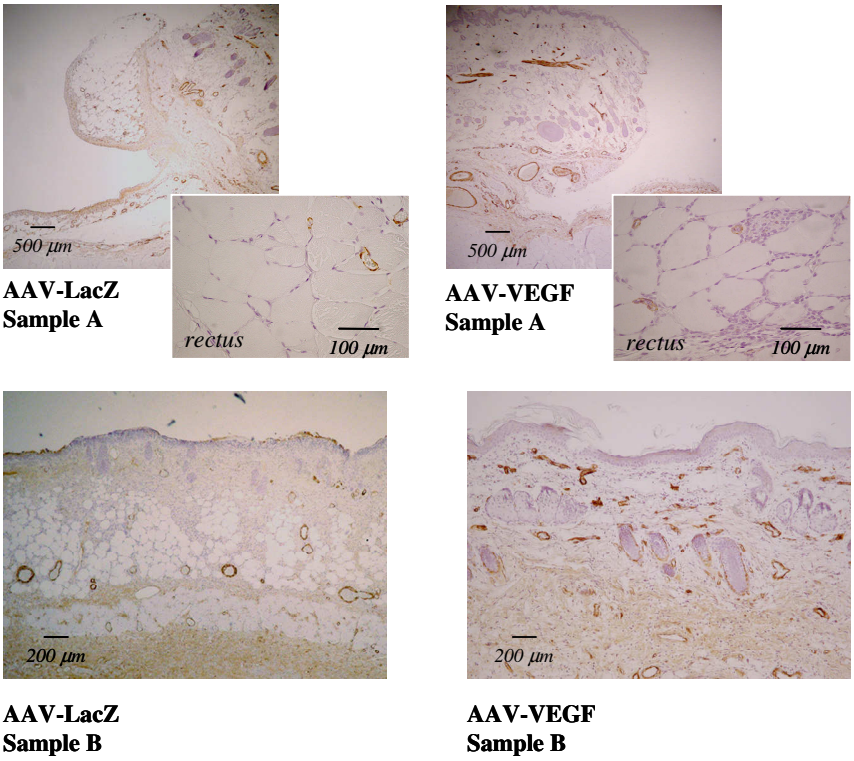


Figure 5.19 VEGF induces endothelial cell proliferation and angiogenesis. The presence of endothelial cells was detected by an anti-CD31 staining. In the VEGF-treated samples proliferating endothelil cells as well as new capillaries, both in close proximity to the injection site (sample A) and in the more distal site (sample B), were detected. Capillaries were mostly present in the level of the panniculus carnosus (p.c.). Quantification of capillaries is shown by the histogram in the lower part, expressed as number of capillaries per 40x field (sample B). * Statistical significance over control $P<0.05$



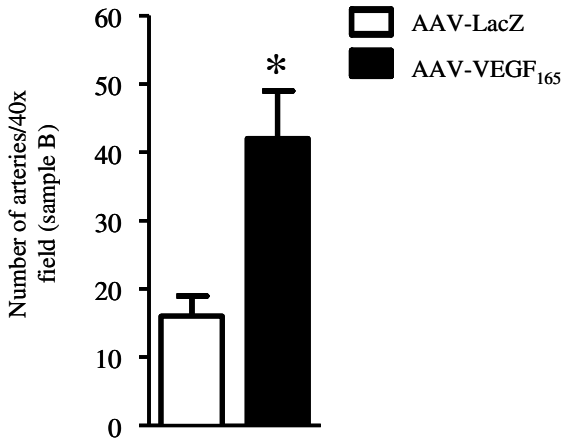


Figure 5.20 VEGF induces the formation of -SMA-positive arteriolae. Expression of VEGF induces the formation of α-SMA positve vessels, assessed by an anti α-SMA immunohistochemistry. A modest effect was detected in sample A whereas a remarkable increase in the number of arteriolae was observed in the more distal sample B. Quantification of the α-SMA positive vessels is shown by the histogram in the lower part, expressed as number of arteries per 40x field (sample B). * Statistical significance over control P<0.05

5.2.2 The artero-venous (AV) loop model

A microsurgical AV-loop is able to sustain new tissue formation

Reconstructive surgery and tissue engineering can both benefit from the development of therapeutic angiogenesis. To further assess the functionality of an AAV-based approach to induce the formation of new vessels in vivo, we developed a rat model for tissue engineering, based on an implanted, microsurgically created artero-venous (AV) vessel loop, in a chamber made of a bi-layered collagen-GAG scaffold (INTEGRA™).

Therefore, we performed a first set of experiments to assess the feasibility of the AV-loop model and to characterize the tissue chamber at 30 days after surgery. An AV-loop was created on the femoral vascular bundle, as documented in Figure 5.21; a vein graft was harvested from one groin, and used as a microvascular loop between the femoral artery and vein on the opposite leg (Figure 5.21, panels a-d). The vein graft was harvested as longest as possible, with a mean length of 9 mm (range 7-15 mm) in order to enhance the angiogenic properties of the loop. After perfusion of the AV-loop, a sheet of INTEGRA™ was folded onto itself, maintaining the outer silicon layer, and secured as a sandwich to include the loop (Figure 5.21, panels e and f).

The presence of the silicon layer ensured the complete isolation of the collagen matrix from the surrounding tissue, blocking possible vessel in-growth from the outside. At day 30 after surgery, the chamber was exposed, and femoral vessels, proximally and distally to the chamber, were assessed for patency. The loop was found patent in the large majority of the animals and the chambers appeared surrounded by a dense connective capsule (Figure 5.21, panel g).

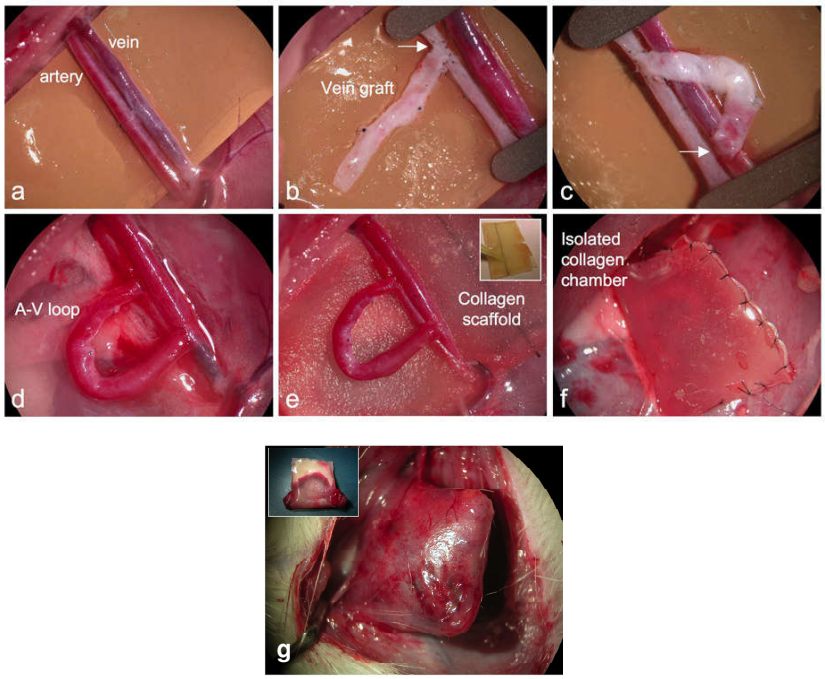


Figure 5.21 Microsurgical creation of an isolated collagen-GAG chamber surrounding an artero-venous loop. In the upper panel, description of the chamber creation. (a) Isolation of the femoral arterovenous (AV) bundle; (b) Microsurgical terminolateral (T-L) anastomosis (arrow) between the femoral artery and the distal end of a vein graft harvested from the controlateral groin; (c) T-L anastomosis of the proximal end of the vein graft with the femoral vein (arrow); (d) AV loop after surgical clamp release; (e) Embedding of the AV loop into the collagen-GAG scaffold; the inset shows a small scaffold island ready for implantation; (f) Folding and suture of the collagen-GAG scaffold on itself; (g), Speciment ready to be withdrawn and after the withdrawal (inset) at day 30 post-surgery; a dense connective tissue enveloping the chamber could be detected.

In order to confirm the connection between the AV-loop and the circulation, perfusion was assessed by laser Doppler imaging at different time-points: immediately after creation of the AV-loop, after closure of the collagen chamber (day 0) and at the end of the experiment (day 30). Measurements performed on naked AV-loops showed that the loops were clearly perfused (Figure 5.22, panel left upper), whereas after suture of the chambers the perfusion signal was almost completely quenched by the scaffold (Figure 5.22, panel left middle). At day 30, the chambers showed intense perfusion signals with a diffuse pattern, suggesting that an extensive neo-vascularization process occurred (Figure 5.22, panel left lower). The quantification of perfusion is reported in the same figure and showed a ~3 fold increase in blood flow (Figure 5.22, right panel).

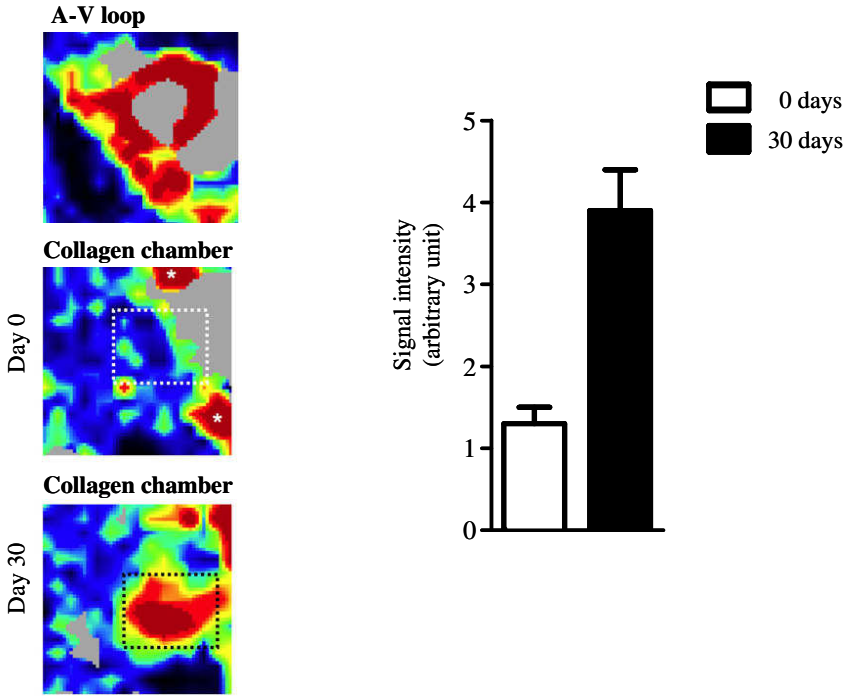


Figure 5.22 Quantification of blood flow by laser Doppler perfusion imaging. In the upper panel, perfusion of the AV-loop before closure of the collagen chamber; in the middle panel, perfusion of the collagen chamber at day 0; in the lower panel, perfusion of the collagen chamber at day 30. The asterisks indicate the proximal and distal regions of the femoral AV-bundle outside of the chambered region. In the histogram quantification of the signal intensity in the boxed regions of interest (ROIs).

To assess whether the blood perfusion might be beneficial for the formation of new tissue within the chamber, Hematoxylin and Eosin staining was performed on the samples and highlighted the presence of abundant newly formed tissue surrounding the AV-loop and filling the vast majority of the available chamber space. An Azan trichromic staining allowed a better visualization of the new tissue, in which several colonizing cells were detected within the collagen scaffold of the chamber (Figure 5.23, panel A). In addition a remarkable number of new vessels were interspersed within the collagen trabeculae of the artificial tissue. This newly formed vasculature included both bigger vessels in the close proximity of the grafted vein wall (Figures 5.23, panel A, inset b) and smaller vessels interspersed in the granulation tissue and dermal template matrix (Figures 5.23, panel A inset a).

To better characterize the vessels present within the new tissue, an immunofluorescence staining using an antibody against α-SMA was performed and confirmed that several of the observed new vessels were indeed small arterioles, which probably sprouted from the vein loop and contributed to the perfusion of the new tissue (Figure 5.23, panels B and C),

in accordance with laser Doppler data. As expected, due to the increased blood flow, we observed that the vein wall underwent a process of remodelling with the presence of an increased layer of smooth muscle cells (Figures 5.23, panel C).

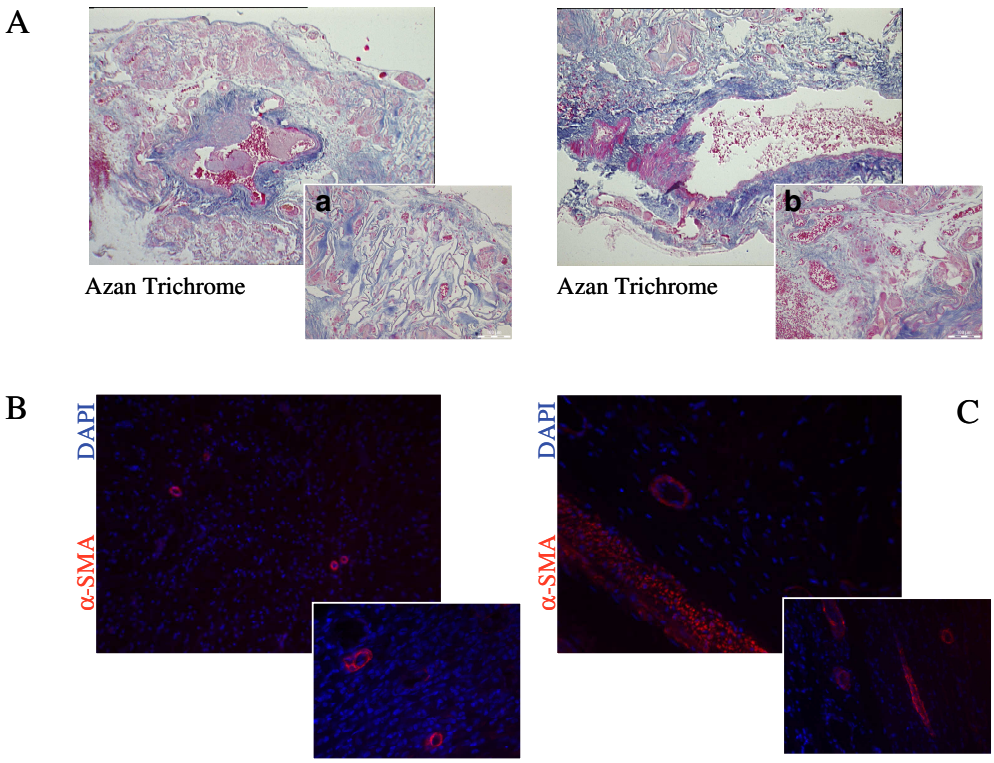


Figure 5.23 The AV-loop induce the formation of new tissue and α-SMA positive vessels. Panel A, histological sections showing closed integration between the collagen matrix and colonizing cells with many large vessels at the periphery and the adventitia of the grafted vein, interspersed inside the granulation tissue and the collagen matrix. In the insets a and b, higer magnification showing the architecture ofthe collagen scaffold and new vessels. Panel B, immunofluorescence staining for α-SMA**,** several of the vessels present in the tissue invading the scaffold showed to be small arteries.

The results obtained in this first set of experiments confirmed that the AV-loop model could be used to induce the formation of new tissue in a collagen-based chamber and could thus represent a potent tool to study and improve novel strategies of tissue engineering.

AAV-VEGF$_{165}$ increases the formation of new tissue through induction of angiogenesis

The use of recombinant proteins to stimulate angiogenesis in engineered tissues has held to partial success, although the final results were limited by the short half life of the growth factors applied in loco (Arkudas et al., 2008; Hirschi et al., 2002; Tanaka et al., 2006).

In order to test whether VEGF$_{165}$ overexpression might result useful in our chamber model, we slightly modified our AV-loop model to introduce a suitable target tissue for AAV transduction within the chamber. Therefore, we inserted a muscular pedicle inside the chamber, derived from the pectineus, which is normally located close to the vascular bundle.

Adult male Wistar rats were divided in three groups (n=7 per group) according to the vector injected (no vector, AAV-LacZ and AAV-VEGF$_{165}$); uninjected animals underwent the same procedure performed in the first set of experiment, without the insertion of muscle tissue. In the AAV treated animals, a muscular pedicle (pectineus muscle flap) was inserted into the sandwich chamber and one or two delicate stitches were tied to hold it to the collagen matrix and to avoid residual contraction. The AV-loop was created as previously described and the isolated muscle flap was injected with either AAV-LacZ or AAV-VEGF$_{165}$ (Figure 5.24, panel A).

After 30 days, dermal implants were excised and analyzed to detect the formation of new tissue and the presence of neo-vessels. Similar to the previous experiment, the samples were encapsulated in a rich fibrous capsula. Haematoxylin and Eosin showed an abundant amount of new tissue, reminiscent of a granulation tissue, filling most of the chamber space. The Azan trichromic staining also allowed the detection of a consistent number of new vessels interspersed within the collagen trabeculae of the artificial tissue (Figure 5.24, panel B). Quantification of the area occupied by the new formed tissue was performed by means of ImageJ software and revealed a larger area of newly formed tissue in both AAV-LacZ and AAV-VEGF$_{165}$ groups (Figure 5.24, panel C). The samples were harvested at 1 month, in order to avoid collagen degradation; indeed, trabeculae were still detectable in all the samples, filling almost 20% of the area. Whereas in the samples with no muscle flap the new tissue filled 65% of the area, in the AAV-LacZ group it raised to 72% and in the AAV-VEGF$_{165}$ group it reached 80% of the area ($P<0.01$ AAV-VEGF$_{165}$ vs control) (Figure 5.24).

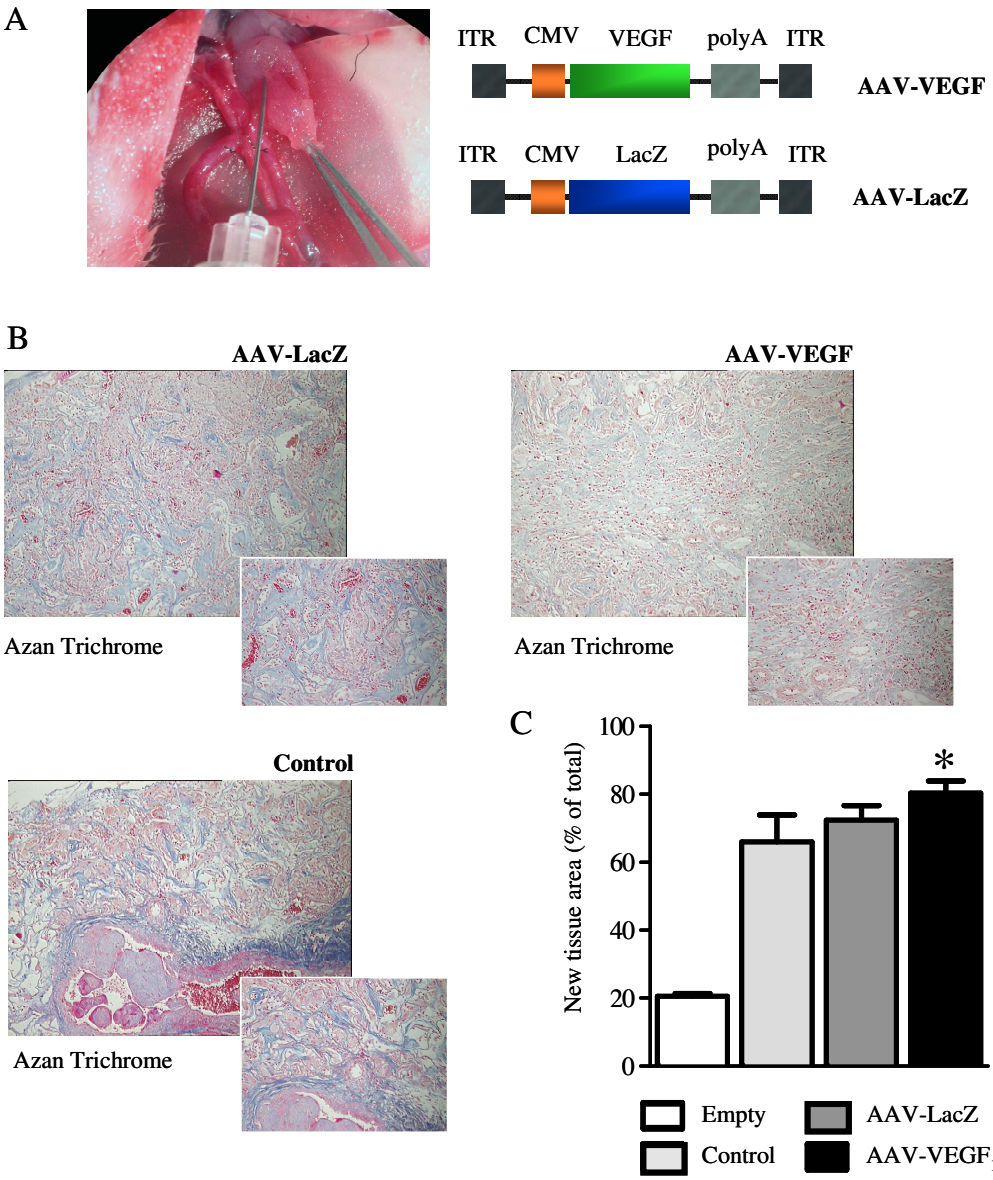


Figure 5.24 The presence of a muscolar flap within the chamber increase the formation of new tissue. Panel A, animal were injected into the pectineus muscular flap with either AAV-LacZ or AAV-$VEGF_{165}$ before closure of the chamber; on the right part schematic rapresentation of the rAAVs. Panel B, Azan trichromic staining showed the formation of new tissue and new vessels within the collagen chamber. Panel C, quantification of the new tissue area, expressed as a fraction of the total area. *Statistical significance over control P<0.01

In order to precisely analyze the number of cells present within the chamber, we quantified the relative area occupied by DAPI nuclear staining by immunofluorescence. Representative images and quantification are reported in Figure 5.25 (panel A and B). This analysis showed an increase in both AAV-treated groups compared to control group (7.8%±3.1% for control samples, 10.37%±2.6% for AAV-LacZ and 13.77%±3.7% for AAV-VEGF$_{165}$; P<0.01 AAV-VEGF$_{165}$ vs control).

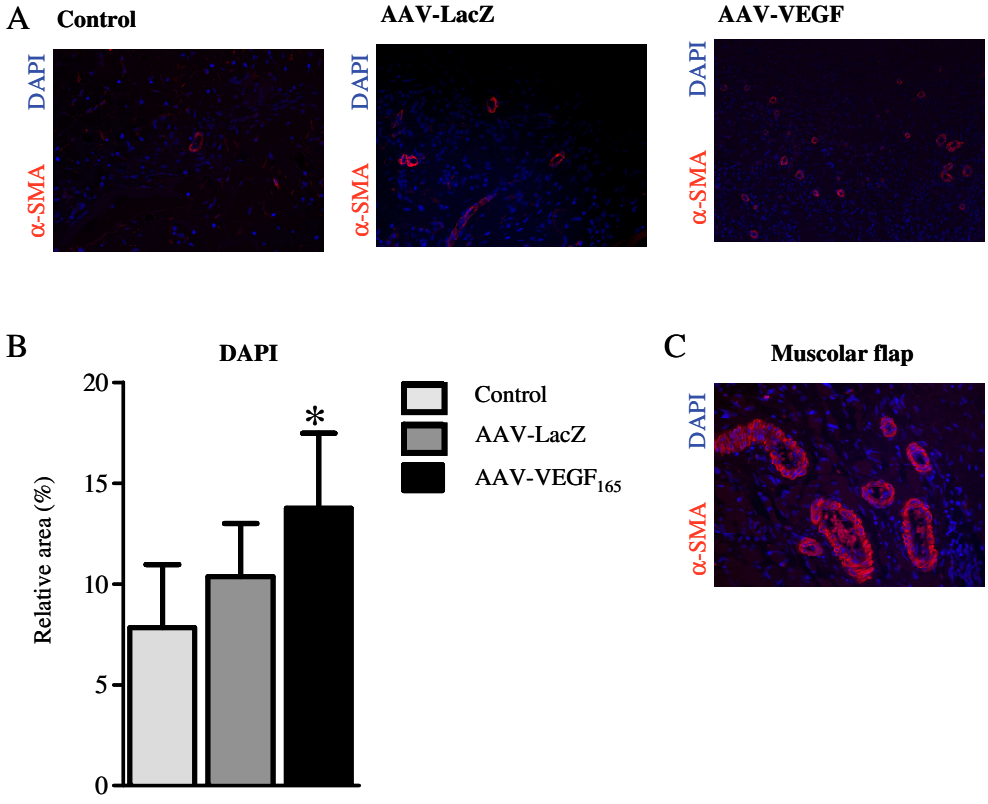


Figure 5.25 VEGF expression in the muscolar flap within the chamber increase the formation of new tissue. Panel A, rapresentative images of tissue chambers withdrawn 30 days after surgery, showing an increasing number of cells within the chamber. Panel B, quantification of the area occupied by cellular nuclei (DAPI staining). Panel C, α-SMA immunofluorescence staining showing the presence of arterial vessels in the muscolar flap, confirming the expression of VEGF by the transduced muscle.* Statistical significance over control P<0.01

Surprisingly, the muscular pedicle injected with AAV-LacZ was able to exert an effect on the chamber environment, which increased the degree of tissue formation but not at the same extent as the transduction with AAV-$VEGF_{165}$. Interestingly, the muscular tissue was preserved intact inside the collagen implant and the effect of $VEGF_{165}$ could be detected in the muscle pedicle as an increase in the α-SMA positive vessels surrounding the muscle fibers (Figure 5.25, panel C).

To assess whether new tissue formation might be associated or even favoured by an increased blood supply to the chamber, we analyzed the presence of α-SMA positive vessels by immunofluorescence staining, which showed that many new small arteries were indeed contributing to the perfusion of the tissue chamber. A quantitative analysis revealed a net increase in the number of arterioles present in the chamber: 2.25 ± 0.35 vessels/section were detected in the control group, while 5.05 ± 0.50 and 8.14 ± 1.90 vessels/section were detected in AAV-LacZ and AAV-$VEGF_{165}$ treated animals, respectively ($P<0.05$ AAV-LacZ vs Control; $P<0.05$ AAV-$VEGF_{165}$ vs Control; $P<0.05$ AAV-LacZ vs AAV-$VEGF_{165}$) (Figure 5.26, panel A). Although not reproducible in all samples, the angiogenic effect in some sections was even more impressive than in the TRAM flap model, especially in close proximity to the vein graft. As reported in Figure 5.26 (panel B), analysis of serial sections revealed that more than 10 vessels/section were detected only in the AAV-$VEGF_{165}$ group, while in the AAV-LacZ group vessels were always less than 10 per section.

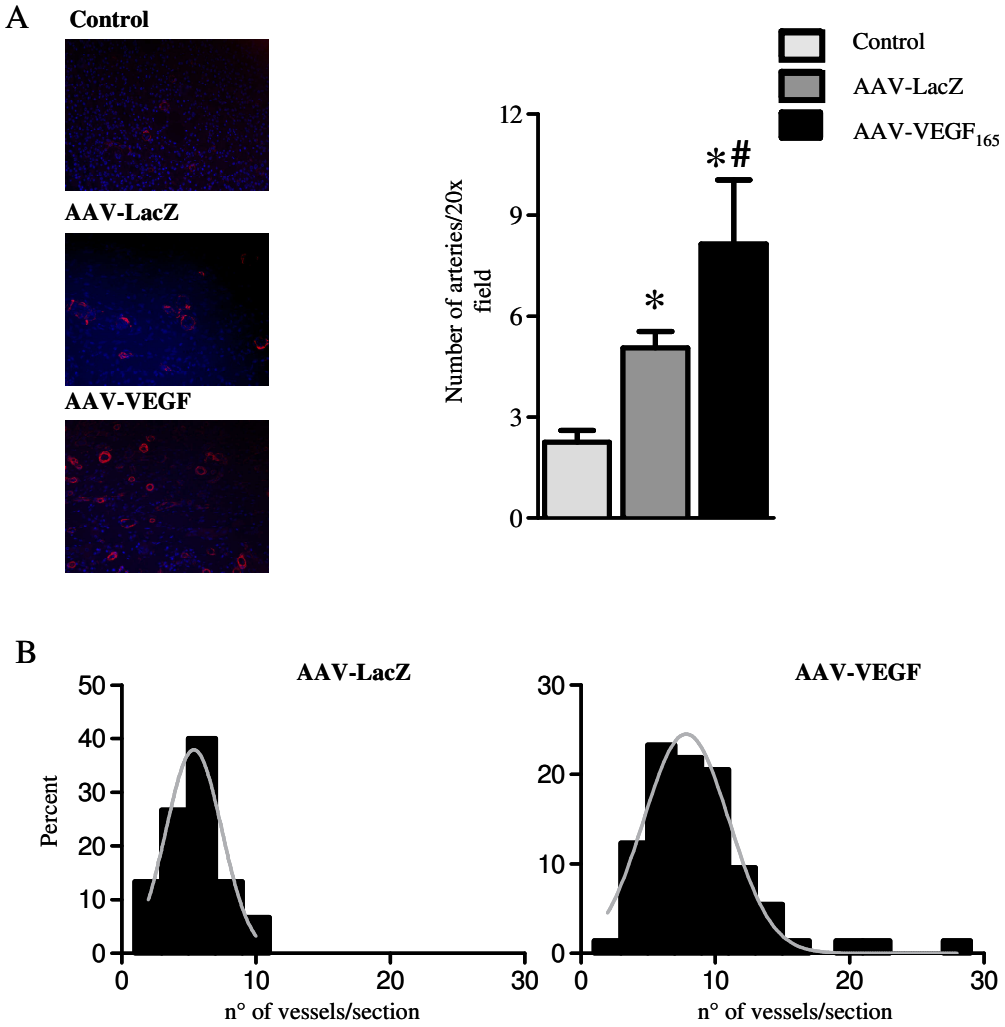


Figure 5.26 VEGF expression in the muscolar flap within the chamber increase the formation of α-SMA positive vessels. In panel A, representative images and quantification of the number of α-SMA positive vessels within the collagen chamber. The transduction with either AAV-LacZ or AAV.VEGF$_{165}$ increases the formation of vessels, compared to control. In panel B, the angiogenic effect was analyzed taking into account the distribution of the vessel per section. The histograms show the distribution of the vessel per section, with a normal distribution curve superimposed. The vessel number per section distribution of the AAV-VEGF-expressing muscles skewed toward the right compared to the AAV-LacZ-treated animals and also included a significant number of section with more than 10 vessel. * Statistical significance over control P<0.05; # statistical significance over AAV-LacZ group

Beside detecting the amount of new vessels, we performed a further morphometric analysis of the newly formed vessels, on α-SMA stained section, and we observed a slight increase in the mean vessel perimeter ($2.2*10^5$ pixels for the control group, $2.1*10^5$ pixels for AAV-LacZ and $2.5*10^5$ pixels for AAV-VEGF$_{165}$) without significant differences in the mean vessel circularity. Indeed, the mean vessel lumen area was higher in the AAV-VEGF$_{165}$ group compared either to control and to the AAV-LacZ group (2539±305 pixel^2 for controls, 2220±720 pixel^2 for AAV-LacZ and 3377±241 pixel^2 for AAV-VEGF$_{165}$;

P<0.05 AAV-VEGF$_{165}$ vs Control; P<0.05 AAV-VEGF$_{165}$ vs AAV-LacZ). The results of this analysis are reported in Figure 5.27.

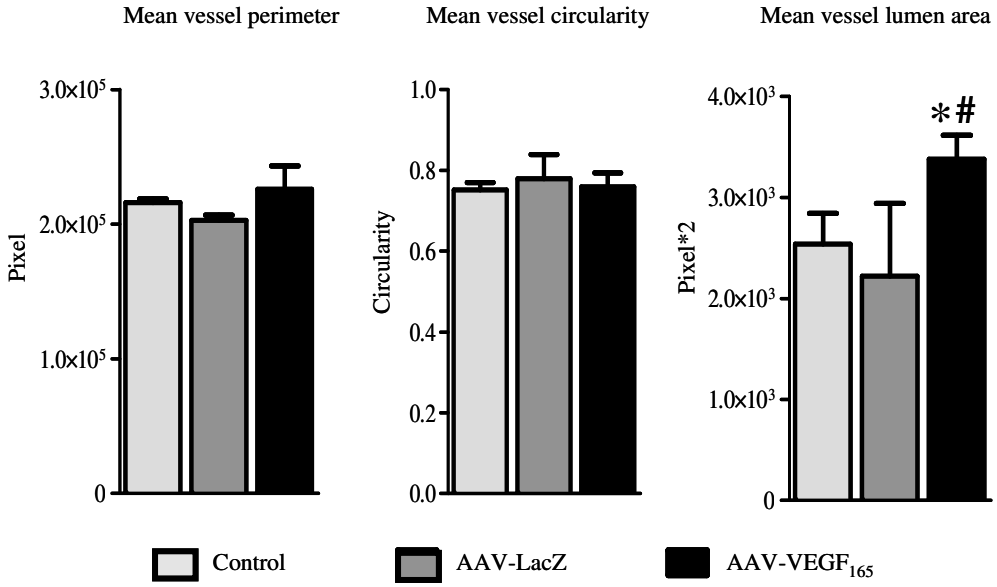


Figure 5.27 Characterization of the α-SMA positive vessels. A further morphometric analysis of the newly formed vessels was performed, no significant differences were observed either for mean vessel perimeter or mean vessel circularity, while a significant increase in the mean lumen vessel area was observed in the AAV-VEGF$_{165}$ group. *Statistical significance over control P<0.05; #statistical significance over AAV-LacZ group

Interestingly, in some of the AAV-VEGF$_{165}$ sample sections, we observed the presence of few enlarged vessels, with a thinner α-SMA cell layer and characterized by an irregular shape, resembling the vascular lacunae, previously described by our group (Arsic et al., 2003) (Figure 5.28).

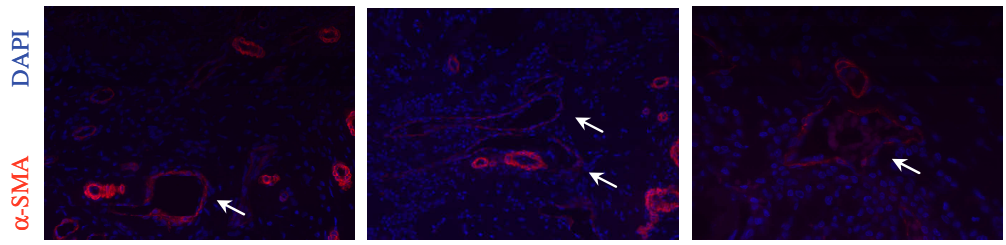


Figure 5.28 VEGF expression induces the formation of enlarged vessels. In some sections of the VEGF$_{165}$ treated animals we observed the presence of enlarged and irregular shape vessels. These vessels are characterized by the presence of a thin layer of α-SMA positive cells and are full of eritrocytes, as showed by the arrow in the image on the right.

The results obtained in this animal model confirmed the possible use of AAV-VEGF$_{165}$ as a tool to induce the formation of new vessels in ischemic conditions, although did not provide any clear cut evidence on the actual functionality of these vessels.

5.3 Modulation of VEGF activity by Angiopoietin-1

A favourable effect of Ang-1 on VEGF-induced vascular leakiness

The results obtained so far indicated that $VEGF_{165}$, by directly stimulating endothelial cell migration and proliferation, is able to trigger angiogenic capillary sprouting and neovascularisation in vivo. Despite the evidence of new blood vessel formation, the information about the functionality of this newly formed vasculature was scanty. We observed that vessels induced by $VEGF_{165}$ are less leaky than the ones induced in response to $VEGF_{121}$ but they still present important abnormalities, including increased permeability and lacunar morphology. These considerations suggested us that they might be not perfectly functional, also considering the complexity of the angiogenic process. More than one growth factor is likely needed in order to obtain a properly organized and functional vascular network. In particular, several concerns have been raised about the widely recognized permeabilizing effect of VEGF, mainly due to the induction of endothelial fenestrations and resulting in edema formation.

Among the factors which have a role in vessel maturation and stabilization, Angiopoietin-1 has been deeply studied and used for this purpose (Jain and Munn, 2000; Thurston et al., 1999).

To start understanding whether a combination approach might improve the functional competence of the VEGF-induced vessels, we analyzed vascular leakiness in mice injected with either $VEGF_{165}$ alone, or with a combination of $VEGF_{165}$ with Angiopoietin-1. As evident in Figure 5.29, muscles expressing $VEGF_{165}$ were remarkably (~ 6 folds) more leaky than controlateral controls at 1 month after treatment. However, this effect was consistently counteracted by the simultaneous delivery of an equal amount of a distinct AAV vector carrying the cDNA for Angiopoietin-1 (AAV-Ang1). The combination of the two factors resulted in an almost complete normalization of the vascular leakiness in the treated muscles (Figure 5.29).

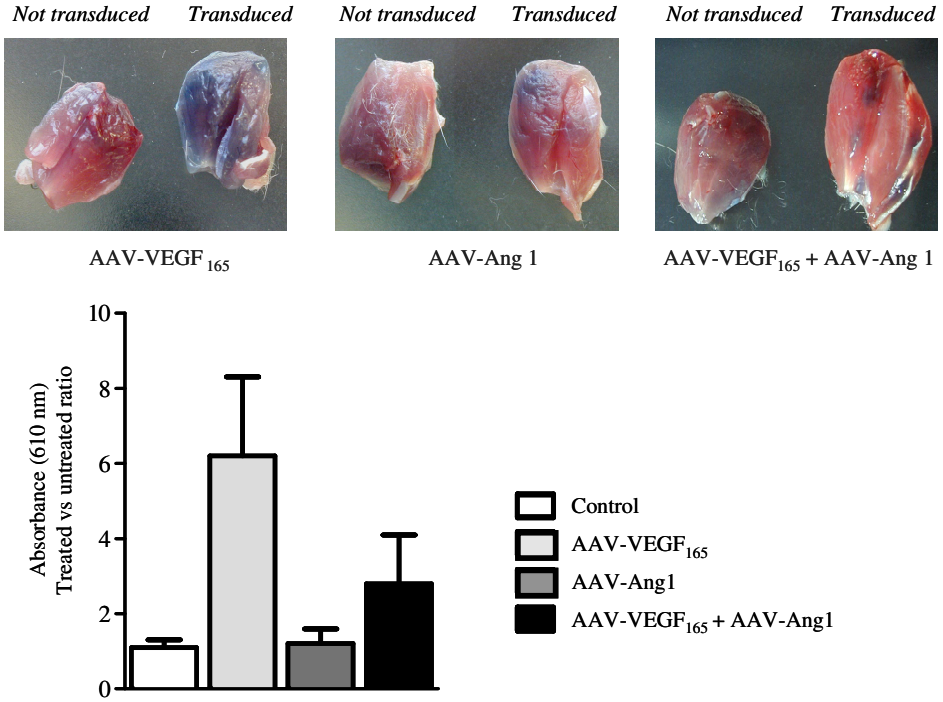


Figure 5.29 Miles' assay for vascular permeability assessment. At 1 month after treatment, a bolus of Evan's blue was injected into the jugular vein and both tibialis anterior muscles were harvested 30 minutes later. In the upper panel some representative images of the harvested muscles (Arsic 2003). The histogram reports spectrophotometric quantification of the dye infiltrated in each muscle, expressed as a ratio between treated and mock-treated legs (shown are means and standard deviations obtained from 6 animals per group).

PET imaging: functional evaluation of the effect of AAV-VEGF$_{165}$ and AAV-Ang1 transduction on muscle perfusion

Given the significant effect of Ang-1 on VEGF$_{165}$-induced vascular leakiness, we wanted to assess whether this also resulted in an improvement of vascular functional competence. Therefore, we set up a model to study muscle perfusion by positron emission tomography (PET) and single photon emission tomography (SPECT), in both resting conditions and after muscle exercise. The general outline of the whole study is schematically shown in Figure 5.30.

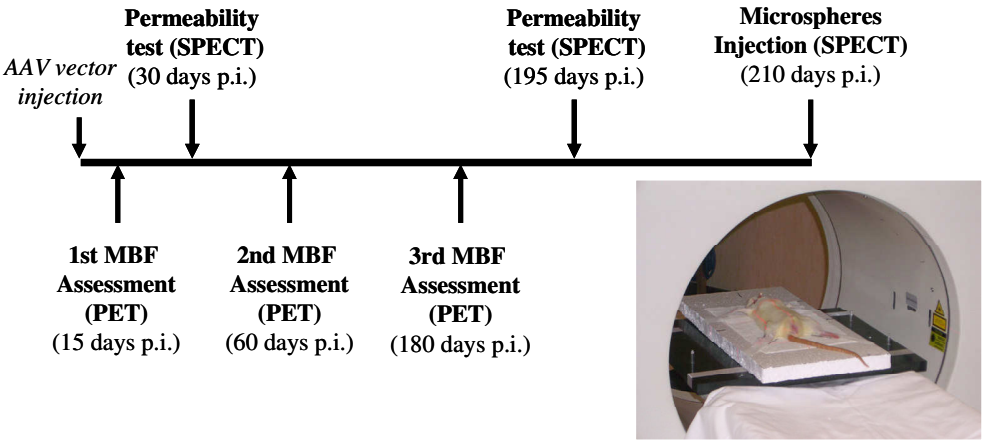


Figure 5.30 PET imaging experimetal set up. All the animals injected with either AAV-LacZ, AAV-VEGF, AAV-Ang-1, or a combination of AAV-VEGF and AAV-Ang1, were subjected to PET imaging for the assessment of muscle blood flow, as well as to SPECT scanning for the sequential measurement of vascular permeability and of artero-venous shunting.

Rats were randomly divided into four experimental groups (n=21 per group), which were all injected in their right hindlimb with an equal amount of vector preparations (AAV-$VEGF_{165}$, AAV-Ang1, AAV-$VEGF_{165}$ + AAV-Ang1 or AAV-LacZ), and with PBS in their left hindlimb, as an internal control. All the animals were repeatedly subjected to PET imaging at different times after treatment up to 6 months (15 days, 2 months and 6 months), by measuring bilaterally an index of muscle perfusion. More precisely, each PET session included two measurements of muscle blood flow (MBF), either in resting condition or after 20 min of pacing-induced bilateral muscle activity. After two weeks from the last PET experiment, all the animals were injected with ^{99m}Tc-labeled DTPA to assess vascular permeability by SPECT. Finally, after two additional weeks, they were evaluated for the presence of artero-venous shunts by the injection of radio-labeled macroaggregates into the abdominal aorta. A representative PET image of a rat at 6 months after AAV-$VEGF_{165}$ injection is shown in Figure 5.31.

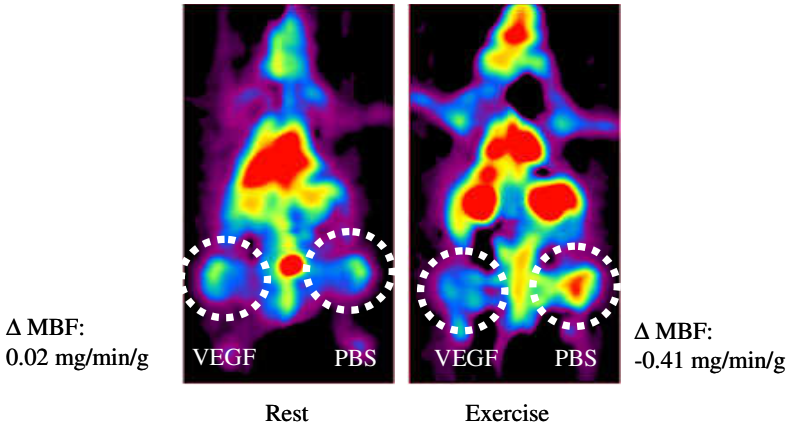


Figure 5.31 PET imaging at 6months after AAV-VEGF injection. The relative perfusion of the different tissues is represented by an artificial color scale. While most organs, including the untreated controlateral leg, underwent a physiological vasodilation during muscle activity, the VEGF165-expressing muscles seemed not able to respond to the increased flow demand.

In contrast to our expectations, the prolonged expression of $VEGF_{165}$ alone did not increase MBF as compared to the control leg, in resting conditions (Figure 5.31, left panel). Even more surprising was the result obtained after exercise (Figure 5.31, panel right), vasodilatation occurred in most organs, including heart, brain, kidney as well as in the left, control leg, whereas the VEGF-treated leg was completely unable to respond to the increased flow demand.

The results obtained in the whole set of animals at three time points after vector delivery are shown in Figure 5.32, expressed as ΔMBF (difference between the flow of the treated and the untreated leg). The unexpected negative effect of $VEGF_{165}$ on muscle perfusion after exercise was already detectable as early as 15 days after vector injection, and became even more evident at the later time points (MBF ratio between the treated and the untreated leg was 0.70±0.13, 0.66±0.02, 0.50±0.02 at 15 days, 2 months and 6 months, respectively, vs 0.89±0.03 control; $P<0.05$ at every time point). In contrast to $VEGF_{165}$ alone, the simultaneous administration of AAV-$VEGF_{165}$ and AAV-Ang1 resulted in a clear improvement in MBF both at baseline and after exercise. Again, this result was already evident at 15 days (MBF ratio: 0.61±0.34 vs 0.33±0.05; $P<0.05$ at rest; 1.20±0.24 vs 0.82±0.06; $P<0.01$ after exercise) and persisted over time, up to 6 months after treatment (1.17±0.42 and 1.16±0.09 at 2 and 6 months, respectively; $P<0.01$ vs control at both times).

The expression of Ang-1 alone had no effect on MBF at any time point. Therefore, the animals treated with Ang-1 were not further considered in the subsequent experiments of this study.

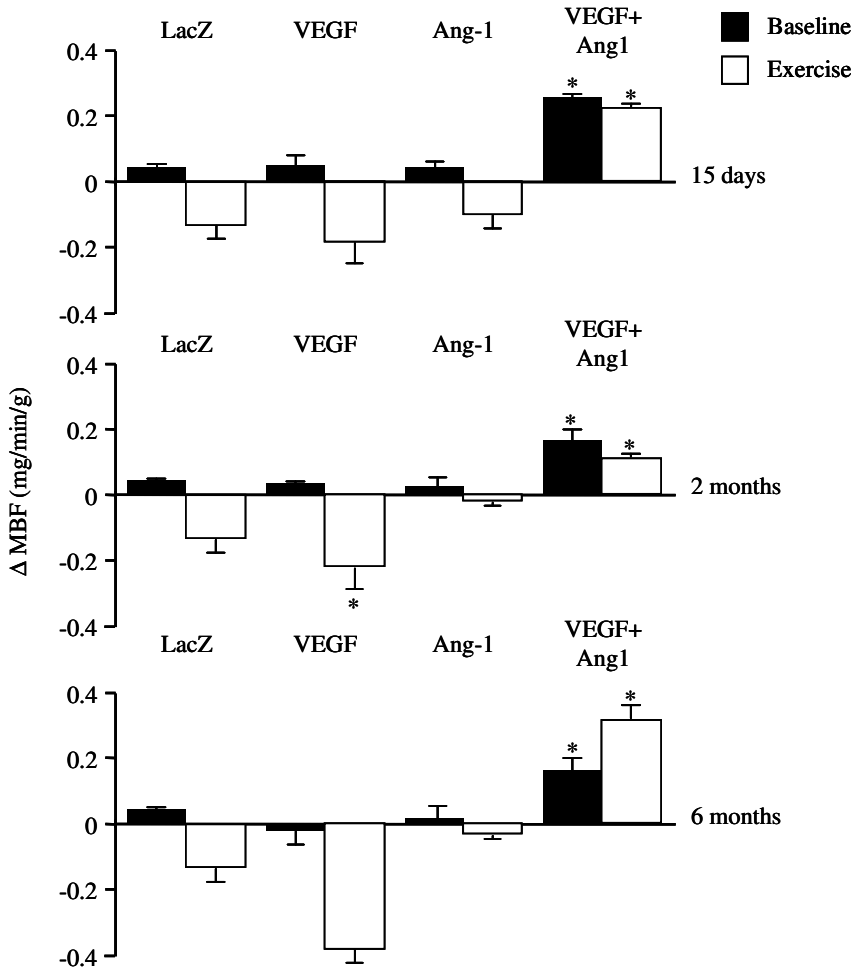


Figure 5.32 PET analysis of MBF after $VEGF_{165}$ and Ang-1 gene transfer. MBF assessment by PET revealed an unexpected drop in MBF in muscle transduced with $VEGF_{165}$ after pacing-induced muscle activity. This negative effect was already detectable at 15 days and persisted up to 6 months after treatment. In contrast, the simultaneous overexpression of Ang-1 resulted in a marked improvement in MBF both in resting conditions and after exercise. * Statistical significance over control

Expression of Ang-1 improves functional maturation of the VEGF-induced blood vessels

In order to start understanding the reason underlying the unexpected drop in MBF after AAV-$VEGF_{165}$ transduction, we simultaneously analyzed the vascular volume and permeability of the treated muscles by the infusion of ^{99m}Tc-DTPA, in the same set of animals previously studied by PET. As expected, the delivery of AAV-$VEGF_{165}$ was found

to determine a remarkable increase in the blood volume of the treated leg, which was evident at 1 month (2.9±0.8 vs 1.0±0.1, P<0.05) and further increased at 6 months after treatment (6.1±1.6 vs 1.3± 0.2, P<0.05) (Figure 5.33). In contrast, the co-injection of AAV-Ang1 significantly counteracted this effect, although at 1 month a modest but not significant increase in vascular volume was still detectable relative to control.

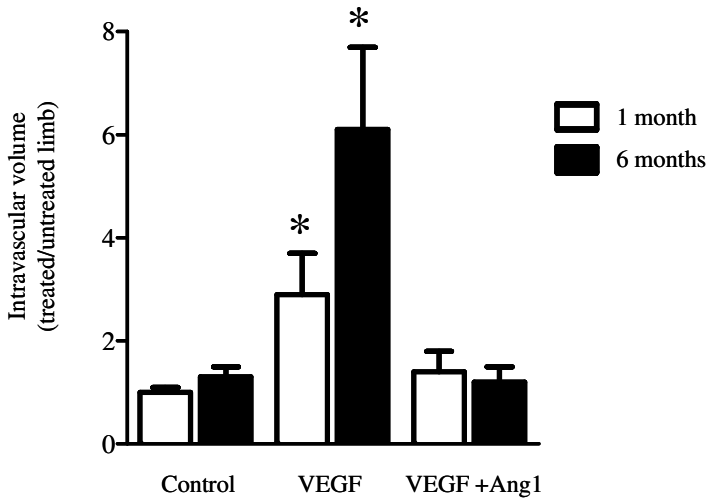


Figure 5.33 Determination of intravascular volume after VEGF165 and Ang-1 gene transfer. An index of the intravascular volume of the treated and the untreated legs was calculated by the systemic injection of 99Tc-DTPA. As evident from the histogram, muscle expressing VEGF165 displayed a remarkable increase in the overall blood content, while almost physiological levels were detected in muscle co-injected with AAV-VEGF and AAV-Ang1. * Statistical significance relative over control.

In the same set of experiments, an index of vascular permeability was also determined, as shown in Figure x. In perfect agreement with the results of the Miles' assay, obtained ex vivo up to 3 months after treatment (see Figure 5.7), the injection of AAV-VEGF $_{165}$ induced a significant increase in the permeability of the treated leg, at both 1 and 6 months, while the simultaneous delivery of AAV-Ang1 resulted in a marked reduction of the VEGF-induced vascular leakiness, achieving an almost complete normalization after 6 months (1.4±0.1 vs 2.2±0.3 at 1 month and 1.1±0.1 vs 1.6±0.2 at 6 months, P<0.05)(Figure 5.34).

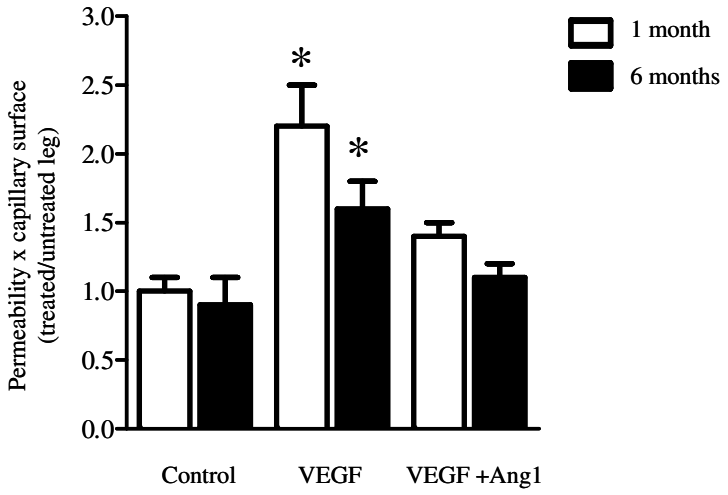


Figure 5.34 Assessment of vascular permeability *in vivo* by infusion of 99Tc-DTPA. An index of vascular permeability for both the treated and the untreated leg was also determined after the systemic injection of 99Tc-DTPA. At 1 and 6 months after AAV-$VEGF_{165}$ injection, the muscles resulted to be significantly more leaky than controls. In contrast, the simultaneous delivery of AAV-Ang1 efficiently counteracted this effect, accounting for an almost complete normalization of the permeability at 6 months after treatment. * Statistical significance over control

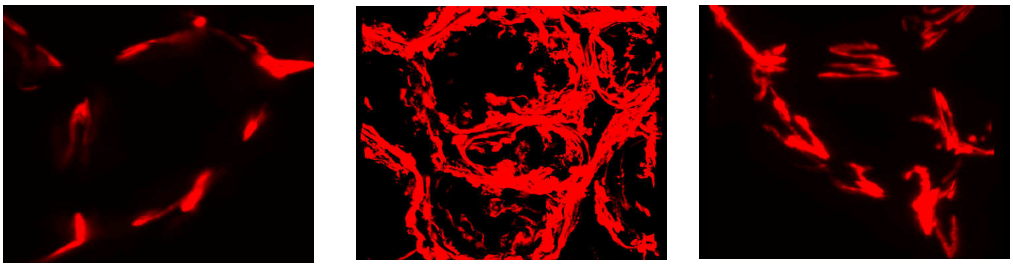
Overall, these functional experiments provided quite contradictory results. In fact, to our surprise, we found a drop in functional muscle perfusion by PET after AAV-$VEGF_{165}$ gene transfer, but an increased intravascular volume, as assessed by both in vivo perfusion with fluorescent microspheres and ^{99m}Tc-DTPA injection.

As already mentioned, one possible explanation for the poor function of the $VEGF_{165}$-induced vessels could be their abnormal permeability, resulting in edema formation and subsequent hemodynamic impairment. In contrast, the significant effect of Ang-1 on vessel maturation, through the reinforcement of the junctions between endothelial cells, extracellular matrix and pericytes, is in prefect agreement with the observed improvement in MBF after the simultaneous delivery of AAV-$VEGF_{165}$ and AAV-Ang1.

Alternatively, as already mentioned before, the documented formation of several large vascular lacunae might constitute a sort of blood reservoir, unable to efficiently flow through the vascular network and thus to actively provide oxygenated blood to muscle fibers. To address this hypothesis, we wanted to further investigate the morphological details of the newly formed vascular structures in a subset of animals injected with AAV-$VEGF_{165}$ or AAV-$VEGF_{165}$ plus AAV-Ang1. Three animals for each experimental group were systemically perfused with a suspension of 0.2 μm red fluorescent microspheres, as already described for mice. After animal sacrifice, thick muscle sections (100 μm) were

scanned, by acquiring at least 20 confocal planes for a 3D-reconstruction of the image. A planar view of a reconstruction for each experimental group is shown in Figure 5.35. In accordance with our previous findings on mouse muscles, the vessels formed in response to $VEGF_{165}$ were found to be abnormally large and poorly organized, with a pronounced tendency to invade the muscle fiber parenchyma.

In contrast, more structured vessels were evident when Ang-1 was expressed together with $VEGF_{165}$ by the skeletal muscle. Interestingly, in the latter group, each muscle fiber appeared to be surrounded by a network of capillaries, each one being larger and more tortuous than those seen in the control.



Control **AAV-$VEGF_{165}$** **AAV-$VEGF_{165}$+AAV-Ang1**

Figure 5.35 Capillary network formed in response to $VEGF_{165}$ or $VEGF_{165}$ + Ang-1. After in vivo perfusion of the animals with fluorescent microspheres, a 3D-reconstruction of the vascular network was obtained by confocal image scanning. As evident in the central panel, $VEGF_{165}$ expression induced the formation of large vascular structures, invading the muscle fiber parenchyma. In contrast, the simultaneous expression of $VEGF_{165}$ and Ang-1 resulted in a more structured capillary network.

Artero-venous shunts after $VEGF_{165}$ and Ang1 overexpression

Considering the abnormal shape and size of the vessels formed by $VEGF_{165}$, a possible explanation for their poor functional performance, alternative or additional to the permeability hypothesis, was to assume the formation of artero-venous shunts that might bypass the capillary system, thus not allowing a proper perfusion of the muscle fibers. In an attempt to verify this hypothesis, a bolus of radioactive macroaggregates in a 50-100 μm diameter range, was injected into the abdominal aorta to ensure their symmetrical distribution to the hindlimbs, bilaterally. The amount of radioactive spheres that were not trapped in the capillary bed and shunted to the lungs was detected by SPECT. As shown in Figure 5.36, a significant amount of radioactivity was found in the lungs of animals treated with AAV-$VEGF_{165}$, indicating the presence of an important artero-venous shunting (11.31±1.21% of abdominal aorta flow shunted to the lungs in the VEGF group vs 3.6±0.44% in the control group, $P<0.01$), while the co-injection of AAV-Ang1 markedly

reduced this phenomenon (6.19±0.61, P<0.01 vs $VEGF_{165}$). A representative SPECT image of a rat for each experimental group is provided in Figure 5.36, in which the histograms represent the means and standard deviations of the same animals previously considered in the perfusion and permeability experiments.

The overall results, concerning the positive effect exerted by Ang-1 on the $VEGF_{165}$-induced vessels, challenged the effectiveness of $VEGF_{165}$ as a sole factor to induce angiogenesis and prompted the use of factor combinations to achieve competent vessel formation.

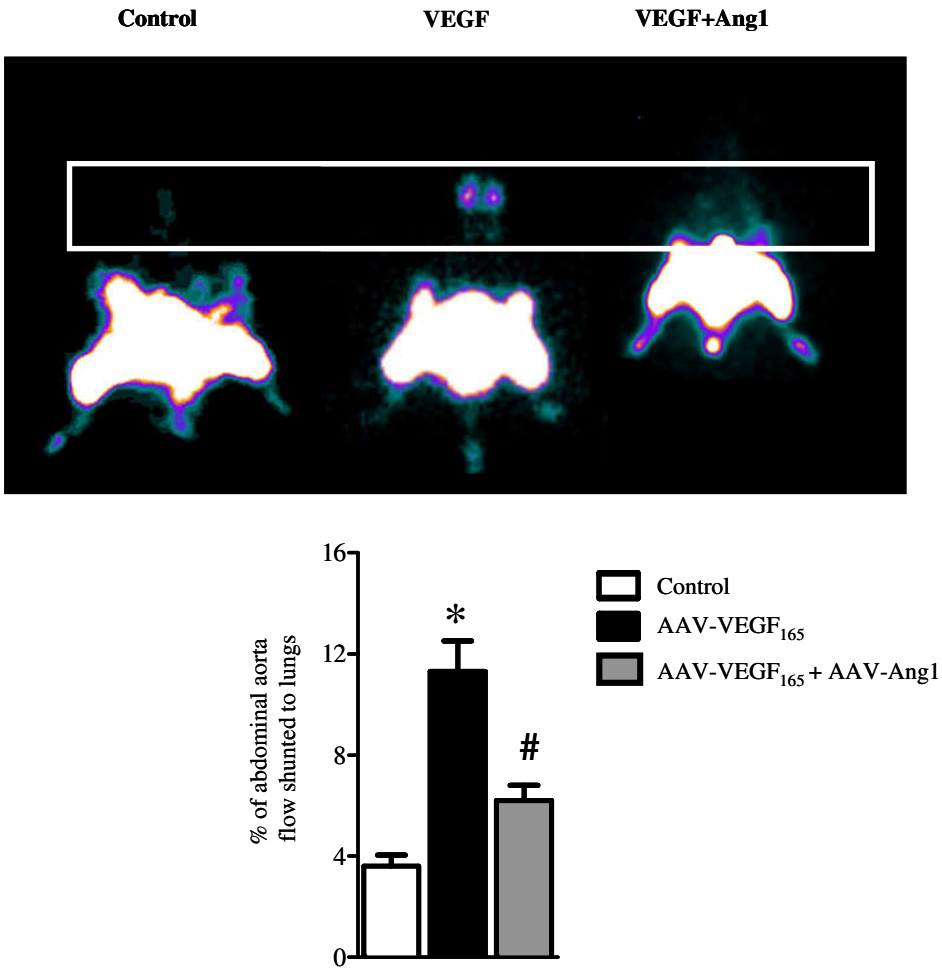


Figure 5.36 Artero-venous shunting assessment. The injection of radioactive macroaggregates into the abdominal aorta allowed the detection of artero-venous shunting activity. The presence of a significant amount of radioactivity in the lungs of animals treated with AAV-$VEGF_{165}$ indicated the formation of an important number of artero-venous shunts, which resulted significantly reduced by the co-injection of AAV-Ang1. * Statistical significance over control, # statistical significance over $VEGF_{165}$-treated group

6. DISCUSSION

6.1 AAV vectors: a tool to study the mechanisms and improve the outcome of therapeutic angiogenesis

The experiments described in this thesis take advantage of the properties of AAV vectors to unravel the mechanisms of vessel formation and maturation.

AAVs can be considered as a unique tool for efficient gene transfer into the cardiovascular system, due to their high and selective tropism for muscle tissues, including skeletal, cardiac and vascular smooth muscle cells, and to their ability to sustain long term transgene expression in the absence of integration into the host genome. Moreover, they are characterized by absence of pathogenicity and they do not elicit any major inflammatory or immune response. As compared to adenoviral vectors, their kinetic of expression lead to a delay and lower level of transgene expression, which however, last for long periods of time, hypothetically for the whole life. These appealing features of recombinant AAV vectors have prompted their use in clinical studies, and to date more than 60 trials have already began (4.1% of worldwide gene therapy clinical trials) (http://www.wiley.co.uk/genmed/clinical).

Beside their use as gene therapy vectors in the clinics, AAV vectors represent a unique tool for research purposes in the cardiovascular field, particularly to study gene function in vivo in adult organisms.

In this thesis AAV have been used to dissect the role of some molecular players, involved in the process of angiogenesis. Although several of these molecules have already been studied and characterized for their angiogenic potential by genetic studies, their exact role and regulation during aniogenesis is still not completely understood, likely explaining the failure of most clinical trials in the cardiovascular field, aimed at the induction of therapeutic angiogenesis.

First, we explored the effects of the most known and potent angiogenetic factors namely various members of the VEGF family. In particular, we compared the biological activities of the $VEGF_{165}$ and $VEGF_{121}$ isoforms, which share the ability to activate endothelial cells but differs in their arteriogenic properties, which are specifically exerted by $VEGF_{165}$ and not by $VEGF_{121}$. Thanks to the sustained expression of the transgene achieved by the rAAV, we could follow the process of new blood vessel formation over time, and realized that only capillaries formed in response to $VEGF_{121}$ whereas small arteries appeared in $VEGF_{165}$-injected muscles. The prolonged expression of $VEGF_{165}$ also resulted in the

appearance of important side effects, such as vascular lacunae and artero-venous shunts, that would not be detected in case of a short time expression, such as the one achievable with adenoviruses or naked DNA. AAV vectors enter into the target cell at high multiplicity of infection, thus allowing to study the effect of the combined expression of two factors involved in vessel formation and maturation: $VEGF_{165}$ and Angiopoietin-1.
All the experiments presented in this thesis were performed by using AAV serotype 2, which has the most used and characterized capsid proteins. Interestingly, over the last few years, other promising serotypes have been discovered and used for gene therapy, also for cardiovascular applications. Serotype 1 was reported to be the most efficient in transducing skeletal muscle cells (Rabinowitz et al., 2002), whereas serotype 8, and even more serotype 9, showed a high tropism for cardiac tissue (Bish et al., 2008; Inagaki et al., 2006; Palomeque et al., 2007).

6.2 The presence of α-SMA positive cells is a first index of vessel maturation

Overall, the results presented in this thesis substantially demonstrate that neo-vascularization is not achievable by the constitutive expression of a single potent angiogenic factor, such as $VEGF_{165}$, and that a fine tuning of its activity is required, in order to obtain functional new blood vessel formation.
The process of vessel maturation is quite complex and several players appears to play in a coordinated manner in every step of the process. As already partially discussed, in the first set of experiments, we compared the angiogenic potential of two main VEGF isoforms: whereas $VEGF_{165}$ overexpression leads to an increased number of both capillaries and small arteries, $VEGF_{121}$ similarly induced capillary sprouting but not arterial formation. The assessment of perfusion confirmed that the formation of new capillaries induces an increase in blood volume, which is lower than the one sustained by the presence of new arterioles. Capillaries and arterioles differ for both their structure and function. Capillaries have a diameter smaller than 10 μm, while small arteries could reach 120 μm. Moreover, the Miles' assay showed that the vessels formed in response to $VEGF_{121}$ are leakier that the one induced by $VEGF_{165}$, although the latter still exerts a potent permeabilizing activity. Vessel permeability is considered a hallmark of immature vessels, whereas the presence of α-SMA coverage is considered a hallmark of vessel maturation. Thus, the vessels induce by $VEGF_{121}$ appear definitely immature, likely unable to sustain an improvement in the blood flow. These considerations are in agreement with the findings of Stalmans and coworkers (Stalmans et al., 2002), who studied the role of different VEGF isoforms in

normal retinal angiogenesis in genetically engineered mice, and found normal arterial development in $VEGF^{164/164}$ mice (which express exclusively the $VEGF_{164}$ isoform), while few arterioles and impaired pericyte recruitment in $VEGF^{120/120}$ mice (which express exclusively the $VEGF_{121}$ isoform). Moreover, hearts from $VEGF^{121/121}$ mice displayed impaired myocardial angiogenesis, with fewer coronary vessels surrounded by α-SMA positive cells, leading to ischemic cardiomyopathy (Carmeliet et al., 1999). How might this difference between the two isoforms be explained? We reported that in the $VEGF_{121}$ treated muscle, beside the absence of small arteries, we did not detect any cellular infiltration and showed that the presence of α-SMA positive vessels correlate with the presence of bone marrow-derived infiltrating cells. Although the hypothesis that these cells might directly contribute to new blood vessel formation through transdifferentiation was attractive, BM transplantation studies have clearly ruled out this possibility (Zentilin et al., 2006). Therefore, VEGF-driven neovascularization appears to essentially depend on classic angiogenic sprouting of locally resident endothelial cells. In this respect, it is worth mentioning that several other authors have recently challenged a relevant incorporation of BM precursors into newly forming vessels (Balsam et al., 2004; Grunewald et al., 2006; Rajantie et al., 2004; Ziegelhoeffer et al., 2004). Therefore, we wondered whether these cells might contribute to vessel maturation by a paracrine effect exerted mainly on mural cells. We better characterized these cells by showing that, beside their bone marrow origin, they are CD11b+, CD45+ and largely NP1+. The expression of the latter receptor helped us in understanding the reason why the two VEGF isoforms do not share the ability to recruit these cells. Indeed, besides the property of binding to heparin (which is unlikely to play any role in the differential angiogenic properties of the two factors), the main difference that distinguishes $VEGF_{165}$ and $VEGF_{121}$ is the ability of the former factor to interact with NP-1.

The role of bone marrow cells at the site of angiogenesis is not completely understood and it might reasonably entail the secretion of factors able to stimulate the proliferation of pericytes, associated with pre-existing vessels, as well as their migration along the newly made vascular sprouts (Benjamin et al., 1998). Consistent with this hypothesis, we observed increased proliferation and migration of SMCs in response to CD11b+ cell-conditioned medium. Obviously, these results open the fundamental question of which are the molecules mediating pericyte/SMC engagement *in vivo*.

In accordance with previously published genetic studies, plausible candidates that are likely to play a major role in this process essentially belong to the Ang1/Tie2, TGF-β/

TGFR-β and PDGF-B/PDGFR-β signalling systems (Hirschi et al., 1999; Hirschi et al., 1998; Leveen et al., 1994; Suri et al., 1996). Of particular interest is the observation that the hearts of $VEGF^{120/120}$ mice express reduced levels of PDGF-B and its type β receptor as compared to $VEGF^{+/+}$ hearts, whereas the levels of Ang-1 are similar (Carmeliet et al., 1999). In accordance with these results, we also observed a differential expression of TGF-β and PDGF-B in the muscles overexpressing $VEGF_{165}$ or $VEGF_{121}$, but no significant differences in the levels of Ang-1. This observation is also reinforced by our striking finding that the simultaneous expression of $VEGF_{121}$ and PDGF-B has a clear arteriogenic effect in the absence of mononuclear cell infiltration. The effect of PDGF-B, however, does not necessarily imply that this is the only factor released by CD11b+ cells to promote *in vivo* artery formation. In light of the complexity of the process, the participation of multiple cytokines and synergistic pathways appears more likely (for instance, an additional plausible candidate is TGF-β, which we also found to be specifically induced by $VEGF_{165}$).

6.3 New vessel formation in ischemic models

The observed increase blood perfusion upon $VEGF_{165}$ in normoperfused muscles confirmed the possible application of AAV vectors as tools to induce angiogenesis *in vivo*. Moreover, the different vessel phenotype induced by the two VEGF isoforms suggested that besides endothelial cell activation, pericyte recruitment is also required for proper vessel maturation. Indeed, $VEGF_{165}$-induced vessels were shown to be more mature and allowed an increased blood supply was compared to those formed in response to $VEGF_{121}$.

These results were produced in normoperfused conditions, while therapeutic angiogenesis would be required in hypoxic conditions, such as myocardial infarction or lower limb ischemia. In order to understand the functional application of the proposed AAV gene transfer method, we tested our gene transfer method in two hypoxic models in rats. Although the major interests for therapeutic angiogenesis are in the field of cardiovascular disorders, the same strategy might result of equal interest in many plastic surgery techniques, where ischemia is often a contributing cause of surgical failure.

Beside reconstructive surgery, tissue engineering is another field that might benefit from the induction of therapeutic angiogenesis, as the increase of blood perfusion of the engineered scaffolds would be likely beneficial (Jain et al., 2005; Rouwkema et al., 2008).

In the TRAM flap model, expression of $VEGF_{165}$ significantly improved flap survival, with a macroscopically evident reduction of the necrotic portion, as well as with a significant

improvement in tissue viability at histological examination. The beneficial effect of AAV-$VEGF_{165}$ on flap survival well correlated with an impressive formation of new blood vessels - both capillaries and arterioles. The clear improvement in tissue viability might be ascribed to a better perfusion through the muscular layer as a consequence of a local angiogenic effect of $VEGF_{165}$. Alternatively, secreted $VEGF_{165}$ might have diffused from the muscle to the skin layer of the more distal portion of the flap, thus promoting angiogenesis within the derma, in agreement with a series of recent reports showing that AAV vectors delivered to the skeletal muscle are able to drive the expression and secretion of different molecules into the circulation (Descamps et al., 1996; Donahue et al., 1999; Hengge and Mirmohammadsadegh, 2000; Kay et al., 2000). The increase in the density of arterioles in the skin, distal to the injection site (sample B) is strongly in favor of the second hypothesis.

In this model we were able to achieve an important therapeutic results, namely increased flap survival, and we demonstrated that this was mainly due to the angiogenic effect induce upon $VEGF_{165}$ expression. These results reinforce the notion that adequate blood supply is an essential requisite for flap survival, and indicate the feasibility of an angiogenic gene therapy approach in plastic and reconstructive surgery.

In tissue engineering the ability to vascularize tissue substitutes would be a significant step forward to improve their survival and to apply these strategies to patients. In this field, several approaches have been attempted over the recent years, such as the use of recombinant proteins (Arkudas et al., 2007; Tanaka et al., 2006) or gene transfer. Studies performed by Tanaka and coworkers showed that, even in absence of growth factors, the presence of a vascular bundle is sufficient to induce the formation of new tissue (Tanaka et al., 2003). Another model that was reported to be able to induce the formation of new vessels was the artero-venous loop (Lokmic et al., 2007; Polykandriotis et al., 2007).

In order to develop a good model to induce the formation of new tissue within a collagen chamber, we choose the AV-loop model. We reported that the presence of the AV-loop was sufficient to induce the formation of new tissue within the tissue chamber, characterized by the spontaneous formation of new vessels, both capillaries and small arteries. The presence of vessels within the engineered tissue or substitute was already reported to be the requisite for new tissue formation and many studies highlighted the importance of an intrinsic vasculature to increase tissue survival or tissue formation. In our model, the chamber was completed isolated from the external tissues, and the only vascular source for angiogenesis was the vein graft. Probably, the hypoxic condition present within

the tissue chamber and shear stress within the AV-loop, represented sufficient stimuli to trigger an angiogenic process (Carmeliet, 2000; Laschke et al., 2006; Risau, 1997).

In order to improve the model, and take advantage of our gene transfer technology, we inserted inside the chamber a muscle pedicle, which was a good candidate for AAV transduction. The presence of the muscle pedicle slightly improved the new tissue area, which was even further increased upon $VEGF_{165}$ expression. The extension of the newly formed tissue area was correlate with the formation of new vessels within the chamber. New vessels were detected both in LacZ and in $VEGF_{165}$ expressing chambers, with a more intense effect in the $VEGF_{165}$ treated animals. In the area within the vein graft we observed a very strong angiogenic effect, not only in close proximity to the transduced muscle, likely sustained by the secreted $VEGF_{165}$, as already reported by some authors (Descamps et al., 1996; Kay et al., 2000). As already mentioned for the other ischemic model, a detail characterization of the new vasculature could not be performed but the achieved final result is a strong indication of the functionality of the new vessels.

In this second model we observed the presence of enlarged, irregular vessels, with a thin α-SMA layer, resembling the vascular lacunae that have already been described in the VEGF expressing muscle(Arsic et al., 2003).These structures were reported to be full of erythrocytes and to contribute to the increased blood volume in the $VEGF_{165}$ expressing muscles. Despite the achievement of good therapeutic results these abnormal structures have to raise serious doubts concerning the degree of maturation of the $VEGF_{165}$ induce vasculature, also considering the failure of the clinical trials based on VEGF overexpression (Kusumanto et al., 2006) (Kastrup et al., 2005).

6.4 Angiopoietin-1: a key player in vessel maturation

The presence of leaky vessels, even if covered by pericytes or by an α-SMA layer, suggested that a further maturation step is required to achieve the formation of a stable and functional vasculature.

Both PET and SPECT imaging provided functional results, complementary to the histological characterization, and suggested a key role for Angiopoietin-1 in the process of vessel maturation. The results obtained in the animals treated with $VEGF_{165}$ alone confirmed the presence of a non-competent vasculature, which was even more evident upon exercise. Indeed, in contrast with the deleterious effects observed after AAV-VEGF165 gene transfer, the co-expression of VEGF165 and Ang-1 resulted in a marked improvement in muscle blood flow, with an almost complete normalization of leakiness

and a significant reduction of the vascular volume. The reduction of the vascular volume was due to the absence of the vascular lacunae observed in the $VEGF_{165}$ treated animals. The pro-maturation effects observed upon Ang-1 co-expression are consistent with literature data supporting a specific ability of Ang-1 to promote vessel maturation. In fact, Ang-1 null embryos die because remodeling and stabilization of the vessels are severely perturbed, while Ang-1 overexpression results in increased vascular branching and resistance to leakage induced by inflammatory agents (Suri et al., 1998; Thurston et al., 1999).

The role of Ang-1 during development was assessed by the use of transgenic animals and Ang-1 was reported to be an essential inducer of new blood vessel formation (Davis et al., 1996). Studies performed in the adult showed that Ang-1 is widely expressed in normal tissues suggesting its role in the stabilization process of pre-existing vessels (Wong et al., 1997). Results from several xenograft models also demonstrated that ectopic expression of Ang-1 results in decreased tumor growth and angiogenesis (Ahmad et al., 2001; Hayes et al., 2000), concomitant with an increased pericyte coverage of the vessels.

The $VEGF_{165}$–induced vessels are characterized by the presence of an α-SMA layer, therefore Ang-1 mechanism of action might consist either effecting smooth muscle cell recruitment or in the strengthening on the endothelial cell-pericyte interaction. Its strong effect in counteracting the vascular permeability induced by $VEGF_{165}$, as observed by SPECT and Miles' assay, would tend to indicate a stabilization of the endothelial-gap junctions. A possible direct effect of Ang-1 in the attraction of mural cells is suggested by the high levels of expression of Tie-2 receptor by mural cells (Asahara et al., 1998; Gamble et al., 2000).

Ang-1 was identified as a Tie-2 receptor agonist, which is mainly expressed on endothelial cell surface. This originally suggested that its pro-maturation activity was exerted through an indirect effect on pericyte recruitment. The discovery of Tie-2 on smooth muscle cell and mural cell precursors suggested a direct role of Ang-1 in the recruitment of pericyte (Metheny-Barlow et al., 2004). The presence of mural cells is postulated to be inhibitory on endothelial cell proliferation. Indeed, several studies have shown that actively proliferating endothelium lacks coverage by mural cells and that the loss of pericytes observed in PDGF-B/PDGFR-β knock-out mice is concomitant with endothelial hyperplasia (Hellstrom et al., 2001). Consistent with this data, during wound healing the recruitment of mural cells coincides with the cessation of vessel growth, suggesting that contact with pericytes might lead to quiescence of endothelial cells (Crocker et al., 1970).

In vitro studies on co-cultures of endothelial and smooth muscle cells using a variety of models further substantiated the decreased growth in response endothelial-smooth muscle cell contact (Orlidge and D'Amore, 1987). Moreover, the absence of a pericyte coverage was reported to lead to major defects in endothelial junction formations (Hellstrom et al., 2001), suggesting a pleiotropic effect exerted by pericytes on vessel growth.

The hypothesis that Ang-1 acts on both pericyte recruitment and endothelail cell-pericyte junctions is in line with our in vivo SPECT data, obtained by the infusion of ^{99m}Tc-DTPA, which showed a clear effect of Ang-1 in reducing the overall vascular permeability of the treated leg, with a concomitant decrease in the intravascular volume. This latter effect might be reasonably due to a specific inhibition of Ang-1 on endothelial cell proliferation, likely mediated by the reinforcement of the contact between endothelial cells and pericytes, and the subsequent prevention of vessel overgrowth. Beside decreased vascular leakiness, our SPECT experiment revealed a strong reduction in the vascular volume in the animals treated with both vectors. This reduction in vascular volume was not expected and was in apparent contradiction with our previous data obtained by PET and ex vivo experiment with fluorescent microspheres (Arsic et al., 2003). In the $VEGF_{165}$ treated animals the increased blood volume was due to the formation of vascular lacunae and their presence could reasonably account for the reduction in muscle perfusion. When the two factors were co-expressed Ang-1 efficiently counteracted the formation of these abnormal vascular structures leading to a reduction in the vascular volume and a simultaneous increase in functional perfusion. The overall results of these imaging experiments suggested that a functional neovascularization does not necessarily requires the development of a great number of new vessels and that a functional test, not restricted to histological or ex vivo quantification, is required to properly characterize the newly formed vasculature. Moreover the results highlighted the feasibility of co-injection of two factors in order to achieve the formation of functional vessels, confirming the notion that the process of vessel formation is complex and involves different players.

6.5 VEGF fine tuning is required for vessel maturation

As also emerged by the aberrant vascular lacunae formed in the response to excessive $VEGF_{165}$, it seems logical that $VEGF_{165}$ is required during the first steps of angiogenesis, while in the later phases it might become unnecessary or even detrimental.

This hypothesis of a two step model suggests to investigate the timing of $VEGF_{165}$ expression, which is required to allow a proper vessel maturation, somehow recapitulating what happens during embryogenesis. The regulated expression of a factors, such as $VEGF_{165,}$ might be addressed by the means of inducible systems, such as the TetON one (Chenuaud et al., 2004; Chtarto et al., 2003; Gossen and Bujard, 1992; Gossen et al., 1995). By this system a fine regulated expression is achievable and would be of interest to study the kinetics of vessel formation induced by VEGF. Moreover due to the fact that the inducible system needs the presence of two transgenes, one coding for the transactivator and on for the protein of interest, the rAAVs might be suitable for this purpose, as already shown by the co-expression of VEGF and Ang-1. Some preliminary results, obtained in our laboratory, confirmed that the inducible system may be applied to the rAAV technology and that it is able to finely regulate the transgene expression both *in vitro* and *in vivo*. By using this inducible system, it was found that transient (less than 15 days) VEGF expression induced the formation of a large number of unstable vessels, with an α-SMA layer, which regressed spontaneously as soon as VEGF production was halted. Thus the presence of the α-SMA layer didn't account for vessel maturation, perhaps because the time of VEGF expression was not sufficient to allow a proper interaction between endothelial cell and pericytes. Indeed, it was found that an overall quenching of the angiogenic phenotype was present at day 30 compared to day 15, concomitant with a progressive stabilization of the interaction between endothelial and mural cells. Moreover, functional results obtained after 30 days of expression, followed by additional 15 days of VEGF withdrawal, allow the maturation of the newly formed vessels. Pinhole scintigraphy using ^{99m}Tc-Tetrofosmin showed that 30 days of VEGF expression led to a not functional vasculature, as expected from the data with the constitutive system, while 30 days of expression followed by 15 days of silencing induced the formation of a fully functional vasculature. The permeability assay confirmed that these two weeks of VEGF withdrawal, following 30 days of VEGF expression, are sufficient to allow the stabilization of the vasculature leading to the complete normalization of vascular permeability.

These results, which are fully consistent with analogous conclusions obtained using transgenic animals (Benjamin and Keshet, 1997; Dor et al., 2002) challenge the usefulness of all gene delivery systems driving short-term transgene expression, such as plasmids and adenoviral vectors, and might explain, at least in part, the overall failure of the gene therapy clinical trials for the induction of therapeutic neovascularization attempted so far (Rissanen and Yla-Herttuala, 2007).

7. CONCLUSIONS

Overall, the findings reported in this thesis reveal that a fine control of the expression of angiogenic factors is needed to achieve the formation of a stable and functional vasculature.

The process of vessel formation and maturation is complex and require the participation of several factors, involved in different steps of the process. We showed that in the early phases VEGF expression is required, endothelial cells, which mainly originate from the local, pre-existing vasculature (Zentilin et al., 2006), become activated to form a set of leaky, immature and irregularly shaped vessels. In the same time interval the recruitment of bone marrow cells, which act in a paracrine manner, induce the pericyte proliferation and migration allowing the formation of a vasculature, less leaky, but not mature. In subsequent phases, the presence of VEGF, or other factors able to induce endothelial cell proliferation, is not required and it was suggested to be detrimental. In this period, a further phase of vessel maturation takes place to ensure the proper acquisition of functional competence of the newly formed vasculature. Indeed, factors that act on pericytes or endothelial cells, for instance PDGF-B or Ang-1, are needed to induce the tightening of the cell-cell or cell-matrix interactions. Moreover, we showed that a detailed histological and functional analysis ex vivo might not be sufficient to characterized the new vasculature, requiring imaging techniques such as PET or SPECT.

These findings might partially explain the reason why some clinical trials for therapeutic angiogenesis failed. The experiments performed with the shortest VEGF isoform, $VEGF_{121}$, showed that this factor is able to induce the formation of new vessels, mostly capillaries, which are leaky and not mature, challenging its use a therapeutic gene in clinical trials. Indeed, two clinical trials, REVASC and RAVE, were not successful and gave contradictory results (Mohler et al., 2003; Stewart et al., 2006). Moreover the experiment of $VEGF_{165}$ and Ang-1 co-expression highlighted the inefficacy of the longer VEGF isoform, $VEGF_{165}$, in inducing the formation of functional vasculature. Therefore, the use of $VEGF_{165}$ as a sole factor in gene therapy might be questionable. In a perspective to translate these results to the clinics, several aspects remain outstanding. Obviously, the clinical translatability of our findings will need further validation in a large animal model, in which important aspects of human pathology can be reproduced, including the occurrence of co-morbidities, such as atherosclerosis and heart failure, which might impact on the efficiency of VEGF-driven therapeutic angiogenesis.

8. REFERENCES

Abramsson, A., Kurup, S., Busse, M., Yamada, S., Lindblom, P., Schallmeiner, E., Stenzel, D., Sauvaget, D., Ledin, J., Ringvall, M., *et al.* (2007). Defective N-sulfation of heparan sulfate proteoglycans limits PDGF-BB binding and pericyte recruitment in vascular development. Genes Dev *21*, 316-331.

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

Adams, R. H., Wilkinson, G. A., Weiss, C., Diella, F., Gale, N. W., Deutsch, U., Risau, W., and Klein, R. (1999). Roles of ephrinB ligands and EphB receptors in cardiovascular development: demarcation of arterial/venous domains, vascular morphogenesis, and sprouting angiogenesis. Genes Dev *13*, 295-306.

Afione, S. A., Conrad, C. K., Kearns, W. G., Chunduru, S., Adams, R., Reynolds, T. C., Guggino, W. B., Cutting, G. R., Carter, B. J., and Flotte, T. R. (1996). In vivo model of adeno-associated virus vector persistence and rescue. J Virol *70*, 3235-3241.

Ahmad, S. A., Liu, W., Jung, Y. D., Fan, F., Wilson, M., Reinmuth, N., Shaheen, R. M., Bucana, C. D., and Ellis, L. M. (2001). The effects of angiopoietin-1 and -2 on tumor growth and angiogenesis in human colon cancer. Cancer Res *61*, 1255-1259.

Allende, M. L., and Proia, R. L. (2002). Sphingosine-1-phosphate receptors and the development of the vascular system. Biochim Biophys Acta *1582*, 222-227.

Allt, G., and Lawrenson, J. G. (2001). Pericytes: cell biology and pathology. Cells Tissues Organs *169*, 1-11.

Arkudas, A., Tjiawi, J., Bleiziffer, O., Grabinger, L., Polykandriotis, E., Beier, J. P., Sturzl, M., Horch, R. E., and Kneser, U. (2007). Fibrin gel-immobilized VEGF and bFGF efficiently stimulate angiogenesis in the AV loop model. Mol Med *13*, 480-487.

Arkudas, A., Tjiawi, J., Saumweber, A., Beier, J. P., Polykandriotis, E., Bleiziffer, O., Horch, R. E., and Kneser, U. (2008). Evaluation of blood vessel ingrowth in fibrin gel subject to type and concentration of growth factors. J Cell Mol Med.

Armulik, A., Abramsson, A., and Betsholtz, C. (2005). Endothelial/pericyte interactions. Circ Res *97*, 512-523.

Arsic, N., Zacchigna, S., Zentilin, L., Ramirez-Correa, G., Pattarini, L., Salvi, A., Sinagra, G., and Giacca, M. (2004). Vascular endothelial growth factor stimulates skeletal muscle regeneration in vivo. Mol Ther *10*, 844-854.

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

Asahara, T., Chen, D., Takahashi, T., Fujikawa, K., Kearney, M., Magner, M., Yancopoulos, G. D., and Isner, J. M. (1998). Tie2 receptor ligands, angiopoietin-1 and angiopoietin-2, modulate VEGF-induced postnatal neovascularization. Circ Res *83*, 233-240.

Asahara, T., and Kawamoto, A. (2004). Endothelial progenitor cells for postnatal vasculogenesis. Am J Physiol Cell Physiol *287*, C572-579.

Autiero, M., Luttun, A., Tjwa, M., and Carmeliet, P. (2003). Placental growth factor and its receptor, vascular endothelial growth factor receptor-1: novel targets for stimulation of ischemic tissue revascularization and inhibition of angiogenic and inflammatory disorders. J Thromb Haemost *1*, 1356-1370.

Baker, A. H. (2004). Designing gene delivery vectors for cardiovascular gene therapy. Prog Biophys Mol Biol *84*, 279-299.

Balasubramaniam, V., Le Cras, T. D., Ivy, D. D., Grover, T. R., Kinsella, J. P., and Abman, S. H. (2003). Role of platelet-derived growth factor in vascular remodeling during pulmonary hypertension in the ovine fetus. Am J Physiol Lung Cell Mol Physiol *284*, L826-833.

Balsam, L. B., Wagers, A. J., Christensen, J. L., Kofidis, T., Weissman, I. L., and Robbins, R. C. (2004). Haematopoietic stem cells adopt mature haematopoietic fates in ischaemic myocardium. Nature *428*, 668-673.

Barleon, B., Sozzani, S., Zhou, D., Weich, H. A., Mantovani, A., and Marme, D. (1996). Migration of human monocytes in response to vascular endothelial growth factor (VEGF) is mediated via the VEGF receptor flt-1. Blood *87*, 3336-3343.

Bartlett, J. S., Wilcher, R., and Samulski, R. J. (2000). Infectious entry pathway of adeno-associated virus and adeno-associated virus vectors. J Virol *74*, 2777-2785.

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

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

Bazzoni, G., and Dejana, E. (2004). Endothelial cell-to-cell junctions: molecular organization and role in vascular homeostasis. Physiol Rev *84*, 869-901.

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

Benjamin, L. E., and Keshet, E. (1997). Conditional switching of vascular endothelial growth factor (VEGF) expression in tumors: induction of endothelial cell shedding and regression of hemangioblastoma-like vessels by VEGF withdrawal. Proc Natl Acad Sci U S A *94*, 8761-8766.

Bergers, G., and Benjamin, L. E. (2003). Tumorigenesis and the angiogenic switch. Nat Rev Cancer *3*, 401-410.

Bish, L. T., Morine, K., Sleeper, M. M., Sanmiguel, J., Wu, D., Gao, G., Wilson, J. M., and Sweeney, L. (2008). AAV9 Provides Global Cardiac Gene Transfer Superior to AAV1, AAV6, AAV7, and AAV8 in the Mouse and Rat. Hum Gene Ther.

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

Bostrom, H., Willetts, K., Pekny, M., Leveen, P., Lindahl, P., Hedstrand, H., Pekna, M., Hellstrom, M., Gebre-Medhin, S., Schalling, M*., et al.* (1996). PDGF-A signaling is a critical event in lung alveolar myofibroblast development and alveogenesis. Cell *85*, 863-873.

Cao, R., Brakenhielm, E., Pawliuk, R., Wariaro, D., Post, M. J., Wahlberg, E., Leboulch, P., and Cao, Y. (2003). Angiogenic synergism, vascular stability and improvement of hind-limb ischemia by a combination of PDGF-BB and FGF-2. Nat Med *9*, 604-613.

Cao, Y., Hong, A., Schulten, H., and Post, M. J. (2005). Update on therapeutic neovascularization. Cardiovasc Res *65*, 639-648.

Cao, Y., Linden, P., Farnebo, J., Cao, R., Eriksson, A., Kumar, V., Qi, J. H., Claesson-Welsh, L., and Alitalo, K. (1998). Vascular endothelial growth factor C induces angiogenesis in vivo. Proc Natl Acad Sci U S A *95*, 14389-14394.

Carmeliet, P. (2000). Mechanisms of angiogenesis and arteriogenesis. Nat Med *6*, 389-395.

Carmeliet, P. (2003). Angiogenesis in health and disease. Nat Med *9*, 653-660.

Carmeliet, P. (2005). Angiogenesis in life, disease and medicine. Nature *438*, 932-936.

Carmeliet, P., and Collen, D. (1999). Role of vascular endothelial growth factor and vascular endothelial growth factor receptors in vascular development. Curr Top Microbiol Immunol *237*, 133-158.

Carmeliet, P., Ferreira, V., Breier, G., Pollefeyt, S., Kieckens, L., Gertsenstein, M., Fahrig, M., Vandenhoeck, A., Harpal, K., Eberhardt, C*., et al.* (1996). Abnormal blood vessel development and lethality in embryos lacking a single VEGF allele. Nature *380*, 435-439.

Carmeliet, P., Moons, L., Luttun, A., Vincenti, V., Compernolle, V., De Mol, M., Wu, Y., Bono, F., Devy, L., Beck, H*., et al.* (2001). Synergism between vascular endothelial growth factor and placental growth factor contributes to angiogenesis and plasma extravasation in pathological conditions. Nat Med *7*, 575-583.

Carmeliet, P., Ng, Y. S., Nuyens, D., Theilmeier, G., Brusselmans, K., Cornelissen, I., Ehler, E., Kakkar, V. V., Stalmans, I., Mattot, V., *et al.* (1999). Impaired myocardial angiogenesis and ischemic cardiomyopathy in mice lacking the vascular endothelial growth factor isoforms VEGF164 and VEGF188. Nat Med *5*, 495-502.

Carvalho, R. L., Jonker, L., Goumans, M. J., Larsson, J., Bouwman, P., Karlsson, S., Dijke, P. T., Arthur, H. M., and Mummery, C. L. (2004). Defective paracrine signalling by TGFbeta in yolk sac vasculature of endoglin mutant mice: a paradigm for hereditary haemorrhagic telangiectasia. Development *131*, 6237-6247.

Chenuaud, P., Larcher, T., Rabinowitz, J. E., Provost, N., Cherel, Y., Casadevall, N., Samulski, R. J., and Moullier, P. (2004). Autoimmune anemia in macaques following erythropoietin gene therapy. Blood *103*, 3303-3304.

Chirmule, N., Propert, K., Magosin, S., Qian, Y., Qian, R., and Wilson, J. (1999). Immune responses to adenovirus and adeno-associated virus in humans. Gene Ther *6*, 1574-1583.

Chtarto, A., Bender, H. U., Hanemann, C. O., Kemp, T., Lehtonen, E., Levivier, M., Brotchi, J., Velu, T., and Tenenbaum, L. (2003). Tetracycline-inducible transgene expression mediated by a single AAV vector. Gene Ther *10*, 84-94.

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

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

Crocker, D. J., Murad, T. M., and Geer, J. C. (1970). Role of the pericyte in wound healing. An ultrastructural study. Exp Mol Pathol *13*, 51-65.

Dafni, H., Landsman, L., Schechter, B., Kohen, F., and Neeman, M. (2002). MRI and fluorescence microscopy of the acute vascular response to VEGF165: vasodilation, hyper-permeability and lymphatic uptake, followed by rapid inactivation of the growth factor. NMR Biomed *15*, 120-131.

Davis, G. E., and Senger, D. R. (2005). Endothelial extracellular matrix: biosynthesis, remodeling, and functions during vascular morphogenesis and neovessel stabilization. Circ Res *97*, 1093-1107.

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

Davis, S., and Yancopoulos, G. D. (1999). The angiopoietins: Yin and Yang in angiogenesis. Curr Top Microbiol Immunol *237*, 173-185.

Daya, S., and Berns, K. I. (2008). Gene therapy using adeno-associated virus vectors. Clin Microbiol Rev *21*, 583-593.

Dejana, E. (2004). Endothelial cell-cell junctions: happy together. Nat Rev Mol Cell Biol *5*, 261-270.

Der Sarkissian, S., Grobe, J. L., Yuan, L., Narielwala, D. R., Walter, G. A., Katovich, M. J., and Raizada, M. K. (2008). Cardiac overexpression of angiotensin converting enzyme 2 protects the heart from ischemia-induced pathophysiology. Hypertension *51*, 712-718.

Descamps, V., Duffour, M. T., Mathieu, M. C., Fernandez, N., Cordier, L., Abina, M. A., Kremer, E., Perricaudet, M., and Haddada, H. (1996). Strategies for cancer gene therapy using adenoviral vectors. J Mol Med *74*, 183-189.

Dickson, M. C., Martin, J. S., Cousins, F. M., Kulkarni, A. B., Karlsson, S., and Akhurst, R. J. (1995). Defective haematopoiesis and vasculogenesis in transforming growth factor-beta 1 knock out mice. Development *121*, 1845-1854.

Dixelius, J., Makinen, T., Wirzenius, M., Karkkainen, M. J., Wernstedt, C., Alitalo, K., and Claesson-Welsh, L. (2003). Ligand-induced vascular endothelial growth factor receptor-3 (VEGFR-3) heterodimerization with VEGFR-2 in primary lymphatic endothelial cells regulates tyrosine phosphorylation sites. J Biol Chem *278*, 40973-40979.

Donahue, B. A., McArthur, J. G., Spratt, S. K., Bohl, D., Lagarde, C., Sanchez, L., Kaspar, B. A., Sloan, B. A., Lee, Y. L., Danos, O., and Snyder, R. O. (1999). Selective uptake and sustained expression of AAV vectors following subcutaneous delivery. J Gene Med *1*, 31-42.

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

Favre, D., Blouin, V., Provost, N., Spisek, R., Porrot, F., Bohl, D., Marme, F., Cherel, Y., Salvetti, A., Hurtrel, B., *et al.* (2002). Lack of an immune response against the tetracycline-dependent transactivator correlates with long-term doxycycline-regulated transgene expression in nonhuman primates after intramuscular injection of recombinant adeno-associated virus. J Virol *76*, 11605-11611.

Favre, D., Provost, N., Blouin, V., Blancho, G., Cherel, Y., Salvetti, A., and Moullier, P. (2001). Immediate and long-term safety of recombinant adeno-associated virus injection into the nonhuman primate muscle. Mol Ther *4*, 559-566.

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

Ferrara, N., Gerber, H. P., and LeCouter, J. (2003). The biology of VEGF and its receptors. Nat Med *9*, 669-676.

Ferrari, F. K., Samulski, T., Shenk, T., and Samulski, R. J. (1996). Second-strand synthesis is a rate-limiting step for efficient transduction by recombinant adeno-associated virus vectors. J Virol *70*, 3227-3234.

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

Fong, G. H., Rossant, J., Gertsenstein, M., and Breitman, M. L. (1995). Role of the Flt-1 receptor tyrosine kinase in regulating the assembly of vascular endothelium. Nature *376*, 66-70.

Forsythe, J. A., Jiang, B. H., Iyer, N. V., Agani, F., Leung, S. W., Koos, R. D., and Semenza, G. L. (1996). Activation of vascular endothelial growth factor gene transcription by hypoxia-inducible factor 1. Mol Cell Biol *16*, 4604-4613.

Fortuin, F. D., Vale, P., Losordo, D. W., Symes, J., DeLaria, G. A., Tyner, J. J., Schaer, G. L., March, R., Snell, R. J., Henry, T. D., *et al.* (2003). One-year follow-up of direct myocardial gene transfer of vascular endothelial growth factor-2 using naked plasmid deoxyribonucleic acid by way of thoracotomy in no-option patients. Am J Cardiol *92*, 436-439.

Fournier, E., Birnbaum, D., and Borg, J. P. (1997). [Receptors for factors of the VEGF (Vascular Endothelial Growth Family)]. Bull Cancer *84*, 397-405.

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

Fuh, G., Garcia, K. C., and de Vos, A. M. (2000). The interaction of neuropilin-1 with vascular endothelial growth factor and its receptor flt-1. J Biol Chem *275*, 26690-26695.

Gaffney, M. M., Hynes, S. O., Barry, F., and O'Brien, T. (2007). Cardiovascular gene therapy: current status and therapeutic potential. Br J Pharmacol *152*, 175-188.

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

Gamble, J. R., Drew, J., Trezise, L., Underwood, A., Parsons, M., Kasminkas, L., Rudge, J., Yancopoulos, G., and Vadas, M. A. (2000). Angiopoietin-1 is an antipermeability and anti-inflammatory agent in vitro and targets cell junctions. Circ Res *87*, 603-607.

Gerber, H. P., Condorelli, F., Park, J., and Ferrara, N. (1997). Differential transcriptional regulation of the two vascular endothelial growth factor receptor genes. Flt-1, but not Flk-1/KDR, is up-regulated by hypoxia. J Biol Chem *272*, 23659-23667.

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

Gerber, H. P., Hillan, K. J., Ryan, A. M., Kowalski, J., Keller, G. A., Rangell, L., Wright, B. D., Radtke, F., Aguet, M., and Ferrara, N. (1999). VEGF is required for growth and survival in neonatal mice. Development *126*, 1149-1159.

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

Gerhardt, H., and Betsholtz, C. (2003). Endothelial-pericyte interactions in angiogenesis. Cell Tissue Res *314*, 15-23.

Gille, H., Kowalski, J., Li, B., LeCouter, J., Moffat, B., Zioncheck, T. F., Pelletier, N., and Ferrara, N. (2001). Analysis of biological effects and signaling properties of Flt-1 (VEGFR-1) and KDR (VEGFR-2). A reassessment using novel receptor-specific vascular endothelial growth factor mutants. J Biol Chem *276*, 3222-3230.

Gossen, M., and Bujard, H. (1992). Tight control of gene expression in mammalian cells by tetracycline-responsive promoters. Proc Natl Acad Sci U S A *89*, 5547-5551.

Gossen, M., Freundlieb, S., Bender, G., Muller, G., Hillen, W., and Bujard, H. (1995). Transcriptional activation by tetracyclines in mammalian cells. Science *268*, 1766-1769.

Grimm, D., Kern, A., Rittner, K., and Kleinschmidt, J. A. (1998). Novel tools for production and purification of recombinant adenoassociated virus vectors. Hum Gene Ther *9*, 2745-2760.

Grines, C., Patel, A., Zijlstra, F., Weaver, W. D., Granger, C., and Simes, R. J. (2003). Primary coronary angioplasty compared with intravenous thrombolytic therapy for acute myocardial infarction: six-month follow up and analysis of individual patient data from randomized trials. Am Heart J *145*, 47-57.

Grines, C. L., Watkins, M. W., Helmer, G., Penny, W., Brinker, J., Marmur, J. D., West, A., Rade, J. J., Marrott, P., Hammond, H. K., and Engler, R. L. (2002). Angiogenic Gene Therapy (AGENT) trial in patients with stable angina pectoris. Circulation *105*, 1291-1297.

Grunewald, M., Avraham, I., Dor, Y., Bachar-Lustig, E., Itin, A., Jung, S., Chimenti, S., Landsman, L., Abramovitch, R., and Keshet, E. (2006). VEGF-induced adult neovascularization: recruitment, retention, and role of accessory cells. Cell *124*, 175-189.

Gu, C., Limberg, B. J., Whitaker, G. B., Perman, B., Leahy, D. J., Rosenbaum, J. S., Ginty, D. D., and Kolodkin, A. L. (2002). Characterization of neuropilin-1 structural features that confer binding to semaphorin 3A and vascular endothelial growth factor 165. J Biol Chem *277*, 18069-18076.

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

Hallock, G. G. (2001). Physiological studies using laser Doppler flowmetry to compare blood flow to the zones of the free TRAM flap. Ann Plast Surg *47*, 229-233.

Hattori, K., Heissig, B., Wu, Y., Dias, S., Tejada, R., Ferris, B., Hicklin, D. J., Zhu, Z., Bohlen, P., Witte, L., *et al.* (2002). Placental growth factor reconstitutes hematopoiesis by recruiting VEGFR1(+) stem cells from bone-marrow microenvironment. Nat Med *8*, 841-849.

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

Hayes, A. J., Huang, W. Q., Yu, J., Maisonpierre, P. C., Liu, A., Kern, F. G., Lippman, M. E., McLeskey, S. W., and Li, L. Y. (2000). Expression and function of angiopoietin-1 in breast cancer. Br J Cancer *83*, 1154-1160.

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

Helbling, P. M., Saulnier, D. M., and Brandli, A. W. (2000). The receptor tyrosine kinase EphB4 and ephrin-B ligands restrict angiogenic growth of embryonic veins in Xenopus laevis. Development *127*, 269-278.

Heldin, C. H., and Westermark, B. (1999). Mechanism of action and in vivo role of platelet-derived growth factor. Physiol Rev *79*, 1283-1316.

Hellstrom, M., Gerhardt, H., Kalen, M., Li, X., Eriksson, U., Wolburg, H., and Betsholtz, C. (2001). Lack of pericytes leads to endothelial hyperplasia and abnormal vascular morphogenesis. J Cell Biol *153*, 543-553.

Hellstrom, M., Kalen, M., Lindahl, P., Abramsson, A., and Betsholtz, C. (1999). Role of PDGF-B and PDGFR-beta in recruitment of vascular smooth muscle cells and pericytes during embryonic blood vessel formation in the mouse. Development *126*, 3047-3055.

Hengge, U. R., and Mirmohammadsadegh, A. (2000). Adeno-associated virus expresses transgenes in hair follicles and epidermis. Mol Ther *2*, 188-194.

Henry, T. D., Annex, B. H., McKendall, G. R., Azrin, M. A., Lopez, J. J., Giordano, F. J., Shah, P. K., Willerson, J. T., Benza, R. L., Berman, D. S., *et al.* (2003). The VIVA trial: Vascular endothelial growth factor in Ischemia for Vascular Angiogenesis. Circulation *107*, 1359-1365.

Herzog, S., Sager, H., Khmelevski, E., Deylig, A., and Ito, W. D. (2002). Collateral arteries grow from preexisting anastomoses in the rat hindlimb. Am J Physiol Heart Circ Physiol *283*, H2012-2020.

Herzog, Y., Kalcheim, C., Kahane, N., Reshef, R., and Neufeld, G. (2001). Differential expression of neuropilin-1 and neuropilin-2 in arteries and veins. Mech Dev *109*, 115-119.

Hirschi, K. K., Rohovsky, S. A., Beck, L. H., Smith, S. R., and D'Amore, P. A. (1999). Endothelial cells modulate the proliferation of mural cell precursors via platelet-derived growth factor-BB and heterotypic cell contact. Circ Res *84*, 298-305.

Hirschi, K. K., Rohovsky, S. A., and D'Amore, P. A. (1998). PDGF, TGF-beta, and heterotypic cell-cell interactions mediate endothelial cell-induced recruitment of 10T1/2 cells and their differentiation to a smooth muscle fate. J Cell Biol *141*, 805-814.

Hirschi, K. K., Skalak, T. C., Peirce, S. M., and Little, C. D. (2002). Vascular assembly in natural and engineered tissues. Ann N Y Acad Sci *961*, 223-242.

Huang, K., Andersson, C., Roomans, G. M., Ito, N., and Claesson-Welsh, L. (2001). Signaling properties of VEGF receptor-1 and -2 homo- and heterodimers. Int J Biochem Cell Biol *33*, 315-324.

Humbert, M., Monti, G., Fartoukh, M., Magnan, A., Brenot, F., Rain, B., Capron, F., Galanaud, P., Duroux, P., Simonneau, G., and Emilie, D. (1998). Platelet-derived growth factor expression in primary pulmonary hypertension: comparison of HIV seropositive and HIV seronegative patients. Eur Respir J *11*, 554-559.

Hungerford, J. E., Owens, G. K., Argraves, W. S., and Little, C. D. (1996). Development of the aortic vessel wall as defined by vascular smooth muscle and extracellular matrix markers. Dev Biol *178*, 375-392.

Ikeda, E., Achen, M. G., Breier, G., and Risau, W. (1995). Hypoxia-induced transcriptional activation and increased mRNA stability of vascular endothelial growth factor in C6 glioma cells. J Biol Chem *270*, 19761-19766.

Inagaki, K., Fuess, S., Storm, T. A., Gibson, G. A., McTiernan, C. F., Kay, M. A., and Nakai, H. (2006). Robust systemic transduction with AAV9 vectors in mice: efficient global cardiac gene transfer superior to that of AAV8. Mol Ther *14*, 45-53.

Isner, J. M. (2001). Still more debate over VEGF. Nat Med *7*, 639-641.

Isner, J. M., and Asahara, T. (1999). Angiogenesis and vasculogenesis as therapeutic strategies for postnatal neovascularization. J Clin Invest *103*, 1231-1236.

Isner, J. M., Walsh, K., Symes, J., Pieczek, A., Takeshita, S., Lowry, J., Rosenfield, K., Weir, L., Brogi, E., and Jurayj, D. (1996). Arterial gene transfer for therapeutic angiogenesis in patients with peripheral artery disease. Hum Gene Ther *7*, 959-988.

Jain, R. K. (2003). Molecular regulation of vessel maturation. Nat Med *9*, 685-693.

Jain, R. K., Au, P., Tam, J., Duda, D. G., and Fukumura, D. (2005). Engineering vascularized tissue. Nat Biotechnol *23*, 821-823.

Jain, R. K., and Munn, L. L. (2000). Leaky vessels? Call Ang1! Nat Med *6*, 131-132.

Jeltsch, M., Kaipainen, A., Joukov, V., Meng, X., Lakso, M., Rauvala, H., Swartz, M., Fukumura, D., Jain, R. K., and Alitalo, K. (1997). Hyperplasia of lymphatic vessels in VEGF-C transgenic mice. Science *276*, 1423-1425.

Joukov, V., Kaipainen, A., Jeltsch, M., Pajusola, K., Olofsson, B., Kumar, V., Eriksson, U., and Alitalo, K. (1997). Vascular endothelial growth factors VEGF-B and VEGF-C. J Cell Physiol *173*, 211-215.

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

Kaipainen, A., Korhonen, J., Mustonen, T., van Hinsbergh, V. W., Fang, G. H., Dumont, D., Breitman, M., and Alitalo, K. (1995). Expression of the fms-like tyrosine kinase 4 gene becomes restricted to lymphatic endothelium during development. Proc Natl Acad Sci U S A *92*, 3566-3570.

Kalluri, R. (2003). Basement membranes: structure, assembly and role in tumour angiogenesis. Nat Rev Cancer *3*, 422-433.

Karkkainen, M. J., Ferrell, R. E., Lawrence, E. C., Kimak, M. A., Levinson, K. L., McTigue, M. A., Alitalo, K., and Finegold, D. N. (2000). Missense mutations interfere with VEGFR-3 signalling in primary lymphoedema. Nat Genet *25*, 153-159.

Karkkainen, M. J., Jussila, L., Ferrell, R. E., Finegold, D. N., and Alitalo, K. (2001). Molecular regulation of lymphangiogenesis and targets for tissue oedema. Trends Mol Med *7*, 18-22.

Karkkainen, M. J., Makinen, T., and Alitalo, K. (2002). Lymphatic endothelium: a new frontier of metastasis research. Nat Cell Biol *4*, E2-5.

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

Karlsson, L., Lindahl, P., Heath, J. K., and Betsholtz, C. (2000). Abnormal gastrointestinal development in PDGF-A and PDGFR-(alpha) deficient mice implicates a novel mesenchymal structure with putative instructive properties in villus morphogenesis. Development *127*, 3457-3466.

Kasahara, Y., Tuder, R. M., Taraseviciene-Stewart, L., Le Cras, T. D., Abman, S., Hirth, P. K., Waltenberger, J., and Voelkel, N. F. (2000). Inhibition of VEGF receptors causes lung cell apoptosis and emphysema. J Clin Invest *106*, 1311-1319.

Kastrup, J. (2003). Therapeutic angiogenesis in ischemic heart disease: gene or recombinant vascular growth factor protein therapy? Curr Gene Ther *3*, 197-206.

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

Kawasaki, T., Kitsukawa, T., Bekku, Y., Matsuda, Y., Sanbo, M., Yagi, T., and Fujisawa, H. (1999). A requirement for neuropilin-1 in embryonic vessel formation. Development *126*, 4895-4902.

Kay, M. A., Manno, C. S., Ragni, M. V., Larson, P. J., Couto, L. B., McClelland, A., Glader, B., Chew, A. J., Tai, S. J., Herzog, R. W.*, et al.* (2000). Evidence for gene transfer and expression of factor IX in haemophilia B patients treated with an AAV vector. Nat Genet *24*, 257-261.

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

Kim, I., Kwak, H. J., Ahn, J. E., So, J. N., Liu, M., Koh, K. N., and Koh, G. Y. (1999). Molecular cloning and characterization of a novel angiopoietin family protein, angiopoietin-3. FEBS Lett *443*, 353-356.

Kitsukawa, T., Shimono, A., Kawakami, A., Kondoh, H., and Fujisawa, H. (1995). Overexpression of a membrane protein, neuropilin, in chimeric mice causes anomalies in the cardiovascular system, nervous system and limbs. Development *121*, 4309-4318.

Kotin, R. M., Siniscalco, M., Samulski, R. J., Zhu, X. D., Hunter, L., Laughlin, C. A., McLaughlin, S., Muzyczka, N., Rocchi, M., and Berns, K. I. (1990). Site-specific integration by adeno-associated virus. Proc Natl Acad Sci U S A *87*, 2211-2215.

Kusumanto, Y. H., van Weel, V., Mulder, N. H., Smit, A. J., van den Dungen, J. J., Hooymans, J. M., Sluiter, W. J., Tio, R. A., Quax, P. H., Gans, R. O.*, et al.* (2006). Treatment with intramuscular vascular endothelial growth factor gene compared with placebo for patients with diabetes mellitus and critical limb ischemia: a double-blind randomized trial. Hum Gene Ther *17*, 683-691.

Lahteenvuo, J. E., Lahteenvuo, M. T., Kivela, A., Rosenlew, C., Falkevall, A., Klar, J., Heikura, T., Rissanen, T. T., Vahakangas, E., Korpisalo, P.*, et al.* (2009). 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 *119*, 845-856.

Lanner, F., Sohl, M., and Farnebo, F. (2007). Functional arterial and venous fate is determined by graded VEGF signaling and notch status during embryonic stem cell differentiation. Arterioscler Thromb Vasc Biol *27*, 487-493.

Laschke, M. W., Harder, Y., Amon, M., Martin, I., Farhadi, J., Ring, A., Torio-Padron, N., Schramm, R., Rucker, M., Junker, D.*, et al.* (2006). Angiogenesis in tissue engineering: breathing life into constructed tissue substitutes. Tissue Eng *12*, 2093-2104.

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

Lee, R. J., Springer, M. L., Blanco-Bose, W. E., Shaw, R., Ursell, P. C., and Blau, H. M. (2000). VEGF gene delivery to myocardium: deleterious effects of unregulated expression. Circulation *102*, 898-901.

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

Leveen, P., Pekny, M., Gebre-Medhin, S., Swolin, B., Larsson, E., and Betsholtz, C. (1994). Mice deficient for PDGF B show renal, cardiovascular, and hematological abnormalities. Genes Dev *8*, 1875-1887.

Lindahl, P., and Betsholtz, C. (1998). Not all myofibroblasts are alike: revisiting the role of PDGF-A and PDGF-B using PDGF-targeted mice. Curr Opin Nephrol Hypertens *7*, 21-26.

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

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

Liu, Y., Cox, S. R., Morita, T., and Kourembanas, S. (1995). Hypoxia regulates vascular endothelial growth factor gene expression in endothelial cells. Identification of a 5' enhancer. Circ Res *77*, 638-643.

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

Lokmic, Z., Stillaert, F., Morrison, W. A., Thompson, E. W., and Mitchell, G. M. (2007). An arteriovenous loop in a protected space generates a permanent, highly vascular, tissue-engineered construct. Faseb J *21*, 511-522.

Losordo, D. W., Vale, P. R., Hendel, R. C., Milliken, C. E., Fortuin, F. D., Cummings, N., Schatz, R. A., Asahara, T., Isner, J. M., and Kuntz, R. E. (2002). Phase 1/2 placebo-controlled, double-blind, dose-escalating trial of myocardial vascular endothelial growth factor 2 gene transfer by catheter delivery in patients with chronic myocardial ischemia. Circulation *105*, 2012-2018.

Losordo, D. W., Vale, P. R., Symes, J. F., Dunnington, C. H., Esakof, D. D., Maysky, M., Ashare, A. B., Lathi, K., and Isner, J. M. (1998). Gene therapy for myocardial angiogenesis: initial clinical results with direct myocardial injection of phVEGF165 as sole therapy for myocardial ischemia. Circulation *98*, 2800-2804.

Maglione, D., Guerriero, V., Rambaldi, M., Russo, G., and Persico, M. G. (1993a). Translation of the placenta growth factor mRNA is severely affected by a small open reading frame localized in the 5' untranslated region. Growth Factors *8*, 141-152.

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

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

Magovern, C. J., Mack, C. A., Zhang, J., Rosengart, T. K., Isom, O. W., and Crystal, R. G. (1997). Regional angiogenesis induced in nonischemic tissue by an adenoviral vector expressing vascular endothelial growth factor. Hum Gene Ther *8*, 215-227.

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

Makinen, K., Manninen, H., Hedman, M., Matsi, P., Mussalo, H., Alhava, E., and Yla-Herttuala, S. (2002). Increased vascularity detected by digital subtraction angiography after VEGF gene transfer to human lower limb artery: a randomized, placebo-controlled, double-blinded phase II study. Mol Ther *6*, 127-133.

Makinen, T., Jussila, L., Veikkola, T., Karpanen, T., Kettunen, M. I., Pulkkanen, K. J., Kauppinen, R., Jackson, D. G., Kubo, H., Nishikawa, S., *et al.* (2001). Inhibition of lymphangiogenesis with resulting lymphedema in transgenic mice expressing soluble VEGF receptor-3. Nat Med *7*, 199-205.

Manno, C. S., Pierce, G. F., Arruda, V. R., Glader, B., Ragni, M., Rasko, J. J., Ozelo, M. C., Hoots, K., Blatt, P., Konkle, B., *et al.* (2006). Successful transduction of liver in hemophilia by AAV-Factor IX and limitations imposed by the host immune response. Nat Med *12*, 342-347.

Marconcini, L., Marchio, S., Morbidelli, L., Cartocci, E., Albini, A., Ziche, M., Bussolino, F., and Oliviero, S. (1999). c-fos-induced growth factor/vascular endothelial growth factor D induces angiogenesis in vivo and in vitro. Proc Natl Acad Sci U S A *96*, 9671-9676.

Marti, H. H., and Risau, W. (1998). Systemic hypoxia changes the organ-specific distribution of vascular endothelial growth factor and its receptors. Proc Natl Acad Sci U S A *95*, 15809-15814.

Maynard, S. E., Min, J. Y., Merchan, J., Lim, K. H., Li, J., Mondal, S., Libermann, T. A., Morgan, J. P., Sellke, F. W., Stillman, I. E., *et al.* (2003). Excess placental soluble fms-like tyrosine kinase 1 (sFlt1) may contribute to endothelial dysfunction, hypertension, and proteinuria in preeclampsia. J Clin Invest *111*, 649-658.

McDonald, D. M., and Choyke, P. L. (2003). Imaging of angiogenesis: from microscope to clinic. Nat Med *9*, 713-725.

Mesri, E. A., Federoff, H. J., and Brownlee, M. (1995). Expression of vascular endothelial growth factor from a defective herpes simplex virus type 1 amplicon vector induces angiogenesis in mice. Circ Res *76*, 161-167.

Metheny-Barlow, L. J., Tian, S., Hayes, A. J., and Li, L. Y. (2004). Direct chemotactic action of angiopoietin-1 on mesenchymal cells in the presence of VEGF. Microvasc Res *68*, 221-230.

Migdal, M., Huppertz, B., Tessler, S., Comforti, A., Shibuya, M., Reich, R., Baumann, H., and Neufeld, G. (1998). Neuropilin-1 is a placenta growth factor-2 receptor. J Biol Chem *273*, 22272-22278.

Miles, A. A., and Miles, E. M. (1952). Vascular reactions to histamine, histamine-liberator and leukotaxine in the skin of guinea-pigs. J Physiol *118*, 228-257.

Minchenko, A., Salceda, S., Bauer, T., and Caro, J. (1994). Hypoxia regulatory elements of the human vascular endothelial growth factor gene. Cell Mol Biol Res *40*, 35-39.

Mingozzi, F., Maus, M. V., Hui, D. J., Sabatino, D. E., Murphy, S. L., Rasko, J. E., Ragni, M. V., Manno, C. S., Sommer, J., Jiang, H., *et al.* (2007). CD8(+) T-cell responses to adeno-associated virus capsid in humans. Nat Med *13*, 419-422.

Mitchell, T. S., Bradley, J., Robinson, G. S., Shima, D. T., and Ng, Y. S. (2008). RGS5 expression is a quantitative measure of pericyte coverage of blood vessels. Angiogenesis *11*, 141-151.

Mohler, E. R., 3rd, Rajagopalan, S., Olin, J. W., Trachtenberg, J. D., Rasmussen, H., Pak, R., and Crystal, R. G. (2003). Adenoviral-mediated gene transfer of vascular endothelial growth factor in critical limb ischemia: safety results from a phase I trial. Vasc Med *8*, 9-13.

Morikawa, S., Baluk, P., Kaidoh, T., Haskell, A., Jain, R. K., and McDonald, D. M. (2002). Abnormalities in pericytes on blood vessels and endothelial sprouts in tumors. Am J Pathol *160*, 985-1000.

Morrison, W. A. (2009). Progress in tissue engineering of soft tissue and organs. Surgery *145*, 127-130.

Nabel, E. G., Plautz, G., and Nabel, G. J. (1990). Site-specific gene expression in vivo by direct gene transfer into the arterial wall. Science *249*, 1285-1288.

Neufeld, G., Cohen, T., Gengrinovitch, S., and Poltorak, Z. (1999). Vascular endothelial growth factor (VEGF) and its receptors. Faseb J *13*, 9-22.

Neufeld, G., Cohen, T., Shraga, N., Lange, T., Kessler, O., and Herzog, Y. (2002). The neuropilins: multifunctional semaphorin and VEGF receptors that modulate axon guidance and angiogenesis. Trends Cardiovasc Med *12*, 13-19.

Nor, J. E., and Polverini, P. J. (1999). Role of endothelial cell survival and death signals in angiogenesis. Angiogenesis *3*, 101-116.

Oike, Y., Yasunaga, K., Ito, Y., Matsumoto, S., Maekawa, H., Morisada, T., Arai, F., Nakagata, N., Takeya, M., Masuho, Y., and Suda, T. (2003). Angiopoietin-related growth factor (AGF) promotes epidermal proliferation, remodeling, and regeneration. Proc Natl Acad Sci U S A *100*, 9494-9499.

Oike, Y., Yasunaga, K., and Suda, T. (2004). Angiopoietin-related/angiopoietin-like proteins regulate angiogenesis. Int J Hematol *80*, 21-28.

Olofsson, B., Korpelainen, E., Pepper, M. S., Mandriota, S. J., Aase, K., Kumar, V., Gunji, Y., Jeltsch, M. M., Shibuya, M., Alitalo, K., and Eriksson, U. (1998). Vascular endothelial growth factor B (VEGF-B) binds to VEGF receptor-1 and regulates plasminogen activator activity in endothelial cells. Proc Natl Acad Sci U S A *95*, 11709-11714.

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

Oosthuyse, B., Moons, L., Storkebaum, E., Beck, H., Nuyens, D., Brusselmans, K., Van Dorpe, J., Hellings, P., Gorselink, M., Heymans, S., *et al.* (2001). Deletion of the hypoxia-response element in the vascular endothelial growth factor promoter causes motor neuron degeneration. Nat Genet *28*, 131-138.

Orlidge, A., and D'Amore, P. A. (1987). Inhibition of capillary endothelial cell growth by pericytes and smooth muscle cells. J Cell Biol *105*, 1455-1462.

Paik, J. H., Skoura, A., Chae, S. S., Cowan, A. E., Han, D. K., Proia, R. L., and Hla, T. (2004). Sphingosine 1-phosphate receptor regulation of N-cadherin mediates vascular stabilization. Genes Dev *18*, 2392-2403.

Palomeque, J., Chemaly, E. R., Colosi, P., Wellman, J. A., Zhou, S., Del Monte, F., and Hajjar, R. J. (2007). Efficiency of eight different AAV serotypes in transducing rat myocardium in vivo. Gene Ther *14*, 989-997.

Pereira, D. J., McCarty, D. M., and Muzyczka, N. (1997). The adeno-associated virus (AAV) Rep protein acts as both a repressor and an activator to regulate AAV transcription during a productive infection. J Virol *71*, 1079-1088.

Peters, A. M., Brown, J., Hartnell, G. G., Myers, M. J., Haskell, C., and Lavender, J. P. (1987). Non-invasive measurement of renal blood flow with 99mTc DTPA: comparison with radiolabelled microspheres. Cardiovasc Res *21*, 830-834.

Petrova, T. V., Makinen, T., and Alitalo, K. (1999). Signaling via vascular endothelial growth factor receptors. Exp Cell Res *253*, 117-130.

Pettersson, P. H., Lindskog, E. A., and Owall, A. (2000). Patient-controlled versus nurse-controlled pain treatment after coronary artery bypass surgery. Acta Anaesthesiol Scand *44*, 43-47.

Plouet, J., Schilling, J., and Gospodarowicz, D. (1989). Isolation and characterization of a newly identified endothelial cell mitogen produced by AtT-20 cells. Embo J *8*, 3801-3806.

Poliakova, L., Kovesdi, I., Wang, X., Capogrossi, M. C., and Talan, M. (1999). Vascular permeability effect of adenovirus-mediated vascular endothelial growth factor gene transfer to the rabbit and rat skeletal muscle. J Thorac Cardiovasc Surg *118*, 339-347.

Poltorak, Z., Cohen, T., and Neufeld, G. (2000). The VEGF splice variants: properties, receptors, and usage for the treatment of ischemic diseases. Herz *25*, 126-129.

Polykandriotis, E., Arkudas, A., Horch, R. E., Sturzl, M., and Kneser, U. (2007). Autonomously vascularized cellular constructs in tissue engineering: opening a new perspective for biomedical science. J Cell Mol Med *11*, 6-20.

Pugh, C. W., and Ratcliffe, P. J. (2003). Regulation of angiogenesis by hypoxia: role of the HIF system. Nat Med *9*, 677-684.

Rabinowitz, J. E., Rolling, F., Li, C., Conrath, H., Xiao, W., Xiao, X., and Samulski, R. J. (2002). Cross-packaging of a single adeno-associated virus (AAV) type 2 vector genome into multiple AAV serotypes enables transduction with broad specificity. J Virol *76*, 791-801.

Rajagopalan, S., Shah, M., Luciano, A., Crystal, R., and Nabel, E. G. (2001). Adenovirus-mediated gene transfer of VEGF(121) improves lower-extremity endothelial function and flow reserve. Circulation *104*, 753-755.

Rajantie, I., Ilmonen, M., Alminaite, A., Ozerdem, U., Alitalo, K., and Salven, P. (2004). Adult bone marrow-derived cells recruited during angiogenesis comprise precursors for periendothelial vascular mural cells. Blood *104*, 2084-2086.

Raper, S. E., Chirmule, N., Lee, F. S., Wivel, N. A., Bagg, A., Gao, G. P., Wilson, J. M., and Batshaw, M. L. (2003). Fatal systemic inflammatory response syndrome in a ornithine transcarbamylase deficient patient following adenoviral gene transfer. Mol Genet Metab *80*, 148-158.

Risau, W. (1997). Mechanisms of angiogenesis. Nature *386*, 671-674.

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

Rissanen, T. T., and Yla-Herttuala, S. (2007). Current status of cardiovascular gene therapy. Mol Ther *15*, 1233-1247.

Riviere, C., Danos, O., and Douar, A. M. (2006). Long-term expression and repeated administration of AAV type 1, 2 and 5 vectors in skeletal muscle of immunocompetent adult mice. Gene Ther *13*, 1300-1308.

Rolny, C., Nilsson, I., Magnusson, P., Armulik, A., Jakobsson, L., Wentzel, P., Lindblom, P., Norlin, J., Betsholtz, C., Heuchel, R., *et al.* (2006). Platelet-derived growth factor receptor-beta promotes early endothelial cell differentiation. Blood *108*, 1877-1886.

Rossant, J., and Howard, L. (2002). Signaling pathways in vascular development. Annu Rev Cell Dev Biol *18*, 541-573.

Rouwkema, J., Rivron, N. C., and van Blitterswijk, C. A. (2008). Vascularization in tissue engineering. Trends Biotechnol *26*, 434-441.

Ruoslahti, E. (2002). Specialization of tumour vasculature. Nat Rev Cancer *2*, 83-90.

Sakoda, T., Kasahara, N., and Kedes, L. (2003). Lentivirus vector-mediated gene transfer to cardiomyocytes. Methods Mol Biol *219*, 53-70.

Sandner, P., Wolf, K., Bergmaier, U., Gess, B., and Kurtz, A. (1997). Induction of VEGF and VEGF receptor gene expression by hypoxia: divergent regulation in vivo and in vitro. Kidney Int *51*, 448-453.

Sato, T. N., Tozawa, Y., Deutsch, U., Wolburg-Buchholz, K., Fujiwara, Y., Gendron-Maguire, M., Gridley, T., Wolburg, H., Risau, W., and Qin, Y. (1995). Distinct roles of the receptor tyrosine kinases Tie-1 and Tie-2 in blood vessel formation. Nature *376*, 70-74.

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

Schermuly, R. T., Dony, E., Ghofrani, H. A., Pullamsetti, S., Savai, R., Roth, M., Sydykov, A., Lai, Y. J., Weissmann, N., Seeger, W., and Grimminger, F. (2005). Reversal of experimental pulmonary hypertension by PDGF inhibition. J Clin Invest *115*, 2811-2821.

Schmidt, M., Voutetakis, A., Afione, S., Zheng, C., Mandikian, D., and Chiorini, J. A. (2008). Adeno-associated virus type 12 (AAV12): a novel AAV serotype with sialic acid- and heparan sulfate proteoglycan-independent transduction activity. J Virol *82*, 1399-1406.

Schnepp, B. C., Jensen, R. L., Chen, C. L., Johnson, P. R., and Clark, K. R. (2005). Characterization of adeno-associated virus genomes isolated from human tissues. J Virol *79*, 14793-14803.

Semenza, G. L. (2000). HIF-1: using two hands to flip the angiogenic switch. Cancer Metastasis Rev *19*, 59-65.

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

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

Shibuya, M., Ito, N., and Claesson-Welsh, L. (1999). Structure and function of vascular endothelial growth factor receptor-1 and -2. Curr Top Microbiol Immunol *237*, 59-83.

Shweiki, D., Itin, A., Soffer, D., and Keshet, E. (1992). Vascular endothelial growth factor induced by hypoxia may mediate hypoxia-initiated angiogenesis. Nature *359*, 843-845.

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

Simari, R. D., and O'Brien, T. (2002). Clinical trials in vascular gene therapy: the missing piece of the puzzle. Arch Pathol Lab Med *126*, 317-319.

Simons, M., Annex, B. H., Laham, R. J., Kleiman, N., Henry, T., Dauerman, H., Udelson, J. E., Gervino, E. V., Pike, M., Whitehouse, M. J., *et al.* (2002). Pharmacological treatment of coronary artery disease with

recombinant fibroblast growth factor-2: double-blind, randomized, controlled clinical trial. Circulation *105*, 788-793.

Sims, D. E. (1986). The pericyte--a review. Tissue Cell *18*, 153-174.

Soker, S., Fidder, H., Neufeld, G., and Klagsbrun, M. (1996). Characterization of novel vascular endothelial growth factor (VEGF) receptors on tumor cells that bind VEGF165 via its exon 7-encoded domain. J Biol Chem *271*, 5761-5767.

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

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

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

Stacker, S. A., and Achen, M. G. (1999). The vascular endothelial growth factor family: signalling for vascular development. Growth Factors *17*, 1-11.

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

Stalmans, I., Ng, Y. S., Rohan, R., Fruttiger, M., Bouche, A., Yuce, A., Fujisawa, H., Hermans, B., Shani, M., Jansen, S., *et al.* (2002). Arteriolar and venular patterning in retinas of mice selectively expressing VEGF isoforms. J Clin Invest *109*, 327-336.

Stewart, D. J., Hilton, J. D., Arnold, J. M., Gregoire, J., Rivard, A., Archer, S. L., Charbonneau, F., Cohen, E., Curtis, M., Buller, C. E., *et al.* (2006). Angiogenic gene therapy in patients with nonrevascularizable ischemic heart disease: a phase 2 randomized, controlled trial of AdVEGF(121) (AdVEGF121) versus maximum medical treatment. Gene Ther *13*, 1503-1511.

Stewart, M., Talks, K., Leek, R., Turley, H., Pezzella, F., Harris, A., and Gatter, K. (2002). Expression of angiogenic factors and hypoxia inducible factors HIF 1, HIF 2 and CA IX in non-Hodgkin's lymphoma. Histopathology *40*, 253-260.

Sundberg, C., Ivarsson, M., Gerdin, B., and Rubin, K. (1996). Pericytes as collagen-producing cells in excessive dermal scarring. Lab Invest *74*, 452-466.

Suri, C., Jones, P. F., Patan, S., Bartunkova, S., Maisonpierre, P. C., Davis, S., Sato, T. N., and Yancopoulos, G. D. (1996). Requisite role of angiopoietin-1, a ligand for the TIE2 receptor, during embryonic angiogenesis. Cell *87*, 1171-1180.

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

Tanaka, Y., Sung, K. C., Fumimoto, M., Tsutsumi, A., Kondo, S., Hinohara, Y., and Morrison, W. A. (2006). Prefabricated engineered skin flap using an arteriovenous vascular bundle as a vascular carrier in rabbits. Plast Reconstr Surg *117*, 1860-1875.

Tanaka, Y., Sung, K. C., Tsutsumi, A., Ohba, S., Ueda, K., and Morrison, W. A. (2003). Tissue engineering skin flaps: which vascular carrier, arteriovenous shunt loop or arteriovenous bundle, has more potential for angiogenesis and tissue generation? Plast Reconstr Surg *112*, 1636-1644.

Terman, B. I., Carrion, M. E., Kovacs, E., Rasmussen, B. A., Eddy, R. L., and Shows, T. B. (1991). Identification of a new endothelial cell growth factor receptor tyrosine kinase. Oncogene *6*, 1677-1683.

Thurston, G., Baluk, P., and McDonald, D. M. (2000a). Determinants of endothelial cell phenotype in venules. Microcirculation *7*, 67-80.

Thurston, G., Rudge, J. S., Ioffe, E., Zhou, H., Ross, L., Croll, S. D., Glazer, N., Holash, J., McDonald, D. M., and Yancopoulos, G. D. (2000b). Angiopoietin-1 protects the adult vasculature against plasma leakage. Nat Med *6*, 460-463.

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

Tomasek, J. J., Gabbiani, G., Hinz, B., Chaponnier, C., and Brown, R. A. (2002). Myofibroblasts and mechano-regulation of connective tissue remodelling. Nat Rev Mol Cell Biol *3*, 349-363.

Tsurumi, Y., Takeshita, S., Chen, D., Kearney, M., Rossow, S. T., Passeri, J., Horowitz, J. R., Symes, J. F., and Isner, J. M. (1996). Direct intramuscular gene transfer of naked DNA encoding vascular endothelial growth factor augments collateral development and tissue perfusion. Circulation *94*, 3281-3290.

Valenzuela, D. M., Griffiths, J. A., Rojas, J., Aldrich, T. H., Jones, P. F., Zhou, H., McClain, J., Copeland, N. G., Gilbert, D. J., Jenkins, N. A., *et al.* (1999). Angiopoietins 3 and 4: diverging gene counterparts in mice and humans. Proc Natl Acad Sci U S A *96*, 1904-1909.

Valtola, R., Salven, P., Heikkila, P., Taipale, J., Joensuu, H., Rehn, M., Pihlajaniemi, T., Weich, H., deWaal, R., and Alitalo, K. (1999). VEGFR-3 and its ligand VEGF-C are associated with angiogenesis in breast cancer. Am J Pathol *154*, 1381-1390.

Veikkola, T., and Alitalo, K. (2002). Dual role of Ang2 in postnatal angiogenesis and lymphangiogenesis. Dev Cell *3*, 302-304.

Veikkola, T., Jussila, L., Makinen, T., Karpanen, T., Jeltsch, M., Petrova, T. V., Kubo, H., Thurston, G., McDonald, D. M., Achen, M. G., *et al.* (2001). Signalling via vascular endothelial growth factor receptor-3 is sufficient for lymphangiogenesis in transgenic mice. Embo J *20*, 1223-1231.

Vincent, K. A., Shyu, K. G., Luo, Y., Magner, M., Tio, R. A., Jiang, C., Goldberg, M. A., Akita, G. Y., Gregory, R. J., and Isner, J. M. (2000). Angiogenesis is induced in a rabbit model of hindlimb ischemia by naked DNA encoding an HIF-1alpha/VP16 hybrid transcription factor. Circulation *102*, 2255-2261.

Waltenberger, J., Claesson-Welsh, L., Siegbahn, A., Shibuya, M., and Heldin, C. H. (1994). Different signal transduction properties of KDR and Flt1, two receptors for vascular endothelial growth factor. J Biol Chem *269*, 26988-26995.

Wang, H. U., Chen, Z. F., and Anderson, D. J. (1998). Molecular distinction and angiogenic interaction between embryonic arteries and veins revealed by ephrin-B2 and its receptor Eph-B4. Cell *93*, 741-753.

Wang, Z., Allen, J. M., Riddell, S. R., Gregorevic, P., Storb, R., Tapscott, S. J., Chamberlain, J. S., and Kuhr, C. S. (2007). Immunity to adeno-associated virus-mediated gene transfer in a random-bred canine model of Duchenne muscular dystrophy. Hum Gene Ther *18*, 18-26.

Wilkinson-Berka, J. L., Babic, S., De Gooyer, T., Stitt, A. W., Jaworski, K., Ong, L. G., Kelly, D. J., and Gilbert, R. E. (2004). Inhibition of platelet-derived growth factor promotes pericyte loss and angiogenesis in ischemic retinopathy. Am J Pathol *164*, 1263-1273.

Wong, A. L., Haroon, Z. A., Werner, S., Dewhirst, M. W., Greenberg, C. S., and Peters, K. G. (1997). Tie2 expression and phosphorylation in angiogenic and quiescent adult tissues. Circ Res *81*, 567-574.

Xiao, X., Li, J., and Samulski, R. J. (1996). Efficient long-term gene transfer into muscle tissue of immunocompetent mice by adeno-associated virus vector. J Virol *70*, 8098-8108.

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

Yamada, Y., Takakura, N., Yasue, H., Ogawa, H., Fujisawa, H., and Suda, T. (2001). Exogenous clustered neuropilin 1 enhances vasculogenesis and angiogenesis. Blood *97*, 1671-1678.

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

Yla-Herttuala, S., Markkanen, J. E., and Rissanen, T. T. (2004). Gene therapy for ischemic cardiovascular diseases: some lessons learned from the first clinical trials. Trends Cardiovasc Med *14*, 295-300.

Zachary, I. (2001). Signaling mechanisms mediating vascular protective actions of vascular endothelial growth factor. Am J Physiol Cell Physiol *280*, C1375-1386.

Zentilin, L., Marcello, A., and Giacca, M. (2001). Involvement of cellular double-stranded DNA break binding proteins in processing of the recombinant adeno-associated virus genome. J Virol *75*, 12279-12287.

Zentilin, L., Tafuro, S., Zacchigna, S., Arsic, N., Pattarini, L., Sinigaglia, M., and Giacca, M. (2006). Bone marrow mononuclear cells are recruited to the sites of VEGF-induced neovascularization but are not incorporated into the newly formed vessels. Blood *107*, 3546-3554.

Zhang, L., Yang, N., Park, J. W., Katsaros, D., Fracchioli, S., Cao, G., O'Brien-Jenkins, A., Randall, T. C., Rubin, S. C., and Coukos, G. (2003). Tumor-derived vascular endothelial growth factor up-regulates

angiopoietin-2 in host endothelium and destabilizes host vasculature, supporting angiogenesis in ovarian cancer. Cancer Res *63*, 3403-3412.

Zhao, J., Pettigrew, G. J., Thomas, J., Vandenberg, J. I., Delriviere, L., Bolton, E. M., Carmichael, A., Martin, J. L., Marber, M. S., and Lever, A. M. (2002). Lentiviral vectors for delivery of genes into neonatal and adult ventricular cardiac myocytes in vitro and in vivo. Basic Res Cardiol *97*, 348-358.

Zhong, T. P., Rosenberg, M., Mohideen, M. A., Weinstein, B., and Fishman, M. C. (2000). gridlock, an HLH gene required for assembly of the aorta in zebrafish. Science *287*, 1820-1824.

Ziegelhoeffer, T., Fernandez, B., Kostin, S., Heil, M., Voswinckel, R., Helisch, A., and Schaper, W. (2004). Bone marrow-derived cells do not incorporate into the adult growing vasculature. Circ Res *94*, 230-238.