Promoting vessel stabilization:
toward a safe mode of therapeutic angiogenesis

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Chapter 1

Angiogenesis in health and disease

BLOOD VESSEL GROWTH AND ROLE OF VEGF IN PHYSIOLOGICAL AND PATHOLOGICAL ANGIOGENESIS

1.1 Mechanisms of angiogenesis

The complex body architecture of vertebrates requires an efficient and constant transport and exchange of nutrients, signalling molecules and cells between tissues and organs. This task is carried out by highly branched networks, the blood vessels. Blood vessels constitute the first organ in the embryo and form the largest network in the adult body. When disregulated, however, the formation of new blood vessels contributes to numerous disorders.

Understanding how blood vessels form can contribute to the development of new therapeutic options for a wide range of diseases many of which are leading causes of mortality in Western society, such as cardiac and peripheral artery diseases, diabetic vascular complications and many cancers.

1.1.1 Embryonic vessel formation

In the developing embryo, endothelial cells arise either from mesodermal precursor cells, the angioblasts, or from progenitors that give rise to both endothelial and blood cell precursors, the hemangioblasts (Fig.1). In the yolk sac, hemangioblasts form aggregates in which the inner part develops into hematopoietic precursors and the outer population into endothelial cells (Carmeliet 2000b).

The key molecular players determining the fate of the hemangioblast are not fully clarified. However, several factors have been identified to play a role; in particular, angioblast differentiation may be promoted by VEGF, FGF-2 and VEGFR-2, while VEGFR-1 has been demonstrated to suppress hemangioblast commitment (Ferrara 2001).

After their commitment to the endothelial lineage, angioblasts assemble into a primitive vascular plexus of veins and arteries, in a process called *vasculogenesis*. This

network will be subsequently refined into a functional network through the process of *angiogenesis*, that will be discussed in the next paragraph (Luttun 2002).

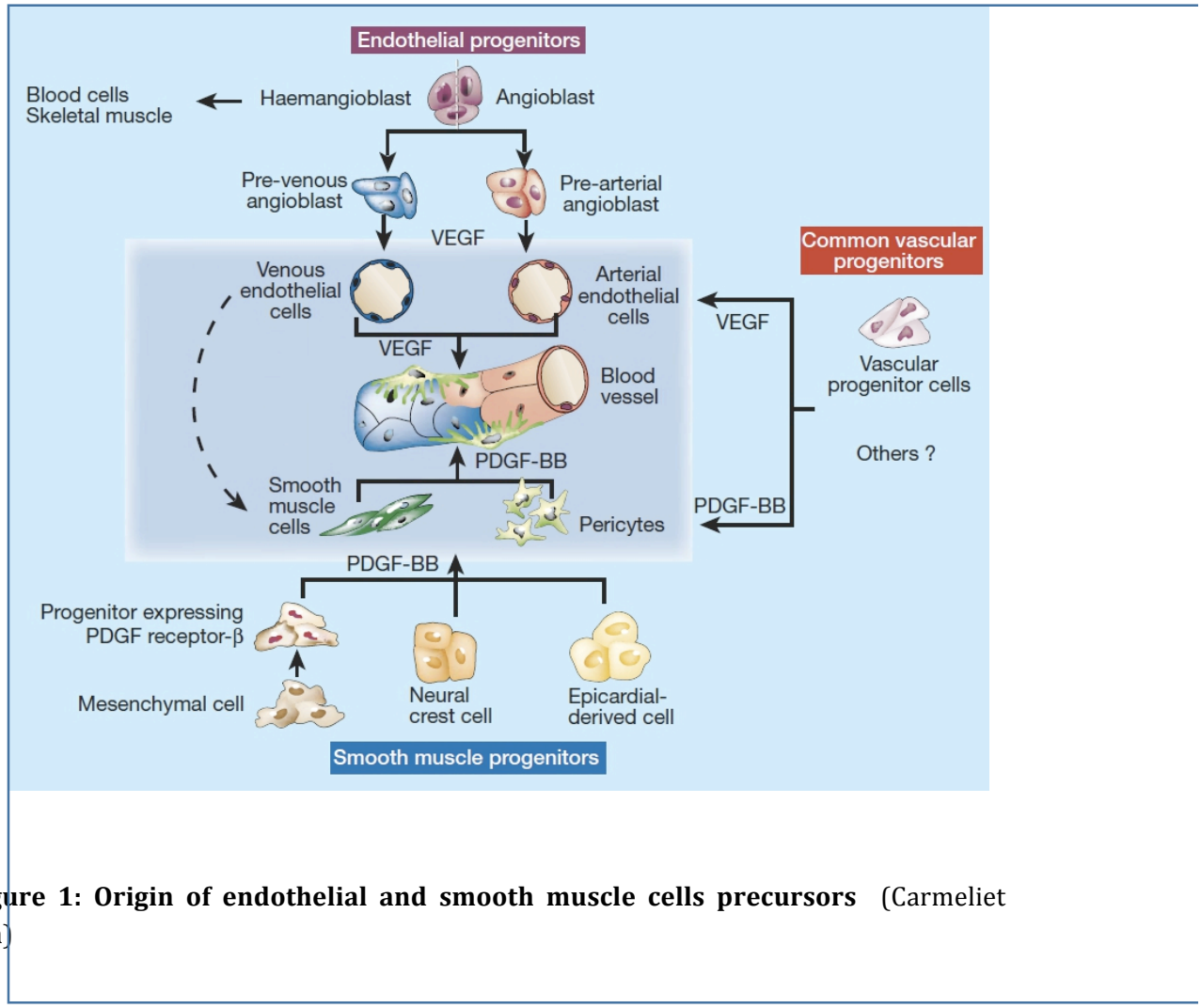

Figure 1: Origin of endothelial and smooth muscle cells precursors (Carmeliet 2000a)

During development of the vascular system, endothelial channels establish contacts with another cellular type, smooth muscle cells (SMCs), that play a fundamental role in the maturation of nascent vasculature, as will be discussed in chapter 2. Coverage by SMCs varies depending on the vessel type: large vessels, such as arteries or veins, are covered by several layers of smooth muscle cells (SMCs), whereas small exchange vessels, such as capillaries, are covered by single cells called pericytes. SMCs also differ in their origin (Fig.1). The first SMC around endothelial tubes in the embryo forms by transdifferentiation from the endothelium in a process that, at least in the heart,

requires Transforming Growth Factor (TGF)-β3 (Nakajima 1997).

TGF-β1, another family member, is involved in the differentiation of mesenchymal stem cells from connective tissue to progenitors that express Platelet Derived Growth Factor Receptor-β (PDGFR-β) (Hellstrom 1999). When the nascent vessels branch out, endothelial cells produce Platelet-Derived Growth Factor-BB (PDGF-BB) that stimulates subsequent growth and differentiation of these precursors.

Pericytes and SMCs of the coronary vessels are derived from a putative progenitor in the epicardial layer of the heart. Cardiac neural crest cells are also a source of SMCs of the large thoracic vessels (Fig.1) (Carmeliet 2000a).

More recently, it has been described a type of embryonic precursor in the mouse that can give rise to both endothelial cells and smooth muscle cells, depending on the growth factor to which it is exposed (Yamashita 2000). In response to PDGF-BB, this precursor differentiates into smooth muscle cells whereas VEGF initiates the developmental pathway to become an endothelial cell (Fig.1).

Important studies revealed the existence of a population of endothelial progenitor cells (EPCs) also circulating in adult peripheral blood. EPCs reside in the bone marrow, in close association with hematopoietic stem cells and the bone marrow stroma but in response to ischemia, vascular trauma or in pathological conditions, they can be mobilized and incorporated into sites of active neovascularization (Asahara 1999) (Luttun 2002).

1.1.2 Blood vessel growth in adults

Vasculogenesis is largely confined to the formation of the primitive vascular structures in the early embryo, although vasculogenic incorporation of precursor cells

may also occur in adults during pathological blood vessel growth, such as in cancer (Rafii 2002). However, the actual contribution of this process to adult vascular growth is still controversial (Purhonen 2008).

The formation of the vast majority of blood vessels during development or in the adult, in both tissue repair and in disease processes, occurs through *angiogenesis*, defined as the formation of new capillaries from pre-existing microvessels (Adams 2010). Microvascular growth by angiogenesis is divided in two phases: first, endothelial cells proliferate and migrate to form a tube-like structure while in a second phase vessels acquire a coverage of mural cells and become mature. Vascular maturation will be discussed in detail in Chapter 2.

Angiogenesis occurs mainly by two broad processes, namely sprouting and intussusceptive angiogenesis; so far, sprouting angiogenesis is the best characterized process at the molecular level.

The angiogenic process starts in response to local ischemia, sensed through the oxygen-sensitive transcription factor hypoxia inducible factor-1α (HIF-1α), which leads to the up-regulation of the expression of many factors involved in the response to hypoxia, among which Vascular Endothelial Growth Factor (VEGF). VEGF activates its receptors in the endothelium and leads to phenotypic changes in some endothelial cells (ECs), called “tip cells” which become motile, invasive and initiate the sprout (Fig.2). Once initiated, the sprouting process is spearheaded by leading endothelial tip cells, that produce long and dynamic filopodia, which are used to probe the environment for directional cues provided by VEGF gradients in the surrounding matrix (Gerhardt 2003). Endothelial cells that follow the tip cells are called “stalk cells”, they proliferate rather than migrating in response to VEGF stimulation and they establish adherent and tight junctions to maintain the integrity of the new sprout (Dejana 2009).

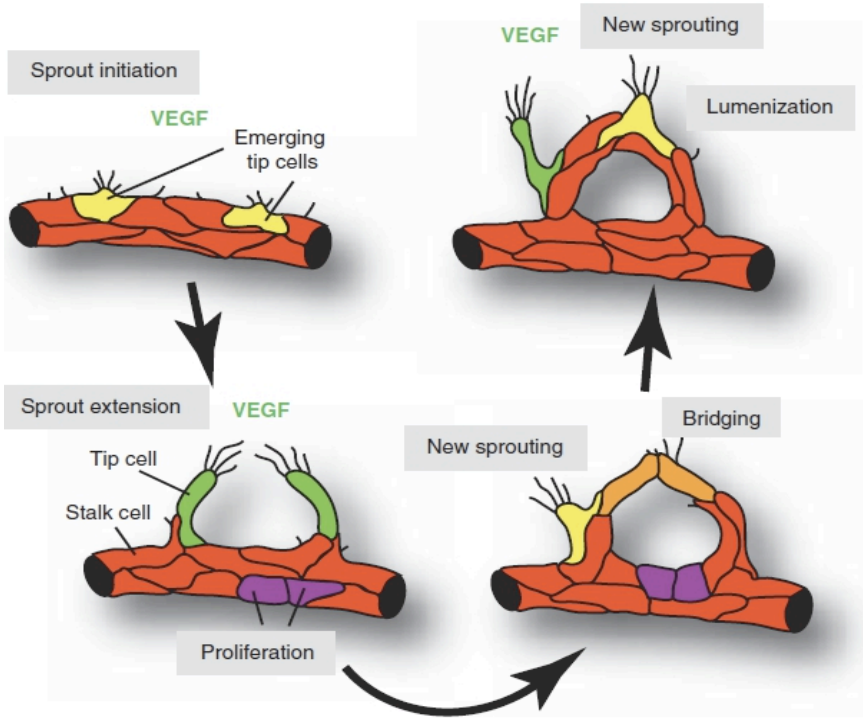

Figure 2: Blood vessel growth by angiogenic sprouting (Adams 2010)

Each new sprout extends until it connects with adjacent sprouts via the respective tip cells to form a continuous lumen that is finally converted into a new blood-carrying tubule (Blum 2008)(Fig.2).

Establishment of blood flow, deposition of a basement membrane and mural cell recruitment all contribute to the subsequent remodelling and maturation of the new vascular connections.

Sprouting is not the only mechanism that contributes to vascular growth; indeed, new vessels can be formed by intussusceptive angiogenesis (IA), the splitting of vessels through the insertion of a tissue pillar (Makanya 2009).

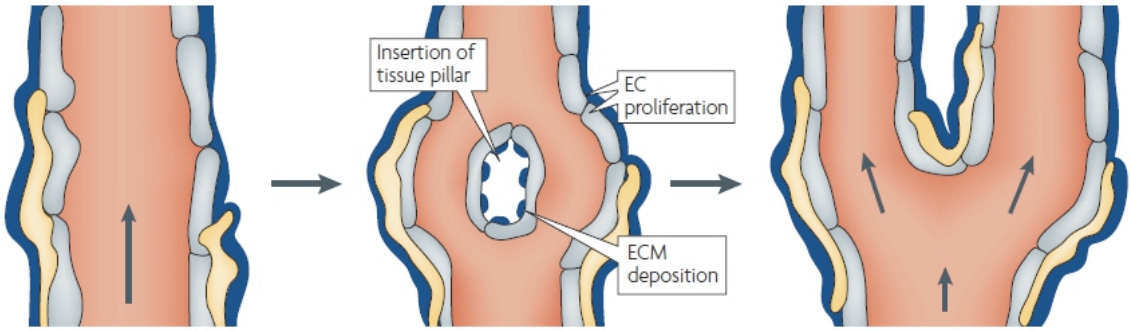

Figure 3: Blood vessel growth by intussusceptive angiogenesis (Adapted from Adams 2007)

The description of intussusceptive angiogenesis is quite recent (Carduff 1986); therefore the molecular control of IA has not been unequivocally elucidated yet.

Probably due to increased shear stress, endothelial cells located directly opposite to each other in the capillary wall protrude into the vessel lumen until they make contact with each other, initiating the formation of a tissue pillar. Such protrusions are followed by endothelial cell contacts, reorganization of endothelial cell junctions and invasion of the pillar core by myofibroblasts and pericytes, which deposit collagen fibrils. In the later remodelling process, pillars enlarge and fuse with adjacent pillars, creating the splitting of the pre-existing vessels into new tubes (Burri 2004).

A temporospatial distribution exists between intussusceptive and sprouting angiogenesis. In fact, IA occurs only on pre-existing vasculature, formed either through sprouting or vasculogenesis. As it has been shown in several studies, particularly in the chicken chorioallantoic membrane (CAM) model, blood vessels initially invade avascular areas by sprouting in the first phase of development, while in the later remodelling phase they grow mainly by IA (Makanya 2009).

The two mechanisms present also some notable differences. Sprouting, on one hand, has the great advantage of being invasive and thus able to bridge vascular gaps, for

example in wound healing. It is, however, a relatively slow process relying largely on cell proliferation. Intussusceptive angiogenesis, on the other hand, is faster, since it occurs within hours or even minutes, and does not initially need cell proliferation (Burri 2004).

1.2 The role of VEGF in angiogenesis

The existence of angiogenic factors was initially postulated on the basis of the strong neovascular response induced by transplanted tumors in transparent chambers (Ide 1939). Independent lines of research converged on the identification of VEGF, finally cloned in 1989, as the potent and diffusible factor responsible for angiogenic growth (Leung 1989) (Keck 1989). Following the hypothesis that this molecule might play a role in the regulation of physiological and pathological growth of blood vessels, the role of VEGF in angiogenesis was the object of intense investigation.

Today it is known that new vessel growth is a highly complex and coordinated process, that can be induced by many factors. Among them, VEGF is the key regulator and the most powerful activator of angiogenesis. For example, it has been shown that the pro-angiogenic effect of the over-expression of other factors such as placental growth factor (PlGF) or hepatocyte growth factor (HGF) is mediated via the up-regulation of VEGF (Korpisalo 2010).

1.2.1 Vascular endothelial growth factors and their receptors

VEGF polypeptides are homodimeric secreted glycoproteins that belong to the PDGF family of growth factors. In mammals, VEGFs are encoded by a family of genes that includes VEGF-A, -B, -C, -D and a related protein, PlGF (Tammela T. 2005). Highly related proteins, called VEGF-E, are encoded by pox viruses of the Orf family and additional variants, collectively called VEGF-F, have been isolated from *Viperinae* snakes venoms.

VEGF-A (commonly reported as VEGF) is the best characterized isoform since it is the most powerful angiogenic growth factor involved in both physiological and pathological angiogenesis, and will be the focus of the next paragraphs.

VEGF-B has a role in coronary artery development; VEGF-C and –D are lymphangiogenic cytokines that can also induce angiogenesis and enhance vascular permeability under certain circumstances. PlGF was originally discovered in the placenta; it is not highly expressed in normal tissues but is expressed by many tumors and in other forms of pathological angiogenesis (Nagy 2007).

VEGF expression is regulated by hypoxia, which stimulates both VEGF mRNA transcription and stabilization. VEGF transcription is under the control of HIF-1, a heterodimeric transcription factor. One of the two subunits, HIF-1α, is rapidly degraded under normoxic conditions; however, under hypoxic conditions, HIF-1α is stabilized, dimerizes with HIF-1β and the complex binds and activates the hypoxia-responsive element in the VEGF promoter (Nagy 2007).

The biological functions of VEGF polypeptides are mediated upon binding to type III receptor tyrosine kinases, VEGFR-1 (Flt-1), VEGFR-2 (KDR/Flk-1) and VEGFR-3 (Flt-4). These receptors are expressed on the cell surface of endothelial cells, vascular smooth muscle cells (VSMCs), bone marrow-derived hematopoietic precursors, macrophages and some malignant cells (Ferrara 2004).

VEGFR-1 and VEGFR-2 are important in blood vascular endothelial cell proliferation, migration and survival. Mice carrying homozigous disruption in either of the two receptors die during early development due to defects in both vasculogenesis and angiogenesis. Embryos lacking functional VEGFR-2 die without mature endothelial or hematopoietic progenitor cells. In contrast, VEGFR-1 deficient mice have normal hematopoietic progenitors cells and endothelial cells that migrate and proliferate but do

not assemble into tubes and functional vessels (Jussila 2002).

VEGFR-2 is expressed in higher copy number than VEGFR-1, but has an approximately ten-fold lower affinity for VEGF-A; however, it is thought that VEGFR-2 is the major receptor transducing VEGF-A signaling in endothelial cells (Ferrara 2004).

In embryos, VEGFR-3 is initially expressed in all vasculature, but during development its expression become restricted to the lymphatic vessels.

VEGFs show distinct patterns of receptor specificity (Fig.4). VEGF-A binds to VEGFR-1 and -2 and to receptor heterodimers, while VEGF-C and -D bind VEGFR-2 and -3.

Receptor-specific interactions have been described for some VEGF variants: PlGF and VEGF-B exclusively bind VEGFR-1 and VEGF-E interacts only with VEGFR-2. VEGF-F variants interact with either VEGFR-1 or -2. VEGF-A, -B and PlGF are predominantly required for blood vessel formation, while VEGF-C and -D are essential for the formation of lymphatic vessels (Cèbe-Suarez 2006).

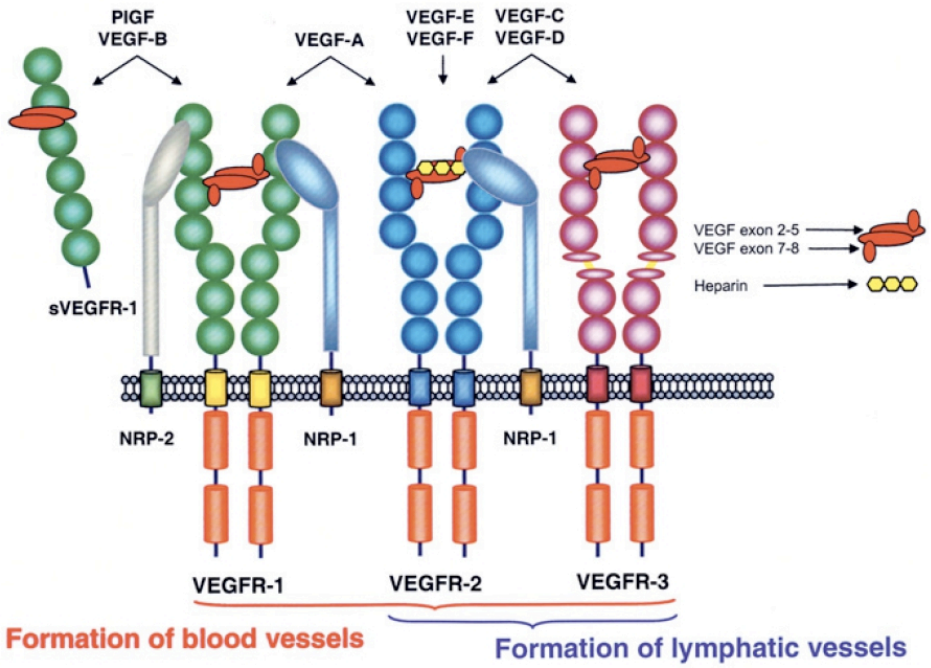

Figure 4: Schematic representation of VEGF family ligands and their receptors (Cèbe-Suarez 2006)

In addition to the VEGF receptor tyrosine kinases, two non kinase receptors, neuropilin-1 and -2 (Nrp-1, Nrp-2) also interact with members of the VEGF family. These receptors are expressed not only on vascular endothelium but also on many types of normal and tumor cells. They have long been known as receptors for the semaphorin/ collapsin family of neuronal guidance mediators (Staton 2007).

Another layer of complexity in the regulation of VEGFs/VEGFRs interactions is added by the fact that VEGF-A is expressed in several isoforms by alternative splicing. These differ by the presence or absence of two domains with affinity for heparin and heparan sulfate proteoglycans and the length of the heparin-binding domain is the molecular basis for the microenvironmental localization of VEGF, fundamental in regulating normal capillary growth and arterio-venous patterning.

$VEGF_{121}$ ($VEGF_{120}$ in mice) lacks both heparin-binding domains and is freely diffusible, while $VEGF_{189}$ ($VEGF_{188}$ in mice) has both heparan sulfate– binding domains and binds tightly to the extracellular matrix (ECM) and the cell surface therefore generating a very steep gradient. $VEGF_{165}$ ($VEGF_{164}$ in mice), that has a single heparin-binding domain, has intermediate affinity for matrix and generates a gradient of intermediate steepness. Transgenic mice have been generated, which selectively express only one of the three isoforms, and have been used to understand how angiogenesis is affected by the microenvironmental distribution of VEGF (reviewed in Banfi 2005).

Mice expressing only $VEGF_{164}$ are viable and show no obvious vascular defects. Conversely, mice expressing only $VEGF_{120}$ exhibit no embryonic lethality, but immediately after birth show severely impaired growth of myocardial capillaries, which were tortuous and dilated. Mice expressing the not diffusible $VEGF_{188}$ isoform, on the other hand, showed a complementary defect with ectopic branching and unusually thin

vessels. Crossing these lines to generate mice expressing $VEGF_{120}$ and $VEGF_{188}$, but not $VEGF_{164}$, abrogated both of these phenotypes and generated normal mice similar to those expressing $VEGF_{164}$ alone (Ruhrberg 2002).

Consistently with these data, expression of $VEGF_{120}$ in the retina is sufficient to drive EC proliferation but not to guide tip cells efficiently. Importantly, however, $VEGF_{120}$ and $VEGF_{164}$ are equally potent at driving EC proliferation in vitro, suggesting that they signal similarly (Gerhardt 2003). This implies that it is the gradient shape that conveys a branching signal to the ECs, rather than qualitative differences in signaling.

1.2.2 Mechanisms of VEGF-induced sprouting angiogenesis

VEGF-A is constitutively expressed at low levels in many normal adult tissues and at higher levels in several types of normal adult epithelium, macrophages and cardiac myocites. It is also expressed at high levels during physiological angiogenesis in development.

The fundamental role of VEGF in embryonic vasculogenesis and angiogenesis was showed in 1996 in two different studies. Both showed that inactivation of a single *Vegf* allele in mice resulted in embryonic lethality between days 11 and 12; mutated embryos exhibited a number of developmental abnormalities, defective vascularization and a reduced number of nucleated red blood cells (Carmeliet 1996) (Ferrara 1996).

VEGF supports both sprouting and intussusceptive angiogenesis. However, since sprouting was described more than 150 years ago, VEGF action is much more characterized in this model of vessel formation rather than in intussusception.

During blood vessel formation through angiogenic sprouting, VEGF, and in particular the formation of its spatial gradients, is fundamental in the selection of tip cells. In the mouse embryonic spinal cord and the in retina, the heparan sulfate-anchored isoform

$VEGF_{164}$ promotes the polarization of tip cells and the directional extension of filopodia. Accordingly, tip cell guidance is defective in mice expressing only the shorter and freely diffusible isoform $VEGF_{120}$ (Gerhardt 2003).

The ability of VEGF to regulate endothelial sprouting is mediated through the Notch signaling pathway, which is well described for its role in cell fate determination and differentiation processes. Notch receptors are transmembrane proteins with large extracellular domains. In mammals, four Notch receptors (Notch1–Notch4) interact with five ligands, namely Delta-like 1, Delta-like 3, Delta-like 4, Jagged1, and Jagged2 (Roca 2007).

VEGF induces Dll4 expression and, because VEGF levels are higher at the vascular front, Dll4 is strongly expressed on tip cells, while Notch signaling activity is greater in stalk cells (Fig.5)

The first cell up-regulating Dll4 becomes a tip cell. Dll4 on the tip cell activates Notch in the neighboring endothelial cells, inducing them to become stalk cells, a process that involves down-regulation of VEGFR-2 expression (Fig.5). As a result, the tip cell prevents the same response to VEGF in stalk cells, which, therefore, lose the competition for the tip position. (Roca 2007).

Once the branch is formed and perfused, and reoxygenation lowers VEGF, maintenance of low VEGF levels secures survival of quiescent endothelial cells and vascular homeostasis.

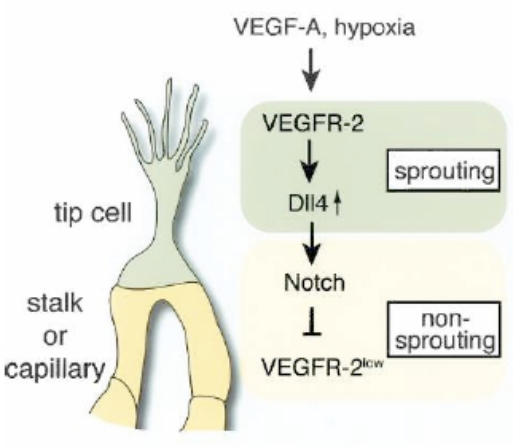

Figure 5: Dll4/Notch and VEGF interaction in tip/stalk cell regulation (adapted from Roca 2007).

1.3 Dose-dependent effects of VEGF

1.3.1 Uncontrolled VEGF over-expression causes pathological angiogenesis

After birth angiogenesis contributes to organ growth but, during adulthood, most blood vessels remain quiescent. However, endothelial cells retain their ability to divide rapidly in response to stimuli, such as hypoxia or inflammation. When this stimulus becomes excessive, blood vessels growth contributes to the pathogenesis of many diseases. VEGF-A, and particularly $VEGF_{165}$, is thought to be the prime cause of pathological angiogenesis and over 26.000 studies have documented its importance in the angiogenic switch in health and disease. Historically, the best know disorders to which excessive angiogenesis contributes significantly are cancer, psoriasis, arthritis and blindness (Carmeliet 2005).

In addition, insufficient vessel growth and abnormal vessel regression not only play a crucial role in the development of heart, brain and limb ischemia, but can also lead to neurodegeneration, hypertension, osteoporosis and other disorders (Carmeliet 2003).

Because of its potency in inducing blood vessel growth, VEGF overexpression with several methods and in different tissues has been investigated to promote therapeutic angiogenesis. Results obtained revealed an intrinsic capacity of VEGF to induce vessels that frequently display morphological and functional abnormalities.

For example, exogenous VEGF administration during embryonic vasculogenesis (Drake 1995) or VEGF overexpression in various tissues in transgenic animals results in malformed, leaky vessels with unusually large and irregular lumens (Dor 2002) (Thurston 1999). The induction of vascular tumors (hemangiomas) as a consequence of excessive VEGF expression was also shown in skeletal muscle (Springer 1998) and subsequently has been reported in myocardium and other tissues, using gene delivery systems such as retrovirally transduced myoblasts (Lee 2000), adenoviral vectors (Pettersson 2000) (Sundberg 2001) and plasmid DNA (Schwarz 2000).

The group of Dvorak rigorously determined the process by which $VEGF_{164}$ induces pathological angiogenesis after over-expression in normal animal tissues from a non-replicative adenoviral vector (Pettersson 2000). Since adenoviral vectors are not integrated into the host genome and are cleared by the host immune system within 10 days, they could follow the effects of $VEGF_{164}$ withdrawal on the different types of newly formed blood vessels.

By using this over-expression platform, they mimiced the induction of pathological angiogenesis and they observed the development of several types of blood vessels. Within 18-24 hours after VEGF expression, they described the formation of “mother vessels” (MVs). MVs are large, thin-walled, serpentine, pericyte-poor vessels and they form from pre-existing vessels by a process that involves vascular basement membrane degradation, pericyte detachment and endothelial cell expansion (Fig.6).

MVs are transient structures that evolve into several different types of daughter vessels; many split into smaller capillary-like structures, others evolve, after 5-7 days of VEGF expression, into glomeruloid microvascular proliferations (GMPs). Like MVs, GMPs require a continuous VEGF supply. Vascular malformations are another type of MVs progeny, characterized by an irregular coating of smooth-muscle cells (Fig.6). Unlike MVs and GMPs, vascular malformations acquire independence from exogenous VEGF.

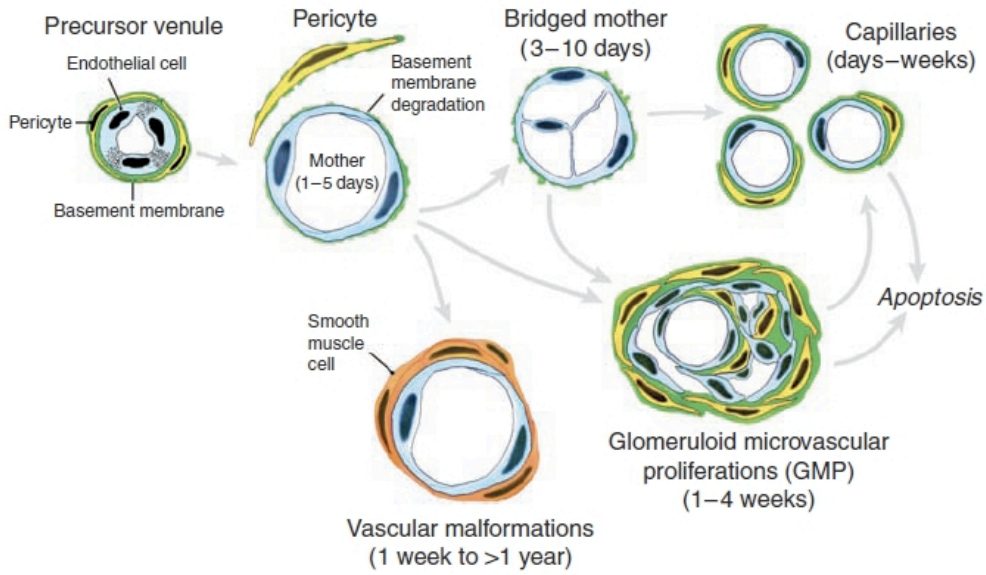

Figure 6: Angiogenic response to VEGF$_{164}$ overexpression in mouse tissue (Nagy 2007)**.**

Mother vessels are commonly observed in tumor angiogenesis and persist indefinitely in some malignant tumors, in vascular telangiectasias and certain hemangiomas. Also the vascular structures into which mother vessels evolve have counterparts in malignant tumors, in vascular malformations, and in benign vascular tumors. In fact, glomeruloid bodies are a feature of glioblastoma multiforme, a highly malignant brain tumor that expresses large amounts of VEGF, and are additionally found in glomeruloid hemangiomas and reactive angioendotheliomas (Nagy 2007).

1.3.2 The control of microenvironmental VEGF dose prevents pathological angiogenesis

Recent evidence from our laboratory indicates that the capacity of VEGF to induce aberrant vasculature is not due to an intrinsically narrow dose-response curve, but rather because the dose delivered must be regulated at the microenvironmental level (Banfi 2005).

To distinguish the effects of the total dose and the level of expression in the microenvironment around each cell, VEGF was delivered to skeletal muscle by using a primary myoblast population that was retrovirally transduced to drive its constitutive expression. Injection of such transduced myoblasts induced the progressive growth of hemangioma-like vessels, even when the total VEGF dose was reduced by serially diluting the cells before implantation (Fig 7a) (Ozawa 2004).

Because retroviral vectors integrate stably into the genome, it was possible to characterize precisely a range of different VEGF expression levels by isolating individual clones in which each cell expressed the same dose. Their implantation *in vivo* made it possible to control VEGF concentration in the microenvironment around each transgenic fiber. This revealed a dose-dependent threshold between normal and aberrant angiogenesis. In fact, microenvironmental VEGF levels varying from 5 to about 70 ng/10^6cells/day induced the growth of stable, uniformly sized capillaries that were associated with pericytes. On the other hand, VEGF levels around 100 ng/10^6/cells/day or higher were always inducing angioma-like vascular structures with few and dysfunctional pericytes, but covered by smooth-muscle actin-positive (SMA^+) mural cells (Fig.7b) (Ozawa 2004).

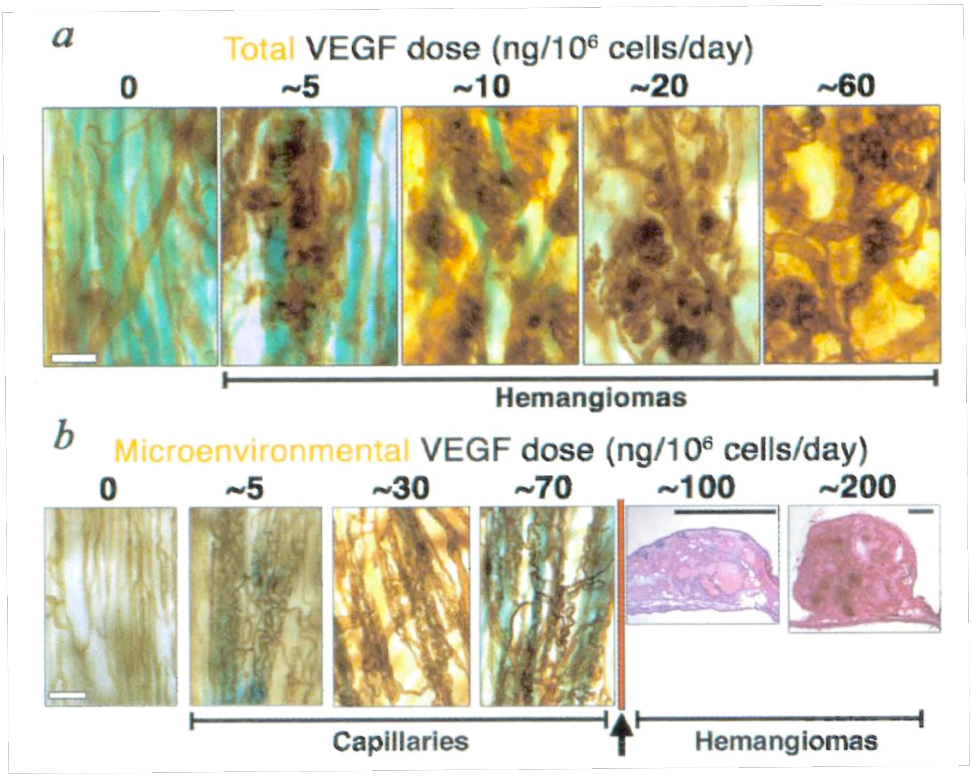

Figure 7: Effect of total versus microenvironmental dose of VEGF (adapted from Ozawa 2004)

The need to control the microenvironmental distribution of VEGF was demonstrated in a murine model of hindlimb ischemia using the same platform of myoblast-mediated gene delivery. Implantation of polyclonal myoblast expressing on average 60 ng/10^6cells/day only moderately increased blood flow, but always induced aberrant leaky vessels. However, when the same total dose was uniformly distributed, after the implantation of a clonal myoblast population, blood flow was fully restored to non-ischemic levels, aberrant vascular growth was completely prevented and the newly induced vessels persisted over 15 months (von Degenfeld 2006).

Therefore, VEGF can induce normal angiogenesis without adverse effects if its expression is tightly controlled at the microenvironmental level, but even few "hotspots" of VEGF expression over the threshold level are sufficient to cause hemangioma formation.

This intrinsic property of VEGF may partly explain the lack of efficacy and the adverse effects of VEGF gene delivery in the first generation of clinical trials for therapeutic angiogenesis, in which only the total dose of vector could be controlled, but not the microenvironmental distribution of expression levels in the tissue (Banfi 2005).

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Chapter 2

Mechanisms of vascular maturation

The importance of pericytes and their cross-talk with endothelium in vascular maturation

2.1 PDGF-BB and pericyte recruitment

The final stage of vessel formation is vascular maturation. The term maturation describes the transition between a growing vascular bed to a quiescent and functional network. This involves the suppression of endothelial proliferation, and the protection against VEGF withdrawal (Adams 2007).

The maturation process starts with investment of nascent endothelial tubes by pericytes. These are vascular mural cells embedded within the endothelial basement membrane that form direct cell-to-cell contact with endothelial cells of capillaries. During vessel sprouting, pericytes are recruited by Platelet-Derived Growth Factor-BB (PDGF-BB), produced by the migrating tip cell.

PDGF was originally purified as a proliferation factor from platelets. The PDGF family consists of four different peptides (PDGF-A, PDGF-B, PDGF-C and PDGF-D) that form five different homo- and heterodimers of PDGF, namely AA, BB, AB, CC and DD.

The expression pattern of PDGFs is complex. PDGF-B is mainly expressed in vascular endothelial cells, megakaryocytes, and neurons. PDGF-A and PDGF-C are expressed in epithelial cells, muscle, and neuronal progenitors. PDGF-D expression is less characterized, but it has been observed in fibroblasts and SMCs at certain locations (Andrae 2008).

PDGFs are potent mitogens and chemoattractants that have critical roles in embryogenesis, angiogenesis and wound repair.

Biological effects of PDGFs are mediated through two tyrosine kinase receptors, PDGFR-α and PDGFR-β. The α receptor binds to chains A, B and C of PDGF, whereas the β receptor binds only the B chain with high affinity. There are *in vivo* evidences that PDGF-AA and PDGF-CC interact with PDGFR-α, while PDGF-BB interacts with PDGFR-β. It is likely that

also PDGF-DD acts through PDGFR-β *in vivo*, but definitive evidence for this is currently lacking (Andrae 2008).

Each of the PDGF-A and -B chains appears in two isoforms, one long and one short. The two isoforms of the PDGF-A chain are generated by alternative splicing, whereas PDGF-B chain isoforms result from a post-translational proteolytic process. Both the A and B long isoforms contain a highly basic amino acid sequence in their C-terminal domains that mediates the interaction between PDGF and components of the extracellular matrix. The matrix major component involved in PDGF binding is likely to be heparan sulfate (Heldin 1999).

During blood vessel sprouting, PDGF-BB expressed by endothelial tip cells acts in a paracrine way and recruits pericytes, which express PDGFR-β.

The fundamental role of the axis PDGF-BB/pericytes in angiogenesis has been demonstrated using different transgenic mouse models with sophisticated manipulations of the PDGF gene or its receptor.

When PDGF-b or PDGFR-β genes are knocked-out, pericyte differentiation is not affected during development, but their recruitment to nascent vasculature is severely impaired, resulting in vascular abnormalities, formation of microaneurysms and bleeding and finally to death soon after birth (Hellstrom 1999) (Lindahl 1997).

The role of PDGF-BB expressed by endothelial cells at the site of active angiogenesis was investigated in mice where the PDGF-b gene was deleted specifically in endothelial cells (pdgf-$b^{lox/-}$). Mice where viable, but displayed a diffuse defect in pericyte recruitment, with pericyte numbers reduced by as much as 90% of normal and microvascular defects in multiple organs (Bjarnegard 2004).

The matrix-binding domain of PDGF-BB regulates its microenvironmental distribution and determines the formation of a steep gradient in the perivascular space. This gradient is fundamental for proper pericyte recruitment and establishment of pericyte/endothelium contact. The physiological importance of this domain was shown using mutant mice in which the PDGF-B retention motif was deleted from the endogenous gene by targeted mutagenesis (pdgf-b$^{ret/ret}$). Such mice developed only about 50% of the normal amount of pericytes and presented defective investment of pericytes in the microvessel wall, severe retinal deterioration, proteinuria and glomerulosclerosis (Lindblom 2003).

The role of pericytes in regulating the morphology and the function of growing vasculature was demonstrated also in conditions of VEGF overexpression. Benjamin et al. demonstrated that, in neonatal retina vascularization, as well as in tumors, association between endothelium and pericytes renders new vessels independent of continued VEGF expression (Benjamin 1998) (Benjamin 1999). In diabetic retinopathy, excessive VEGF production leads to the formation of pericyte-poor and leaky vessels, resulting in blindness (Hammes 2002). Continuous uncontrolled VEGF overexpression in skeletal muscle, by retrovirally-transduced myoblasts, also induced aberrant vessels that were not covered by pericytes and failed to stabilize, remaining dependent on VEGF signaling for survival (Ozawa 2004).

2.2 Cross-talk between endothelium and pericytes

Pericyte recruitment by PDGF-BB is not the only process involved in vascular maturation. In fact, in order to exert their regulatory function, pericytes establish with endothelial cells a complex interaction that involves several pathways. The two best known

and understood are Transforming-Growth Factor (TGF)-β and its receptors and angiopoietins 1 and 2 (Ang-1 and -2) and their receptor Tie2. Other more recently discovered and less characterized signals between pericytes and endothelium include the S1P, Ephrin and Notch pathways.

2.2.1 TGF-β and its multiple role in vascular stabilization

TGF-β is a family of multifunctional cytokines, including three TGF-β isoforms (β1, β2, β3), activins, and bone morphogenetic proteins (BMPs). TGF-β family members have critical and specific roles during embryogenesis and in maintaining the homeostasis of adult tissues. Alterations in their signaling pathways have been linked to a diverse set of developmental disorders and diseases, including cancer, fibrosis, autoimmune and cardiovascular diseases (Goumans 2009).

In particular, several *in vitro* and *in vivo* studies elucidate the role of TGF-β1 signaling pathway in both vasculogenesis and angiogenesis. TGF-β1 regulates basic functions of endothelial cells, such as cell proliferation and differentiation, through ALK1 and ALK5 receptors and their downstream signaling pathways, involving Smad1/5 and Smad2/3, respectively (von Tell D. 2006).

Both endothelial cells and pericytes express TGF-β1 and its receptors; however, only when a cell-to-cell contact is established, TGF-β1, which is secreted in an inactive form, can undergo the cleavage of the latency-associated peptide (LAP) by plasmin and be activated (Hirschi 2003).

TGF-β promotes vessel stabilization in multiple ways. First, TGF-β1 inhibits endothelial cell proliferation and migration, stimulates mural cell differentiation and is required for the

formation of capillary structures (Darland 2002).

Secondly, it has a direct stimulatory effect on the synthesis and deposition of extracellular matrix components. It may also inhibit degradation of the provisional matrix around nascent vessels by inducing plasminogen activator inhibitor 1 in endothelial cells (von Tell 2006).

Generation of mice lacking *Tgfb1* gene or its receptors has revealed its critical role in vascular development. Knock-out mice result in 50% embryonic lethality at E9.5-E10.5 because of defective vasculogenesis. Similarly, knocking-out *Alk1* or *Alk5* genes result in embryonic lethality with the same phenotype. Moreover, targeted deletions in *Alk1* or *Alk5* genes in mice result in vascular abnormalities resembling those described in patients with hereditary hemorrhagic telangiectasia-1 and -2, an autosomal dominant vascular disorder characterized by fragile blood vessels with impaired mural cell coverage (Goumans 2009) (Matthew 2008).

2.2.2 Angiopoietins in vascular stabilization

Angiopoietins (Ang) are the ligands for the endothelium-specific tyrosin kinase receptor Tie-2. The two best characterized members of the family are Ang1 and Ang2.

Ang1 is expressed by pericytes, acts as an agonist for the Tie2 receptor and it reduces vascular permeability in the skin, tumors and in an *in vitro* model of blood–brain barrier (von Tell 2006). Moreover, Ang-1 promotes vascular stabilization by facilitating pericyte recruitment (Hawighorst 2002).

It has been further shown that Ang1 may promote pericytes recruitment by having a direct effect on undifferentiated precursors, since Tie2 expression was identified on a rare

population of mesenchymal cells present in tumor stroma, which may constitute a source of tumor vessel pericytes (De Palma 2005).

The importance of the Ang1-Tie2 signaling pathway was demonstrated in loss-of-function studies: Tie2-deficient embryos die between E10.5 and E12.5 because the primitive capillary plexus fails to remodel and mature, and the same happens with Ang1-deficient mice (Augustin 2009).

Ang2 is a context-dependent ligand of Tie2 receptor and is expressed mainly by endothelial cells at sites of active angiogenesis. In the presence of VEGF, Ang2 promotes sprouting of new blood vessels and remodeling of the vasculature, since it induces the dissociation of pericytes from endothelial cells. In the quiescent vasculature, where Tie2 is constitutively activated by a basal Ang1 expression, Ang2 acts as a functional antagonist of Ang-1, since it binds preferentially to Tie-2 without inducing signal transduction, therefore destabilizing mature vessels (Maisonpierre 1997). The vessel-destabilizing effect of Ang2 has been also demonstrated after transgenic overexpression of Ang2 in a normal retina (Hammes 2004).

Although the loss of Ang2 is not lethal in the embryo, Ang2-deficient mice display a perturbed vessel regression of the hyaloid vessels in the embryonic lens (Augustin 2009).

In contrast to the mild phenotype of Ang-2-deficient mice, mice transgenically overexpressing Ang-2 have an embryonic lethal phenotype similar to the Ang-1-null and Tie-2-null phenotypes (Maisonpierre 1997). The similarity of the Ang-1 loss-of-function phenotype with the Ang-2 gain-of-function phenotype confirms the antagonistic concept of Ang-1 and Ang-2 functions.

2.3 Role of bone marrow recruited cells in vascular stabilization

Pericytes and endothelial cells are not the only two cell types present in the site of active VEGF-induced angiogenesis. In fact there are several evidences that recruited bone marrow-derived circulating cells (RBCCs) home to the site of angiogenesis and contribute to the sprouting of endothelial cells and the stabilization of newly-formed vessels (Murdoch 2008).

$VEGF_{164/5}$ overexpression drives the recruitment of this heterogeneous mix of myeloid cells, as was demonstrated in conditional transgenic animals (Grunewald 2006) or after injection of viral vectors (Zentilin 2006).

Together with VEGF, the chemokine SDF-1 (stroma-derived factor-1) contributes to the site-specific homing pattern of bone-marrow derived cells. The action of VEGF and SDF-1 in the recruitment of RBCCs has been extensively described in pathological conditions, such as in tumors (reviewed in Schmid 2010).

Briefly, in the tumor environment, hypoxia induces the expression of the factors SDF-1, VEGF and granulocyte macrophage colony stimulating factor (GM-CSF), which trigger RBCCs and results in their mobilization, trans-endothelial migration and homing to the tumor (Fig.8) (Shojaei 2007).

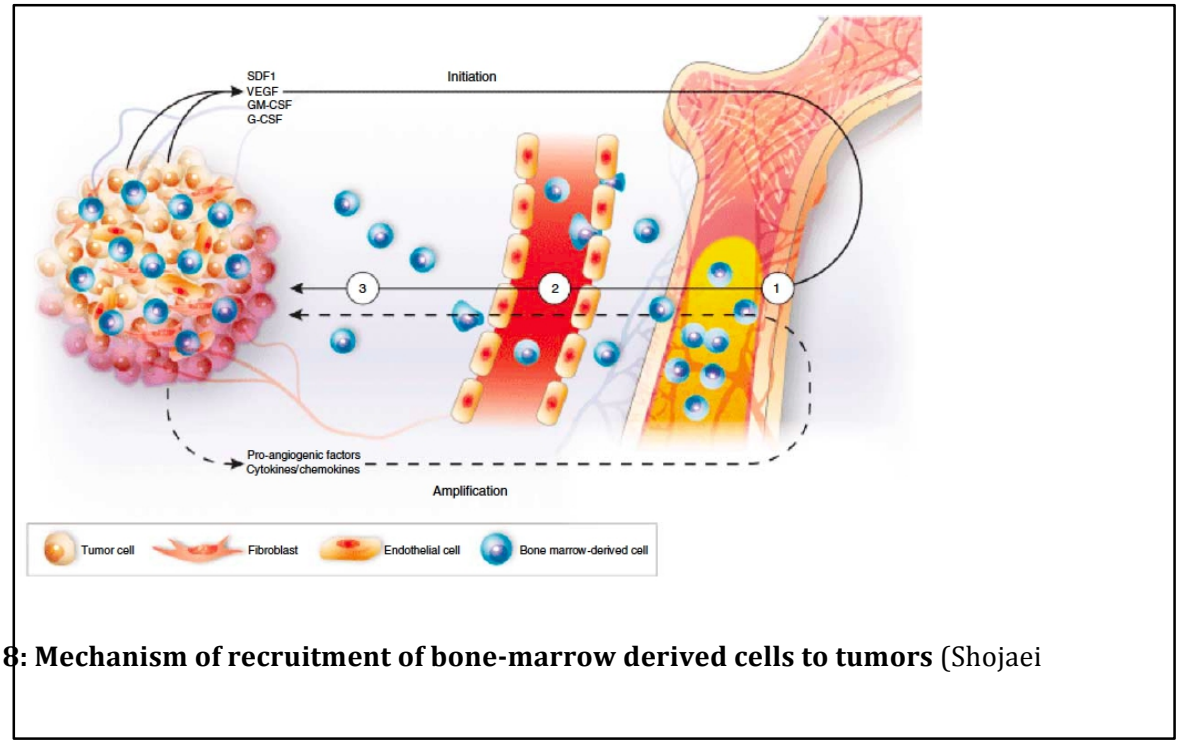

Figure 8: Mechanism of recruitment of bone-marrow derived cells to tumors (Shojaei 2007)

Recently, the role of VEGF and SDF-1 in this process has been also investigated in a non-pathological adult neovascularization. Using a mouse model of inducible overexpression, it has been shown that VEGF initiates a massive infiltration of RBCCs that are trapped by SDF-1 and positioned in a perivascular position. Further, RBCCs have been described to be angio-competent, since they release MMP-9 that facilitates endothelial cell sprouting, and they can augment neovascularization when transplanted in skin wounds (Grunewald 2006).

Further investigations demonstrated a role for another population of bone marrow recruited cells in the process of vascular maturation. Zacchigna et al. demonstrated that cells infiltrating the site of $VEGF_{165}$-induced angiogenesis in skeletal muscle are mainly CD11b+ cells expressing neuropilin-1, defined as NEMs (Nrp1-Expressing Mononuclear cells). NEMs were shown to promote vessel maturation in a paracrine fashion, by

expression of Ang1, TGF-β and PBGF-BB. Therefore, the authors proposed a model in which mature vessel formation relies on the occurrence of two events: the activation of endothelial cells, through the canonical VEGF pathway, and the recruitment of bone marrow-derived cells through the NP-1 receptor. These cells in turn facilitate pericyte or SMC investment of the new vessels (Fig. 9) (Zacchigna 2008).

Furthermore, it has been recently demonstrated that purified NEMs injected into a mouse tumor model improve pericyte coverage of tumor vessels, leading to better vascular function and restoring normoxia (Carrer 2010).

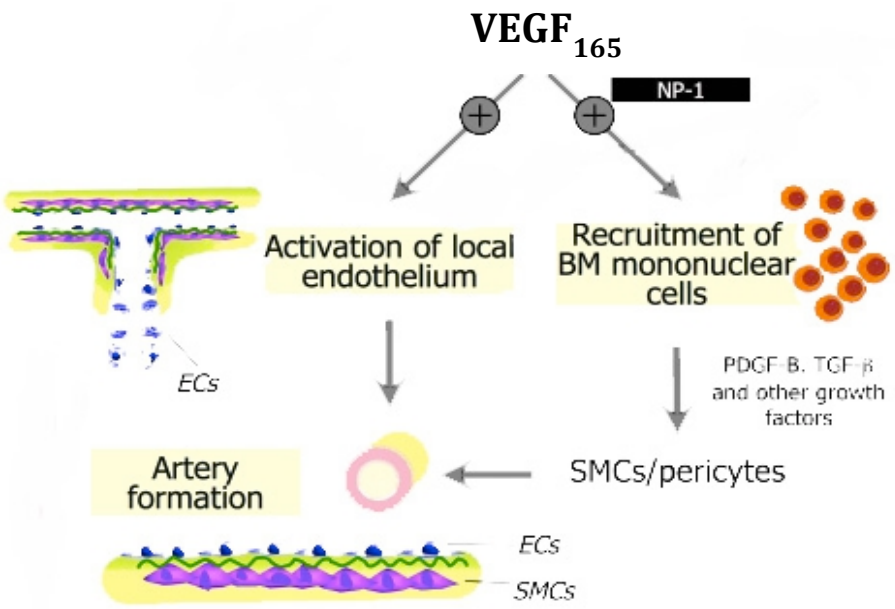

Figure 9: Proposed mechanism to explain the role of bone-marrow recruited cells in vascular maturation (adapted from Zacchigna 2008)

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Chapter 3

Strategies for therapeutic angiogenesis

Current status and limitations of therapeutic angiogenesis for the treatment of ischemic diseases

3.1 Therapeutic angiogenesis for occlusive vascular diseases

Occlusive vascular diseases, which include coronary artery diseases (CAD) and peripheral artery diseases (PAD), are the leading cause of death in the western world, despite advances in medical and surgical therapy. Individuals with initial symptoms are treated with drugs such as vasodilators and antiplatelet agents. As the disease progresses, patients undergo invasive vascular procedures, like coronary artery bypass or catheter-based interventions.

However, current drug treatments often have limited impact on the outcome of advanced lower extremity PAD and in many patients interventional revascularization is not possible because of diffuse vessel disease or unacceptably high operative morbidity and mortality or it has poor long-term results (Vincent 2007).

The increasing knowledge on the biological bases of blood vessels growth led to the idea of restoring the blood supply in ischemic tissues by the delivery of growth factors that control the formation of new vasculature. This strategy, defined as therapeutic angiogenesis, has emerged over the last decade and a half as a very attractive application of gene therapy with the potential to impact the treatment of a large number of patients with end-stage peripheral arterial disease that are not amenable to other options.

Several angiogenic factors have been delivered in a variety of animal models of coronary or limb ischemia, alone or in different combinations, using gene therapy vectors or progenitors cells modified in order to express the desired growth factors. This has uncovered several limitations in the first approaches to therapeutic angiogenesis, which will need to be addressed to fully exploit its potential.

3.1.1 Gene therapy vectors for angiogenesis

The initial studies on gene transfer for therapeutic angiogenesis employed naked plasmid DNA. Plasmid DNA is very easy to produce. However, since only a small amount of plasmid is taken into a cell, gene transfer efficacy *in vivo* is very low and the half-life of the plasmid is short. Carrier molecules have been used to increase the efficacy and the use of plasmid vectors for clinical trials has been approved in several countries. Nevertheless, their clinical relevance is not clear (Rissanen 2007).

Viral vectors have been used to achieve effective gene transfer *in vivo*. The most commonly used, listed in table 1, are adenoviruses (AVs), adeno-associated virus (AAVs), retroviruses and lentiviruses.

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

Table 1: Viral vectors commonly used in angiogenic gene therapy (Adapted from Rissanen 2007)

Adenoviruses are easy to produce at high titers, have a high transgene capacity and can transduce multiple cell type, both replicating and quiescent. Therefore AVs have been

widely used in several gene therapy applications in the past years (http://www.wiley.co.uk/genetherapy/clinical/). AVs produce a very high initial level of gene expression, with a peak few days post-injection. However, the expression ceases after 10-14 days due to their strong immunogenicity which also precludes repeated administration of AVs of the same serotype (Rissanen 2007).

Despite their versatility, the use of AVs in adult skeletal muscle is limited due to their scarce efficacy in transducing adult myofibers. This is mainly due to a structural property of the extracellular matrix surrounding myofibers that has pores with a diameter of around 40 nm. Therefore AVs, which have a size of about 70-100 nm, cannot access the target cells (van Deutekom 1998).

AAVs are small vectors with a limited transgene capacity, but with many advantages that make them one of most used vectors nowadays. AAVs exist in different serotypes with different tropism for target tissues. In particular serotypes 1, 6, 8 and 9 have been reported to be very effective in the adult skeletal muscle and myocardium (Wu 2006). AAVs, like AVs, do not integrate into the host genome but, differently from AVs, they generate only a limited inflammatory reaction, therefore ensuring a long-term gene expression that can last for several months. Importantly, maximal gene expression is not as high as the one induced by AVs, but transgene expression gradually increases over the first 60 days post-injection (Fisher 1997).

Retroviruses were among the first vectors to be used for *in vivo* cardiovascular gene therapy. However, concerns regarding their oncogenic potential have reduced the interest in these vectors in favor of the safer lentiviruses.

Since retro- and lentiviruses integrate stably in the host genome, they both allow indefinite gene expression. On the other hand, they have a low transduction efficiency *in vivo*. For these reasons these vectors are mostly used for *ex vivo* gene transfer into progenitors cells (Karvinen 2010).

3.1.2 Cell-based gene therapy for angiogenesis

The main target tissue for the treatment of peripheral artery disease is skeletal muscle. Muscle tissue can be targeted easily with traditional gene therapy vectors, such as AAVs. However, it has a number of properties that make it a suitable target also for cell-based gene therapy. Firstly, myogenic precursor cells, myoblasts, that are located between the basal lamina and the plasma membrane of myofibers, are relatively easy to isolate and culture *in vitro*; secondly, they can be genetically engineered with high efficiency *in vitro* using retroviral vectors; thirdly, upon intramuscular injection, myoblasts stably fuse with resident adult muscle fibers (Fig. 10) (von Degenfeld 2003). This cell based approach, that provides a rapid method of "adult transgenesis", presents several advantages compared to other gene delivery systems. Myoblast-mediated gene transfer is well suited to study combinations of angiogenic growth factors directly in skeletal muscle, as multiple gene products can be inserted, through multiple sequential infections without loss of myoblast ability to fuse into myofibers *in vivo*. Since the injected myoblasts fuse with pre-existing myofibers, transgene expression is sustained in the long-term. Furthermore, since myoblasts are engineered *in vitro*, the expression of the transgene can be measured and selected, making it a useful tool for the investigation of dose-dependent effects of angiogenic growth factors on vessel phenotypes (von Degenfeld 2003).

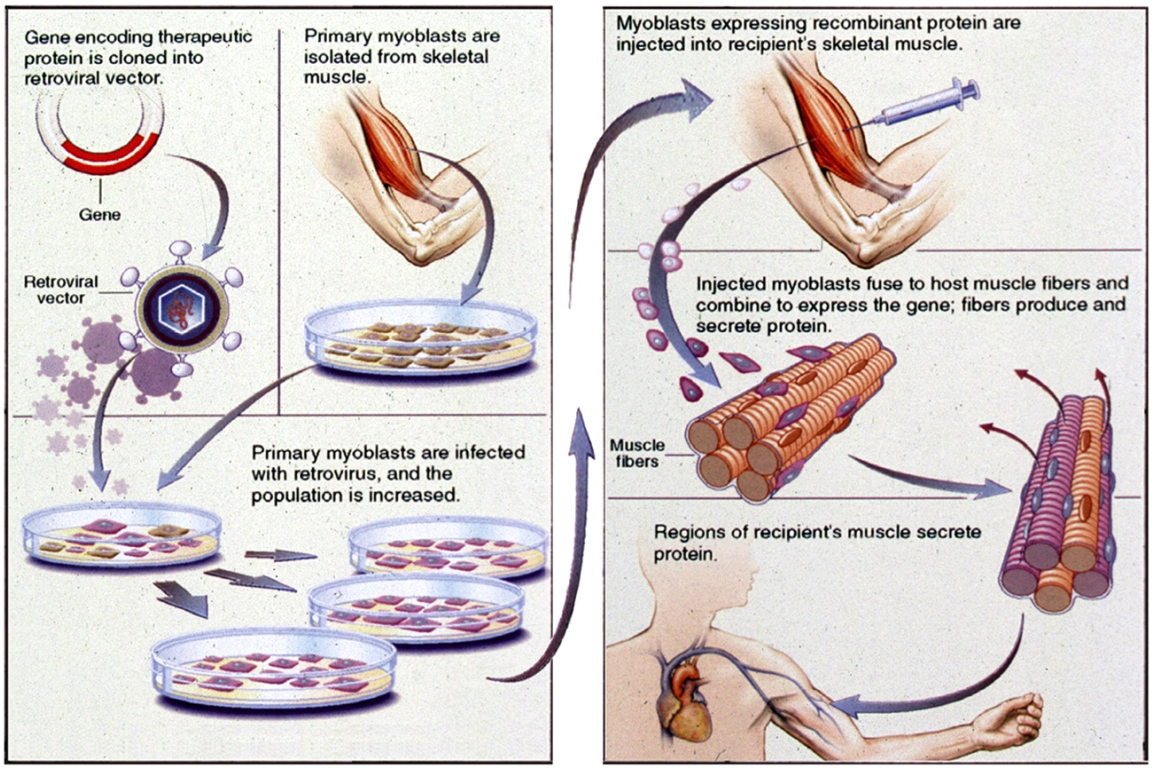

Figure 10: Myoblast-mediated gene therapy (adapted from Blau 1995)

Myoblast-mediated gene transfer was instrumental in elucidating the effects of long-term VEGF delivery in the skeletal muscle and in the heart, in particular the potential of uncontrolled VEGF expression to induce hemangioma-like structures (Springer 1998) (Lee 2000). Interestingly, *ex-vivo* transduction of myoblasts allowed us to quantify the levels of gene expression in individual cells and to isolate clonal populations expressing homogeneous specific levels of VEGF. Using these unique populations, it was possible to rigorously study the dose-dependent effects of VEGF and to distinguish the role of total and microenvironmental doses in inducing normal or aberrant angiogenesis (see section 3.2 for more details) (Ozawa 2004).

Therefore, myoblast-mediated gene transfer is a unique tool to achieve controlled expression of one or more angiogenic factors in skeletal muscle, providing a valuable model to elucidate the factors controlling therapeutic angiogenesis in a clinically relevant tissue.

3.2 Limitations of VEGF in therapeutic angiogenesis

VEGF-A, is the master regulator of both physiological and pathological angiogenesis and therefore has been used in several gene therapy clinical trials with the aim to induce therapeutic blood vessel growth in peripheral and myocardial ischemia. $VEGF_{165}$ was initially delivered as naked plasmid DNA through intramyocardial injections and resulted in the improvement of myocardial perfusion, collateral growth and myocardial contractile function. The safety of VEGF delivery to human peripheral arteries and skeletal muscle was also tested with adenovirus, giving positive results in phase I clinical trials (Yla-Herttuala 2007).

However, despite these encouraging preclinical and early clinical evidences, the results of placebo-controlled clinical trials of VEGF gene therapy were disappointing and did not shown convincing improvements in clinical outcomes.

Retrospective analyses have indicated several possible explanations for this poor therapeutic efficacy (Korpisalo 2010). Indeed, VEGF has a very narrow therapeutic window in vivo. At low doses, VEGF is mostly vasculoprotective and have none or little angiogenic effect. On the other hand, high VEGF doses become rapidly unsafe.

Previous work from our group rigorously addressed the therapeutic window of VEGF, investigating the dose-dependent effect of VEGF in the skeletal muscle. Taking advantage of the *ex vivo* selection and characterization of retroviral-transduced myoblasts described

above, it was possible to isolate and expand single myoblasts from the heterogeneous transduced population, stably producing specific amounts of $VEGF_{164}$ from about 10% to about 325% of the average of the parent population. Therefore, it was possible to distinguish the effect of the total VEGF dose delivered against the effect of microenvironmentally controlled VEGF dose. When the total VEGF dose was reduced by implanting lower numbers of the heterogeneous VEGF myoblast population, the aberrant vascular morphology could not be avoided. On the contrary, when clonal populations of myoblasts homogeneously expressing different levels of VEGF were implanted a threshold between normal and aberrant angiogenesis was found. Clonal myoblasts that expressed low to medium levels of VEGF induced growth of stable and pericyte-coated capillaries while clones that expressed high levels of VEGF induced hemangiomas. Therefore, the induction of normal or aberrant angiogenesis by VEGF depends strictly on its microenvironmental amount secreted and not on the total dose delivered (Ozawa 2004). Furthermore, only homogeneous controlled VEGF levels below that threshold were able to induce functional improvement in a murine model of hindlimb ischemia (von Degenfeld 2006).

These findings importantly suggest that the mode of VEGF delivery is fundamental to achieve therapeutic efficacy. Traditional gene therapy vectors used in clinical trials, like adenoviruses, do not allow the control of the distribution of microenvironmental levels of VEGF *in vivo*, but only the total dose administered by changing the titer. Therefore, easily VEGF expression is too low to induce efficient angiogenesis or “hotspots” of excessive VEGF expression make the treatment unsafe. This can partially explain the lack of success of clinical trials conducted so far.

Another crucial point to be considered when delivering VEGF for therapeutic

angiogenesis is the duration of expression. Although long-term transgene expression can be desirable when replacing a missing functional protein, it might be harmful in case of growth factors delivery.

On the other hand, in a transgenic system of conditional switching of VEGF expression, it has been shown that short-term VEGF expression of about 2 weeks led to unstable vessels that promptly regressed following cessation of the angiogenic stimulus. Upon expression longer then 4 weeks, remodelling occurred and new vessels persisted for months after VEGF withdrawal (Dor 2002). VEGF-dependence of newly induced vessels has been demonstrated also injecting inducible AAVs in skeletal muscle, confirming that sustained VEGF expression for at least 1 month was needed to induce the formation of stable vessels (Tafuro 2009). Furthermore, abrogation of VEGF signalling with a recombinant receptor-body (VEGF-Trap) does not cause regression of new vessels only 4 weeks after implantation of VEGF-expressing myoblasts in skeletal muscle. Interestingly, aberrant vessels induced by high VEGF levels never acquired VEGF-independence (Ozawa 2004).

The need for at least 4 weeks of sustained VEGF expression in order to have stable vessels is a challenge for the use of short-term gene delivery systems, such as plasmids and adenoviral vectors, and might provide a further reason for the failures of clinical trials for therapeutic angiogenesis.

3.2.1 Vascular maturation as therapeutic target

The increasing knowledge of molecular regulation of blood vessel growth emphasizes the importance of the vascular maturation phase, in which vessels acquire proper pericyte coverage, stabilize and become fully functional. Therefore, targeting vascular maturation

has emerged as an attractive strategy to overcome VEGF limitations that became evident in the first generation of clinical trial.

To target vascular maturation, it is necessary to co-deliver a factor involved in vascular stabilization together with an angiogenic factor, such as VEGF. Among factors involved in vascular stabilization, Ang-1 and TGF-β1 exert their effect directly through endothelial cell receptors, without the involvement of other cell types. Differently, PDGF-BB stimulates maturation indirectly through the recruitment of pericytes, which establish cell-to-cell contact with endothelial cells and provide a coordinated array of signals (Fig.11).

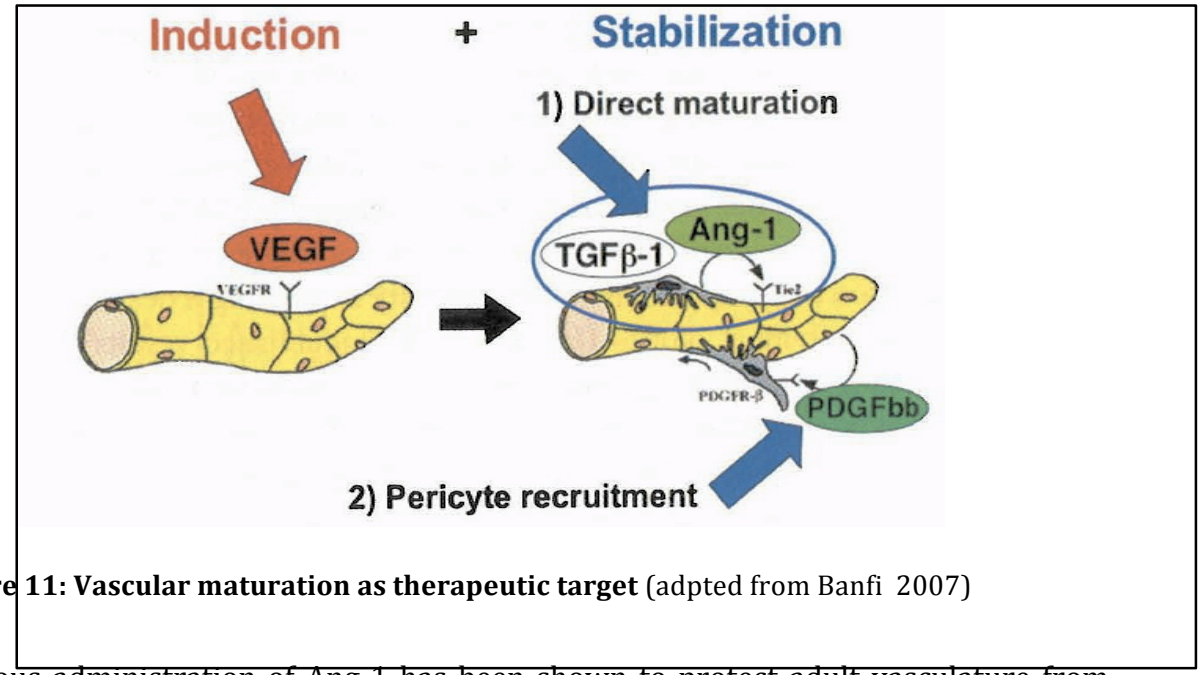

Figure 11: Vascular maturation as therapeutic target (adpted from Banfi 2007)

Exogenous administration of Ang-1 has been shown to protect adult vasculature from leakage and to counteract the side-effects of VEGF and inflammation (Thurston 2000). Co-delivery of VEGF and Ang-1 was also shown to reduce VEGF-induced leakage in rat hindlimb ischemia (Jiang 2006) and in normal rat muscle 3 months after AAV injection (Arsic 2003). Moreover, adenoviral delivery of VEGF and Ang-1 induced vessels that were

more mature and persisted after 4 weeks, while the effect induced by VEGF alone disappeared at the same time point (Zhou 2004).

Considering the fundamental role of pericytes in controlling vascular maturation, previous work from our group focused on the co-delivery of VEGF and PDGF-BB with the aim to correct the aberrant angiogenic effects of VEGF through the global actions mediated by recruited pericytes.

The possibility to modulate angiogenesis by PDGF-BB co-delivery has been tested in previous studies. Sustained delivery of recombinant VEGF and PDGF-BB proteins from a polymeric biomaterial has been reported to lead to larger and more mature vessels compared to VEGF alone subcutaneously and in ischemic skeletal muscle. (Richardson 2001). The combination of PDGF-BB with FGF-2 (fibroblast growth factor-2), another potent angiogenic growth factor, as recombinant proteins, has been reported to induce angiogenic synergy and increased stabilization in the mouse cornea and in rat and rabbit hindlimb ischemia (Cao 2003). More recently, Korpisalo et al. showed that the co-injection into rabbit skeletal muscle of two different adenoviruses carrying VEGF-A and PDGF-BB (AdVEGF-A and AdPDGF-BB) resulted in a prolonged angiogenic response. However, this effect was mostly mediated via paracrine effect from recruited mononuclear cells rather than an increased pericyte coverage (Korpisalo 2008).

Previous data from our group, using myoblast-mediated gene transfer, showed that aberrant vessels induced by high level of VEGF correlate with a loss of normal pericytes, leading to the hypothesis that aberrant angiogenesis might depend on the imbalance between endothelial activation and insufficient vascular maturation. Indeed, the growth of aberrant vessels by uncontrolled VEGF levels was completely abrogated and replaced by

homogeneous mature capillary network, when VEGF and PDGF-BB were co-expressed in the skeletal muscle (Fig 12) (Banfi, manuscript submitted)

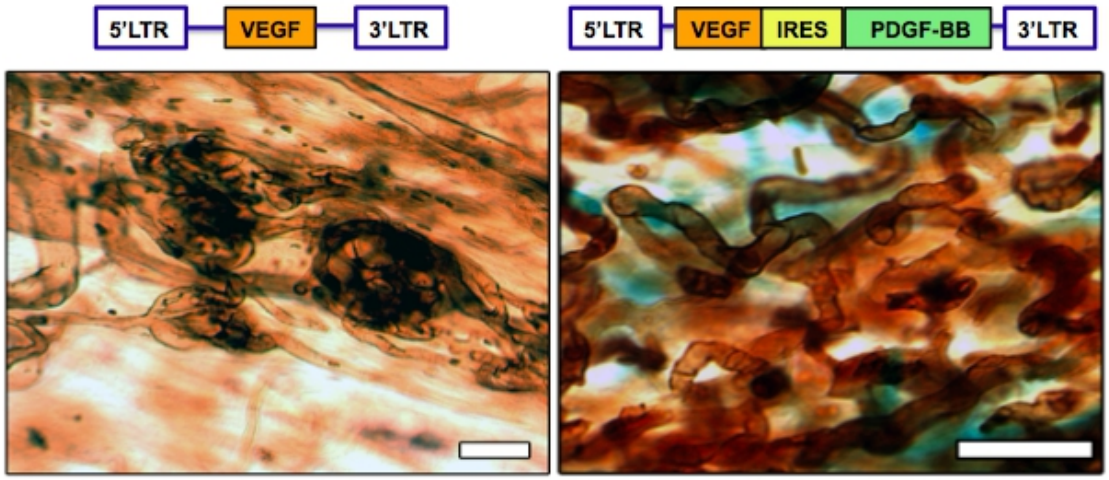

Figure 12: PDGF-BB co-expression prevents the induction of aberrant vessels by high uncontrolled levels of VEGF (adpted from Banfi, manuscript submitted)

However, this effect required that the gradients of the two factors were precisely co-localized in the microenvironment at fixed relative levels, through coordinated co-expression from a single bicistronic construct. In fact, inconsistent gradient co-localization by expression from independent cell sources, or random relative levels by expression from separate viral construct, failed to completely prevent aberrant angiogenesis. Furthermore, gain- and loss-of-function experiments with clonal populations showed that the transition between normal and aberrant angiogenesis is not a fixed property of VEGF dose, but rather depend quantitatively on the relative activation of VEGF and PDGF-BB signaling (Banfi, manuscript submitted).

The fact that the two growth factors were expressed from the same bicistronic cassette ensures that the microenvironmental levels of VEGF and PDGF-BB around each transduced fiber are fixed regardless of the possible variability in viral infection efficiency and transcriptional activity.

These findings have important implications for the design of novel approaches to therapeutic angiogenesis. In fact, co-expression of PDGF-BB normalized the aberrant vessel phenotype despite uncontrolled and high VEGF levels and, therefore, overcame the need to tightly control VEGF dose at the microenvironmental level. As a consequence, coordinated VEGF and PDGF-BB co-delivery from a single bicistronic vector might provide a convenient tool to increase safety and efficacy of viral-based delivery of VEGF.

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Aim of the thesis

Despite its fundamental role in inducing new blood vessels, VEGF failed to show clear clinical efficacy in trials for therapeutic angiogenesis.

As described in the previous sections, this can be partially explained considering two intrinsic properties of VEGF:

1) VEGF can induce normal or aberrant angiogenesis depending on its level around each producing cell and not on its total delivered dose. Therefore, in order to exploit its therapeutic window, its dose must be controlled in the microenvironment level and not simply at the total level, to avoid spots of excessive and unsafe production.

2) The duration of VEGF expression is another crucial variable to take into account, since newly induced vessels requires at least 4 weeks of sustained VEGF expression in order to mature and consequentially become independent from the exogenous source of growth factor.

Myoblast-mediated gene delivery system allows the selection and the characterization *in vitro* of myoblasts expressing specific VEGF levels and ensures a stable expression of the transgene over time, fulfilling the requirements for an efficient VEGF-based therapeutic angiogenesis. However, this method has a limited clinical relevance, since it would require excessive time and costs to generate and select therapeutic populations of autologous cells for every patient. Moreover, the long-term sustained expression of a growth factor such as VEGF raises serious safety concerns.

On the other hand, traditional gene therapy vectors, such as adenoviruses, would represent a convenient and off-the-shelf treatment. However, they do not allow the distribution of VEGF doses to be controlled *in vivo* and have a short duration of expression

(around 2 weeks) that, although being desirable, is not sufficient to generate stable and mature vessels.

Therefore, a strategy to modulate the deleterious effects of uncontrolled VEGF expression *in vivo* and to accelerate vascular stabilization would be desirable to make short-term expression gene therapy vectors a convenient, safe and efficient tool to promote therapeutic angiogenesis.

PDGF-BB regulates vascular maturation through the recruitment of pericytes. Moreover, previous results from our group demonstrated that PDGF-BB, when co-expressed at a fixed ratio with VEGF, can re-establish the balance between endothelial cell activation and vessel maturation, preventing the formation of aberrant vessels even at high and uncontrolled VEGF levels. Therefore, here we aimed to establish whether PDGF-BB co-expression could also accelerate the stabilization of VEGF-induced vessels. For this reason, in chapter 4 we investigated the time-course of vascular stabilization after induction by VEGF alone or together with PDGF-BB, abrogating VEGF signalling at clinically relevant time-points after myoblast injection by systemic treatment with a VEGF-Trap recombinant protein. Furthermore, taking advantage of the possibility to select clonal populations expressing specific VEGF levels, we dissected the role of VEGF dose in the kinetic of vascular stabilization and how this is modulated by PDGF-BB co-expression.

In chapter 5, we aimed to apply these findings to a viral based delivery system and we tested the hypothesis that co-expression of VEGF and PDGF-BB from a single bicistronic adenoviral vector could induce normal vessels that stabilize within the short duration of expression afforded by adenoviruses, providing a convenient, safe and efficient gene therapy approach for therapeutic angiogenesis.

Chapter 4

Coordinated co-expression of PDGF-BB accelerates stabilization of $VEGF_{164}$-induced vessels in a dose-dependent fashion

4.1 Introduction

The induction of new blood vessels by administration of vascular growth factors to restore blood supply in ischemic tissues, defined as therapeutic angiogenesis, is an attractive strategy to treat many patients suffering from advanced occlusive vascular disorders that are not amenable to traditional surgical revascularization (Korpisalo 2010).

VEGF-A is the master regulator of physiological and pathological angiogenesis in adults (Ferrara 2003) and has been used in pre-clinical models and clinical trials of therapeutic angiogenesis. However, results obtained have been so far disappointing and add support to the concern that VEGF-based gene therapy has some limitations due to the biological properties of VEGF itself (Yla-Herttuala 2007).

Indeed, it has been demonstrated that unregulated and long-term expression of VEGF determines the formation of abnormal and pathological vessels in skeletal muscle (Springer 1998) and myocardium (Lee 2000), after the delivery of retrovirally transduced myoblast, or in a rabbit model of hind-limb ischemia after the delivery of AAVs (Zacchigna 2007).

If long-term expression of VEGF can be harmful, on the other hand, it has been shown, that a sustained expression of VEGF inferior to 4 weeks leads to unstable vessels that regress after cessation of the angiogenic stimulus (Dor 2002). Therefore, the ideal strategy would be to provide VEGF expression for such a period of time. However, gene therapy vectors available until now for therapeutic angiogenesis allow either an indefinite expression of the transgene, like retroviral and lentiviral vectors or AAV vectors, or a transient expression of about two weeks due to the clearance of the host immune system, like adenoviruses.

To overcome the safety concerns of prolonged VEGF over-expression, it would be desirable to achieve stable vessels within a short time of expression. Therefore a strategy to reduce the time required for nascent vessels to stabilize and became VEGF-independent is needed.

In physiological vascular development, VEGF-independence is achieved through pericyte attachment to the vessel wall, a process that is mediated by PDGF-BB produced by activated endothelial cells (von Tell 2006). Studies of vascular regression in diabetic retinopathy and in tumors demonstrated that association with pericytes renders vessels independent of continued VEGF expression (Hammes 2002) (Benjamin 1999).

In this study, we tested the hypothesis that co-expression of PDGF-BB could accelerate stabilization of VEGF-induced vessels.

We took advantage of a well-characterized myoblast-mediated gene transfer system to achieve controlled and long-term expression of VEGF, PDGF-BB or both in skeletal muscle (von Degenfeld 2003). VEGF and PDGF-BB expression in vivo was induced by implantation into adult mouse ear and limb muscles of myoblasts transduced with a retroviral construct expressing VEGF alone or the two factors at a fixed ratio, as previously described (Banfi, manuscript submitted). VEGF signal was abrogated *in vivo* at specific time points after myoblasts delivery by systemic treatment with VEGF-Trap, a recombinant receptor-body made by fusing extracellular domains of VEGFR-1 and VEGFR-2 and the human immunoglobulin Fc portion, that tightly binds and sequesters all VEGF isoforms (Holash 2002).

To mimic the expression conditions in a clinical application of gene therapy, in which the delivered VEGF dose cannot be controlled, we first analyzed stabilization of vessels induced by heterogeneous levels of VEGF alone or together with PDGF-BB.

Subsequently, to rigorously define the role of VEGF dose on vessel stabilization, we delivered clonal populations of transduced myoblasts, expressing homogeneous and well-characterized VEGF levels, and we assessed the time-course of vascular stabilization in the absence or presence of PDGF-BB co-expression. Finally, we sought to determine the underlying mechanisms by investigating the regulation of different factors involved in vascular maturation in the different conditions.

We found that PDGF-BB co-expression can significantly accelerate stabilization of vessels induced by heterogeneous and high VEGF levels already after 2 weeks of expression, providing a convenient strategy to induce persistent vessels with a short-term expressing vector. Furthermore, mechanistically we determined that VEGF negatively regulates vascular stabilization in a dose-dependent fashion, with vessels induced by low VEGF levels stabilizing faster than those induced by higher doses. Interestingly, PDGF-BB accelerate vascular stabilization selectively at high VEGF doses, but not at low ones, possibly by restoring physiological levels of Semaphorin 3A and TGF-β signaling.

4.2 Materials and methods

Cell culture

Primary myoblasts isolated from C57BL/6 mice were transduced to express the β-galactosidase marker gene (lacZ) from a retroviral promoter (Rando 1994) and over-infected at high efficiency (Springer 1997) with retroviruses carrying the cDNA of murine $VEGF_{164}$, or human PDGF-BB or both linked through an IRES sequence (Internal-Ribosome-Entry-Site). The isolation and characterization of early passage myoblast clones homogeneously expressing specific VEGF levels have been previously described (Ozawa 2004). Briefly, myoblast clones were isolated using a FACS Vantage SE cell sorter (Becton Dickinson) and single cell isolation was confirmed visually. The stability of the VEGF secretion was assessed periodically by ELISA. All myoblast populations were cultured in 5% CO_2 on collagen-coated dishes with a growth medium consisting of 40% F10, 40% DMEM low glucose (1000 mg glucose/liter) and 20% fetal bovine serum, supplemented with 2.5 ng/ml basic fibroblast growth factor (FGF-2), as previously described (Banfi 2002).

Implantation of myoblasts into mice

6-8 week-old, male SCID CB.17 mice (Charles River Laboratories, Sulzfeld, Germany) were treated in accordance with the Swiss Federal guidelines for animal welfare, after approval from the Veterinary Office of the Canton of Basel-Stadt (Basel, Switzerland). SCID mice were used to avoid an immunologic response to myoblasts expressing xenogenic proteins. Myoblasts were dissociated in trypsin and resuspended in PBS with 0.5% BSA. $5x10^5$ myoblasts in 5 µl were implanted into the tibialis anterior muscle in the calf or into the posterior auricular muscle, midway up the dorsal aspect of the

external ear, and into the tibialis anterior and gastrocnemius muscles in the calf, using a syringe with a $29^1/_2$-gauge needle.

VEGF-Trap$_{R1R2}$

VEGF-Trap$_{R1R2}$, which consists of portions of extracellular domain of VEGFR-1 and VEGFR-2 coupled to human Fc, is a soluble form of VEGF receptors that can be used to deplete active VEGF in vivo (Holash 2002). VEGF-Trap$_{R1R2}$ was used to test the dependence of the vascular structures induced by different VEGF levels on continued VEGF production.

Mice were treated with VEGF-Trap$_{R1R2}$ (25 mg/kg; 100 µl intraperitoneally) in PBS (40 mM phosphate and 20 mM NaCl, pH 7.4) or with vehicle (PBS; 100 µl intraperitoneally) 2 and 4 days before tissue harvest. On days 14 and 21, the vasculature was stained by injection of biotinylated *L. esculentumlectin* i.v. to examine the morphological changes (n= 5 mice per group).

Tissue staining

The entire vascular network of the ear could be visualized following intravascular staining with a biotinylated *Lycopersicon esculentum* lectin (Vector Laboratories) that binds the luminal surface of all blood vessels, as previously described (Ozawa 2004). Mice were anesthetized, lectin was injected intravenously and 4 minutes later the tissues were fixed by vascular perfusion of 1% paraformaldehyde and 0.5% glutaraldehyde in PBS pH 7.4. Ears were then removed, bisected in the plane of the cartilage, and stained with X-gal staining buffer (1 mg/ml 5-bromo-4-chloro-3-indoyl-β-D-galactoside, 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 0.02% Nonidet P-40, 0.01% sodium deoxycholate, 1 mM $MgCl_2$ in PBS pH 7.4). Tissues were

stained using avidin-biotin complex-diaminobenzidine histochemistry (Vector Laboratories), dehydrated through an alcohol series, cleared with toluene and whole-mounted on glass slides with Permount embedding medium (Fisher Scientific). Vascular morphology was analyzed at 2 and 3 weeks post-injection. To study vessel perfusion in vivo fluorescein isothiocyanate (FITC)-labeled *Lycopersicon esculentum* lectin (50 µg in 50 µl; Vector Laboratories) was injected into the femoral vein and allowed to circulate for 4 hours before perfusion of fixative.

For tissue sections, mice were anesthetized and fixed by vascular perfusion of 1% paraformaldehyde in PBS pH 7.4. *Tibialis anterior* and *gastrocnemious* muscles were harvested, embedded in OCT compound (Sakura Finetek), frozen in freezing isopentane and cryosectioned. Tissue sections were then stained with X-gal (20 µm sections) or with H&E (10 µm sections). Immunofluorescence was performed on cryosection of 10 µm in thickness; section were permeabilized by incubation with 0.3% Triton (Sigma-Aldrich) and 2% goat serum (Invitrogen) in PBS for 1 hour at room temperature. The following primary antibodies and dilutions were used: rat monoclonal anti-mouse PECAM-1 (BD-Pharmingen) at 1:100; mouse monoclonal anti-mouse α-SMA (clone 1A4, MP Biomedicals) at 1:400; rabbit polyclonal anti-NG2 (Millipore) at 1:200. Fluorescently labeled secondary antibodies (Molecular Probes, Invitrogen) were used at 1:200. Antibodies incubation were performed at room temperature for 1 hour.

Vessel measurements

Vessel length density was measured in whole mounts of ears stained with *L. esculentum* lectin. Vessel length density was measured on 3-6 fields per ear and 4 ears per group by tracing the total length of vessels in the acquired field (20x objective) and dividing it by the area of the fields. All image measurements were performed with AnalySIS D

software (Soft Imaging System). Area Fraction (AF) of endothelial structures were measured in 3-6 fields in immunostained sections of hindlimb muscles (n=3). ImageJ software (http://rsb.info.nih.gov/ij) was used for image analysis.

Quantitative Real-Time PCR

For RNA extraction, muscle previously injected with transgenic myoblasts were freshly harvested and disrupted using a Qiagen Tissue Lyser (Qiagen) in 1 ml of PBS+ 1% Trizol (Invitrogen). RNA was extracted according to manufacturer's instruction.

Total RNA was reverse transcribed into cDNA with the Omniscript Reverse Transcription kit (Qiagen) at 37 °C for 60 minutes. Quantitative Real-Time PCR (qRT-PCR) was performed on an ABI 7300 Real-Time PCR system (Applied Biosystems). Expression of genes of interest was determined using commercial TaqMan gene expression assays (Applied Biosystems). The cycling parameters were: 50°C for 2 minutes, followed by 95°C for 10 minutes and 40 cycles of denaturation at 95°C for 15 seconds and annealing/extension at 60°C for 1 minute. Reactions were performed in triplicate for each template, averaged, and normalized to expression of the GAPDH housekeeping gene.

Statistics

Data are presented as mean±standard error. The significance of differences was evaluated using analysis of variance (ANOVA) followed by the Bonferroni test (for multiple comparisons), or using a Student's t test (for single comparisons); $p < .05$ was considered statistically significant.

4.2 Results

PDGF-BB accelerates stabilization of vessels induced by heterogeneous VEGF levels

We initially sought to express heterogeneous and uncontrolled levels of angiogenic factors in skeletal muscle *in vivo*, to mimic conditions after direct delivery of viral gene therapy vectors.

Myoblast populations retrovirally transduced to express $mVEGF_{164}$ (VEGF), hPDGF-BB (PDGF) or both (VIP) were implanted into the posterior auricular muscle of adult SCID mice. To assess VEGF-dependence of the induced vasculature at different time points, mice were systemically treated with saline or VEGF-Trap for 4 days before vessels length density (VLD) was evaluated 2 and 3 week after myoblast implantation.

PDGF-BB alone did not alter the pre-existing capillary network at any time point (Fig 1a, 1c, 1c and 1j). After 2 weeks of expression, lectin intravascular staining revealed that heterogeneous VEGF levels induced a mixed angiogenic response consisting of both normal capillaries and aberrant bulbous vascular structures as expected, depending on the different VEGF expression levels achieved in the microenvironment around each transduced fiber (Fig 1e). Coordinated co-expression of VEGF and PDGF-BB induced only morphologically normal capillaries (Fig 1g), as previously described (Banfi, manuscript submitted).

Both the aberrant structures and the normal vessels induced by VEGF alone were completely eliminated by VEGF-Trap treatment (Fig 1f). However, a portion of the homogeneous normal capillaries induced by VIP cells were already VEGF-independent after 2 weeks (Fig 1h). VEGF-Trap treatment did not alter the pre-existing capillary bed (Fig 1 b and 1d).

Vascular maintenance or regression after VEGF-Trap treatment was quantified by

Vessel Length Density (VLD) measurements (Fig 1i). In areas remote from the site of myoblast implantation in saline-treated mice, the mean VLD of pre-exisiting muscle capillaries was 80±4.5 mm/mm^2. PDGF-BB alone did not induce angiogenesis nor affect the stability of pre-existing vasculature at either time point.

We analyzed the two angiogenic phenotypes induced by VEGF alone separately, and we found that, as expected, areas of aberrant vessels had a reduced VLD compared to controls, due to the described fusion of pre-existing vessels in response to high VEGF levels (Drake 1999). These aberrant vessels were completely VEGF-dependent because they all disappeared after VEGF-Trap treatment.

On the other hand, new normal vessels induced by VEGF alone caused a 50% increase in VLD compared to controls (117±5.3 vs 80±4.5 mm/mm^2). However, VEGF-Trap treatment after 2 weeks caused also all new normal vessels to disappear, reducing VLD to the same value as the pre-existing capillary network. VLD in areas of VIP myoblast implantation was similarly increased of about 40 mm/mm^2 compared to control area (120±5.9 vs 80±4.5 mm/mm^2) but it was only partially reduced to 100±5.4 mm/mm^2 after VEGF-Trap treatment. Therefore, about 50% of the new normal capillaries induced by VIP co-expression became VEGF-independent already after 2 weeks of expression.

After 3 weeks, aberrant structures induced by heterogeneous levels of VEGF alone were still completely sensitive to VEGF-Trap treatment, whereas a portion of the new normal vessels induced by VEGF alone persisted after VEGF-Trap injection (Fig. 1n and 1o). Coordinated co-expression of VEGF and PDGF-BB induced a dense network of normal capillaries, which were almost unaffected by VEGF-Trap treatment (Figure 1p and 1q).

Quantification showed that aberrant VEGF-induced vessels were still completely VEGF-dependent. Areas of normal angiogenesis induced by VEGF alone showed an

increase of about 55 mm/mm^2 in VLD compared to control (138±8.9 vs 84±5.9 mm/mm^2). Contrary to the 2-weeks time point, the VLD was still increased by about 15 mm/mm^2 after treatment compared to controls, indicating that about 25% of normal VEGF-induced vessels were VEGF-independent by 3 weeks.

However, the homogeneous network of VIP-induced capillaries yielded a VLD of 170±8.7 mm/mm^2 and VEGF-Trap caused only a slight reduction of about 10 mm/mm^2. This shows that almost 90% of the vessels induced by coordinated co-expression of VEGF and PDGF-BB were already stable after 3 weeks.

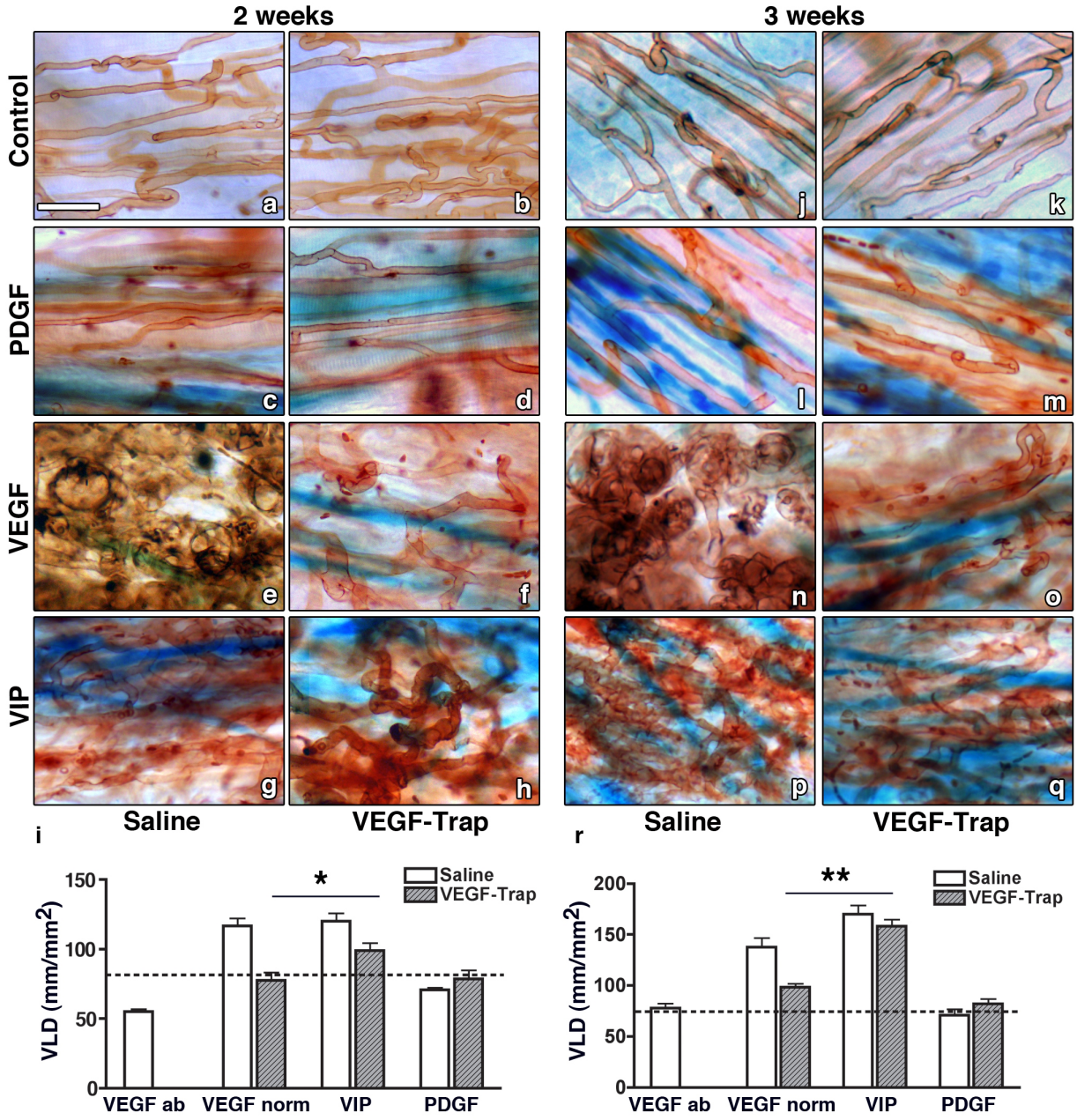

Figure 1. **PDGF-BB co-expression accelerates vascular stabilization of VEGF-induced vessels both at 2 and 3 weeks**. Myoblasts expressing VEGF, PDGF-BB, or both (VIP) were implanted in ear muscles. Mice were treated either with VEGF-Trap or saline 2 and 3 weeks post-implantation. Blood vessels were visualized in whole-mount preparations after intravascular lectin perfusion (in brown) at 2 weeks (a-h) and three weeks (j-q). Implanted myoblasts were identified by X-gal staining (blue). Quantification of vascular length density revealed that around 50% of VIP-induced vessels were stable after 2 weeks (i) and almost the 90% after 3 weeks (r). VEGF alone induced both aberrant vascular structures which were completely dependent on VEGF stimulus at every time point, and some normal capillaries that started to stabilize at 3 weeks. VEGFab=aberrant angiogenesis by VEGF alone; VEGFnorm=normal angiogenesis by VEGF alone; VIP=VEGF+PDGF-BB; PDG= PDGF-BB alone. Size bar= 40μm. *= $p<0.05$.

PDGF-BB restores pericyte coverage and prevents aberrant unstable angiogenesis

Next we asked whether the difference in stabilization of vessels induced by VEGF alone or with PDGF-BB correlated with a differential recruitment of pericytes. Myoblasts expressing VEGF alone, PDGF-BB or the two factors together were injected into hindlimb muscles of SCID mice. Vascular structures were analyzed by immunostaining of frozen sections for CD31 (endothelial cells), α-smooth muscle actin (α-SMA, smooth muscle cells) and nerve-glial antigen-2 (NG-2, pericytes).

After 2 weeks of expression, aberrant structures induced by VEGF alone were covered by α-SMA$^+$ smooth muscle cells, while normal vessels induced by VEGF alone or by VEGF with PDGF-BB were both surrounded by pericytes (Fig 2b and 2c). The same mural cell-coverage pattern was present also after 3 weeks post-implantation (Fig 2j and 2k). PDGF-BB alone did not induce the growth of new capillaries, but only a massive recruitment of NG-2$^+$pericytes.

After VEGF-Trap treatment, α-SMA$^+$ aberrant vessels induced by VEGF alone disappeared completely both at 2 and 3 weeks (Fig 2f and 2n), while a portion of vascular structures covered by pericytes were still present in muscles injected with VIP expressing myoblasts at both time points (Fig 2g and 2o). Some pericyte-covered normal vessels induced by VEGF alone were still present after VEGF-Trap treatment at 3 weeks, but not at 2 weeks (Fig 2n and 2f, respectively).

The NG-2$^+$ cells recruited by PDGF-BB were not affected by VEGF-Trap treatment at either time point.

Quantification of the Area Fraction (AF) of endothelial structures was used to measure the proportion of stabilized vessels in each condition (Fig 2h and 2p).

In areas remote from the sites of myoblast implantation the mean area occupied by pre-existing endothelial structures was about 3% of the total. PDGF-BB alone had no

effect at any time point. Aberrant vessels induced by VEGF alone only slightly increased this value, while normal angiogenesis by VEGF alone increased the endothelial AF to 6%. Both kinds of vascular structures disappeared completely after VEGF-Trap treatment, confirming the VEGF-dependence of all vessels induced by VEGF alone after 2 weeks (Fig 2f).

The dense and homogeneous network of VIP-induced capillaries, instead, covered an AF of 15% and still about 8% after VEGF-Trap treatment. Therefore, about 40% of new VIP-induced endothelial structures in leg muscle was already VEGF-independent after 2 weeks (Fig 2h). This percentage increased to 75% while only about 50% of normal angiogenesis induced by VEGF alone was stable and all aberrant vessels completely regressed (Fig. 2p).

Taken together, these results show that the persistent VEGF-dependence of aberrant VEGF-induced structures correlates with a failure to acquire normal pericytes, substituted by smooth muscle cells. However, the faster stabilization of vessels induced by PDGF-BB co-expression does not depend on a differential pericyte recruitment, because normal vessels induced by VEGF alone, which stabilize slowly, and by both VEGF and PDGF-BB which stabilize faster, were similarly covered by normal pericytes.

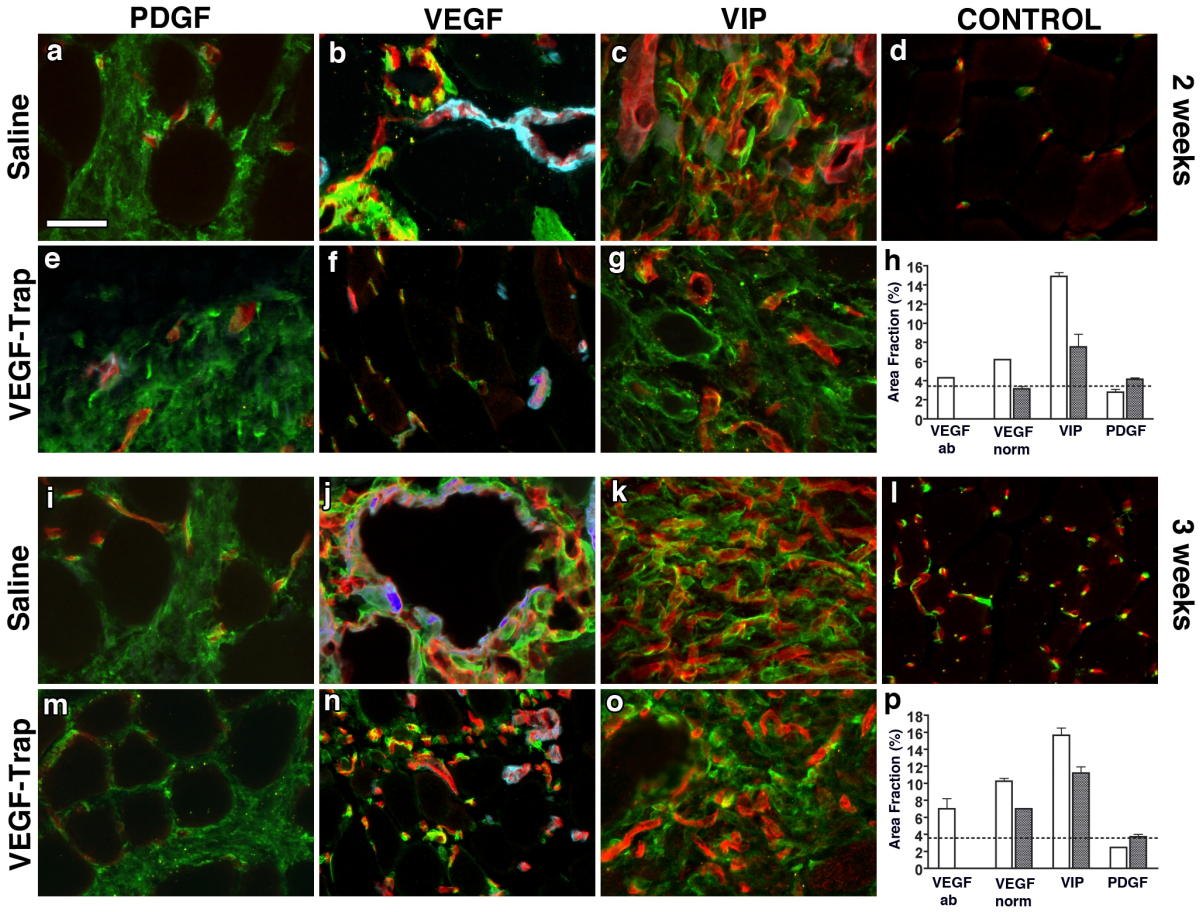

Figure 2. **In skeletal muscle, PDGF-BB co-expression induces normal pericyte-covered vessels that are partially VEGF-independent already after 2 weeks of expression**. Immunofluorescent staining of endothelium (red), pericytes (green) and smooth muscle actin (blue) in crysections of skeletal muscle injected with VEGF, PDGF-BB, VIP or control cells and treated with saline or VEGF-Trap at 2 (a-g) and 3 weeks (i-o) post-injection. Quantifications of Area Fraction of endothelial cells at 2 (h) and 3 (p) weeks confirmed the stabilization trends previously found in the ear muscle; VEGFab=aberrant angiogenesis by VEGF alone; VEGFnorm=normal angiogenesis by VEGF alone. Size bar= 40μm

Vascular stabilization is inversely dependent on VEGF dose

In the previous experiments, although more reflective of the situation in a clinical gene therapy application, it was impossible to determined which vessels were induced by specific VEGF levels.

Therefore, in order to rigorously determine the role of VEGF dose on the kinetics of vessel stabilization and the role of PDGF-BB in accelerating it at different VEGF levels,

we took advantage of previously isolated and characterized clonal populations of transduced myoblasts, in which every cell expressed a specific level of VEGF or VEGF and PDGF-BB in a fixed ratio from the VIP construct (Misteli 2010; Ozawa 2004; Gianni-Barrera, manuscript submitted). We selected six clonal populations based on their *in vitro* VEGF production quantified by ELISA: low (≈ 10 ng/10^6 cells/day), medium (≈ 60 ng/10^6 cells/day) and high (>130 ng/10^6 cells/day) VEGF levels (V-low, V-med and V-high respectively), and clones expressing similar VEGF doses but with a fixed ratio of PDGF-BB (VIP-low, VIP-med and VIP-high respectively), as reported in table 1.

V clones **(VEGF alone)**	**VIP clones** **(VEGF + PDGF)**
V-low V=(11,0± 0,4 ng /10^6 cells/day)	VIP-low V=(9,8± 2, ng/10^6 cells/day) P =(2,3± 0,7 ng/10^6 cells/day)
V-med V=(61,0± 2,9 ng /10^6 cells/day)	VIP-med V=(52,1± 4,9 ng/10^6 cells/day) P =(12,9± 0,8 ng/10^6 cells/day)
V-high V=(133,2± 9,7 ng /10^6 cells/day)	VIP-high V=(156,5± 2,0 ng/10^6 cells/day) P =(48,2± 3,4 ng/10^6 cells/day)

Table 1. **V clones and VIP clones and relative amount of VEGF of VEGF and PDGF-BB expressed**.

Myoblast populations were implanted into the posterior auricular muscle of adult SCID mice, which were treated 2 and 3 weeks later with saline or VEGF-Trap systemic administration for 4 days. Vessel stability was determined quantifying the vessel length density in ear whole–mounts after vascular lectin staining.

After two weeks, as expected, normal and aberrant vascular structures induced by V-med and V-high, respectively, had not stabilized. Interestingly, however, 35% of the vessels induced by the lowest VEGF dose (V-low) remained after VEGF-Trap treatment (Fig. 3a)

At the same time point, PDGF-BB co-expression did not significantly increase vessel stabilization at low and medium levels compared to VEGF alone (35% for VIP-low and 10% for VIP-med). However, whereas none of the vessels induced by high VEGF levels were stable, this was dramatically increased to 44% by PDGF-BB co-expression (Fig. 3b).

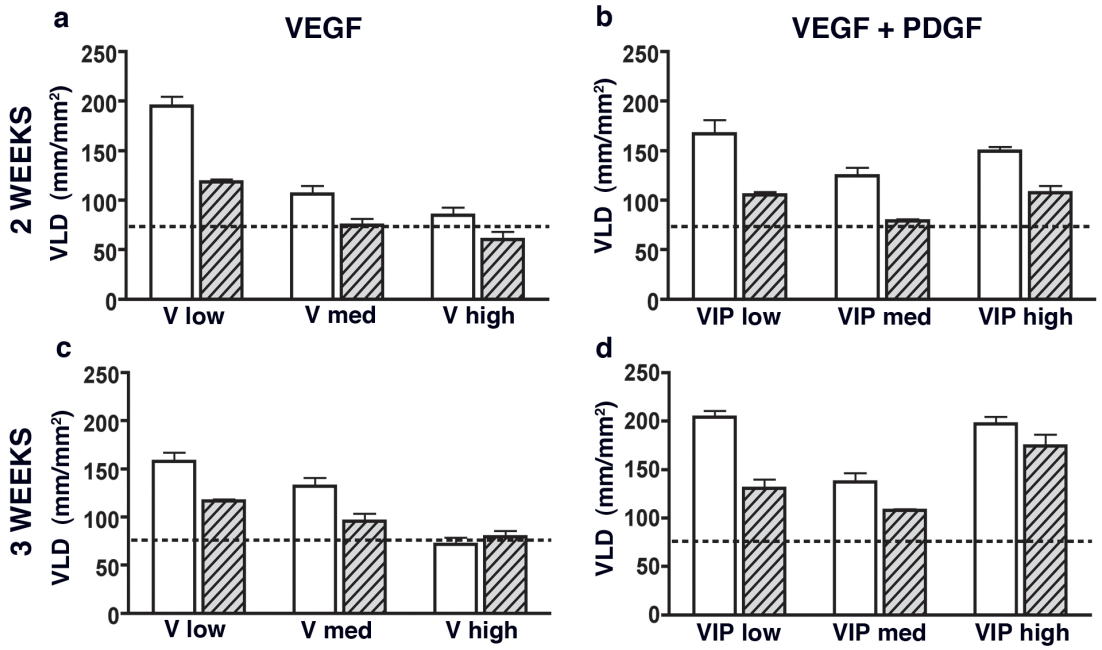

Figure 3. **Vascular stabilization of VEGF- and VIP-induced vessels is dependent on VEGF dose.** Myoblasts expressing clonal levels of VEGF alone or VEGF and PDGF-BB were injected in the ear muscle. Vessel length density was quantified after intravascular lectin staining. Only low VEGF level induced partially stable vessels at 2 and 3 weeks (a, c). However, the highest degree of VEGF-independence was obtained with co-expression of high VEGF and PDGF-BB levels both at 2 and 3 weeks post-injection (b and d). White bars= saline treatment; striped bars= VEGF-Trap treatment; dotted line= vessel length density of non-injected areas.

At three weeks, vessel stabilization gradually increased for V-low and V-med (50% and 10% respectively), while aberrant vascular structures induced by high VEGF were still completely dependent on VEGF signaling (Fig. 3c). PDGF-BB co-expression again did not affect stabilization at low VEGF level, but stabilization of vessels induced by medium and high VEGF levels was significantly increased by PDGF-BB co-expression to 41% and 81% respectively.

Therefore, at both time points considered, PDGF-BB co-expression caused a

significant acceleration in vascular stabilization only at high VEGF level and partially at medium levels, whereas at the lowest VEGF doses it did not have any effect on the time-course to VEGF independence of the induced vessels.

Vessels induced by different doses of both VEGF alone and with PDGF-BB are fully perfused

The establishment of blood flow through new vascular structures has been shown to favour their stabilization. Therefore, different stabilization kinetics might be due to different perfusion of the new vessels. We asked whether vessels induced by the different VEGF doses alone or with PDGF-BB might be functionally connected to circulation in different proportions. The perfusion of vessels induced by different V and VIP clones was assessed by injecting mice 4 hours before sacrifice with a fluorescein-labelled lectin (FITC-lectin), which binds to the lumen of all vessels connected to the general circulation. Perfused endothelial structures were identified by immunostaining as double-positive for CD31 and FITC-lectin. As shown in figure 4, two weeks after myoblast implantation, all new vascular structures induced by all V and VIP clones were functionally perfused. Even aberrant bulbous vessels induced by high VEGF levels were filled with the fluorescent lectin.

Very few non-perfused endothelial structures were visible in every condition with similar frequency. Therefore, vessels induced by all doses of VEGF alone or with PDGF-BB were similarly perfused, suggesting that establishment of functional flow was not responsible for the different stabilization rates.

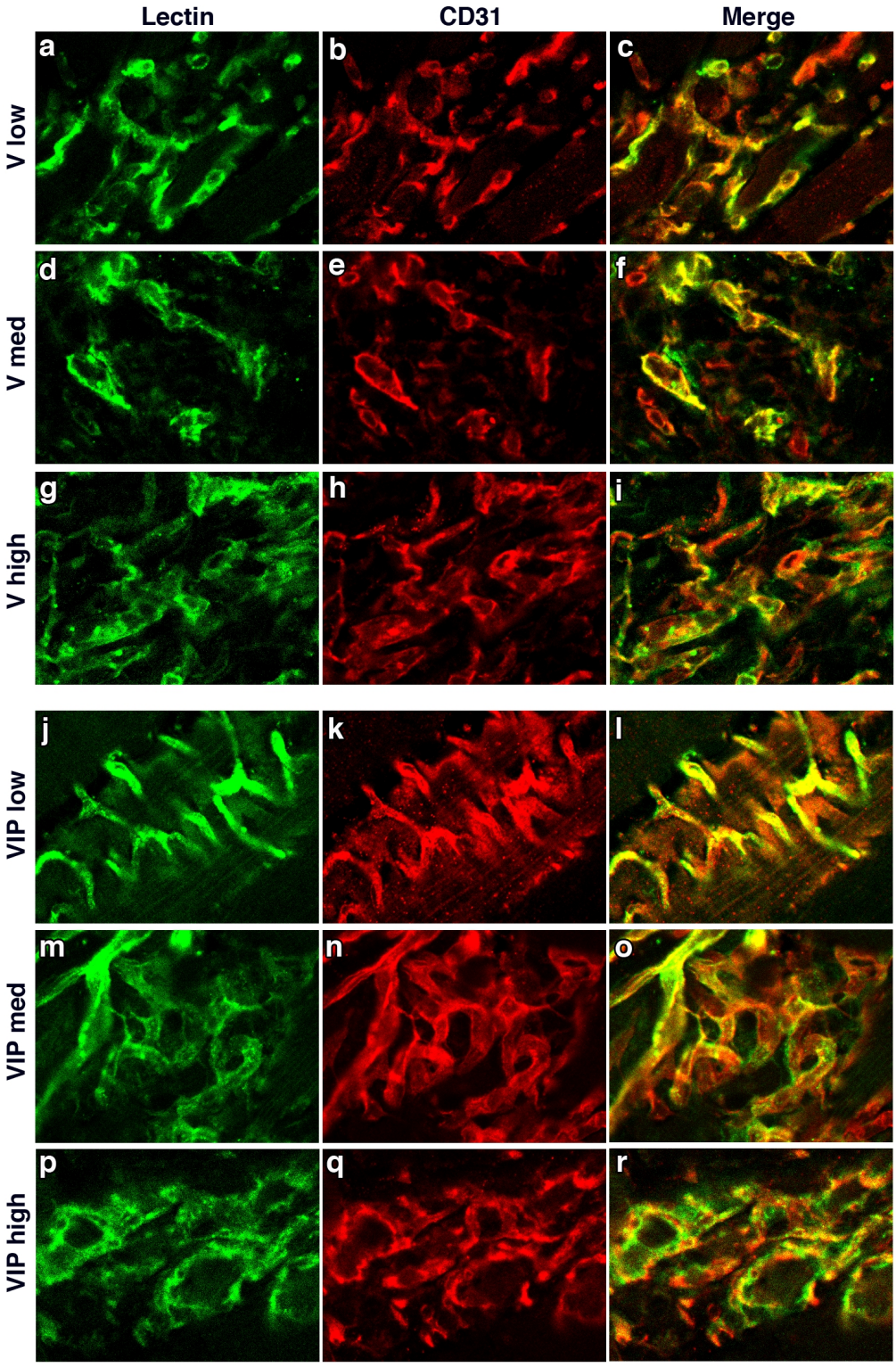

Figure 4. **Vessels induced by different VEGF or VIP doses are equally perfused at 2 weeks**. Mice previously injected with clonal myoblast populations were perfused with FITC-lectin 2 weeks post-injection. Endothelial structures were immunostained in frozen sections (in red, CD31) and compared with relative lectin perfusion (in green, lectin). No differences in perfusion rate were detectable at different VEGF doses, with or without PDGF-BB.

Vascular stabilization is not directly dependent on pericyte coverage

We next asked whether the different stabilization kinetics induced by increasing VEGF levels were due to a different pericyte coverage. In particular, we determined whether vessels induced by low and medium VEGF levels, which are both morphologically normal capillaries but stabilize with clearly different rates, were associated with a qualitatively or quantitatively different pericyte coverage and whether exogenous PDGF-BB could modify it.

Therefore, we analyzed by immunofluorescent staining the phenotype and the spatial distribution of mural cells around new vessels induced by clonal VEGF- and VIP-expressing myoblasts at the two weeks time-points.

Capillary networks induced by low and medium VEGF levels were similarly covered by pericytes, closely in contact with endothelial structure (Fig 5a and 5c). Moreover, at the same VEGF doses, PDGF-BB co-expression did not affect mural cells recruitment and distribution, since vessels were covered by a comparable amount of pericytes with similarly normal morphology and endothelial contact (Fig 5b and 5d).

As previously described, aberrant vessels associated with high VEGF production were surronded by α-SMA-positive mural cells and not by pericytes (Fig 5e) (Ozawa 2004).

PDGF-BB co-expression with the same high VEGF level led to a homogeneous capillary network covered by normal pericytes (Fig 5f). However, although the total pericyte amount in the area of effect was higher compared to the other conditions, as a consequence of high PDGF-BB expression, the amount and morphology of pericytes wrapped around the new vessels was not different from the normal vessels induced by the other VEGF doses alone or with PDGF-BB (Fig 5f).

Therefore, all normal capillaries induced by different VEGF or VIP doses displayed a similar pericyte coverage, despite different stabilization rates.

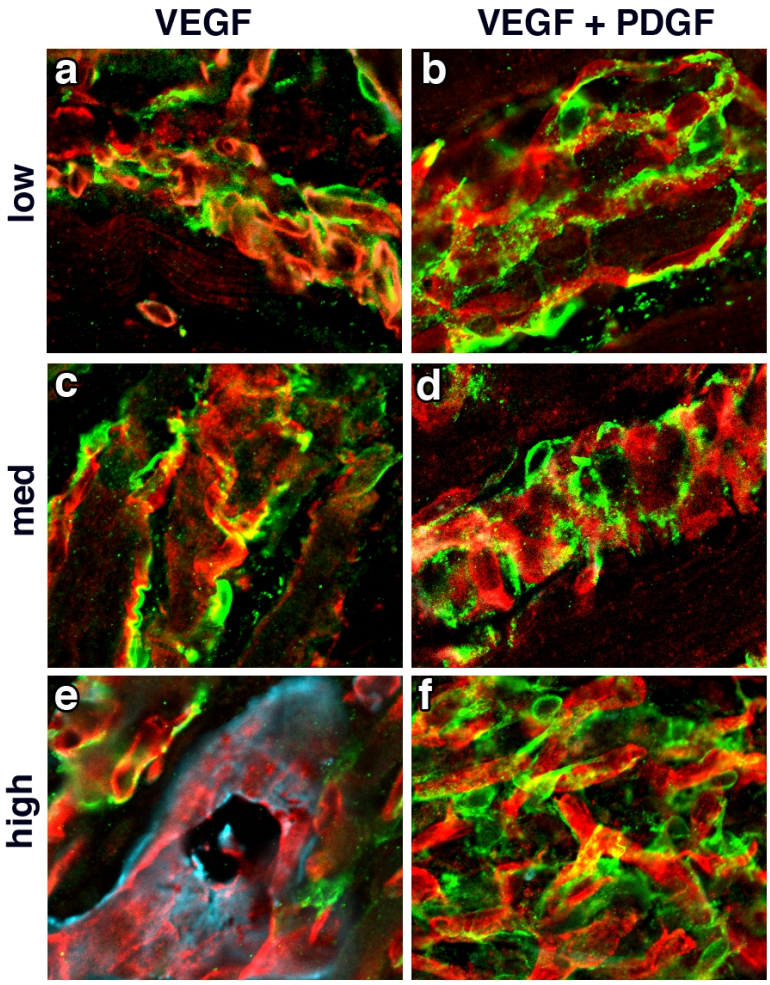

Figure 5. **Pericyte coverage of normal vessels induced by different VEGF or VIP doses at 2 weeks is not differentially regulated**. Immunofluorescent staining of endothelium (red), pericytes (green) and smooth muscle actin (blue) of cryosections of skeletal muscle injected with VEGF, PDGF-BB, or both (VIP) 2 weeks before. All vessels were surrounded by normal pericytes, in close association with the endothelial structures (a-d and f). Only high VEGF induced aberrant structure covered by SMA^+ cells.

Consistently with the similar pericyte coverage of vessels induced by low and medium VEGF levels, despite different stabilization kinetics, the expression the endogenous *Pdgfb* gene was also similar between these two conditions (Fig 6). Because the effects described at 2 and 3 weeks are likely to depend on changes taking place previously, we analyzed gene expression at 7 and 14 days.

After 2 weeks of VEGF expression, endogenous *Pdgfb* expression at all VEGF levels was not increased compare to the control. However, at 7 days *Pdgfb* expression was up-

regulated 2-fold by all VEGF levels; the slightly higher expression in the V-low condition was not significant (Fig.6).

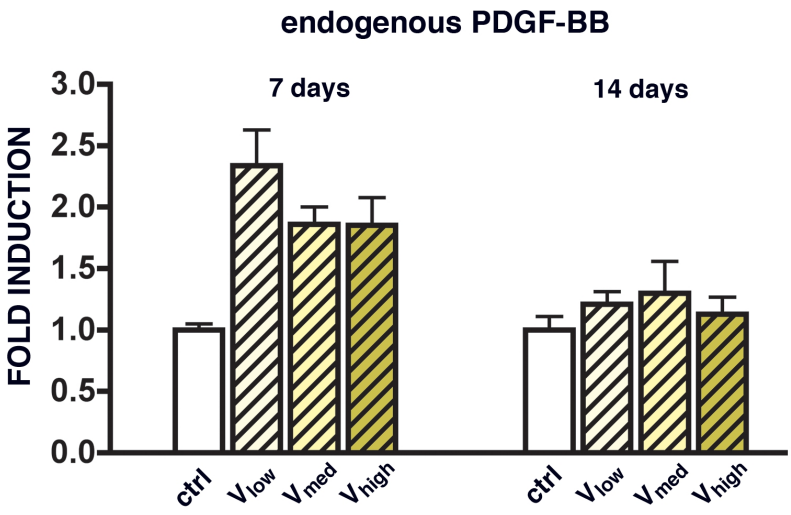

Figure 6. **Endogenous PDGF-BB is not differentially expressed at different VEGF levels**. Muscles were harvested 7 and 14 days after implantation of V clones or control cells (ctrl). Relative mRNA expression of endogenous PDGF-BB was quantified by qRT-PCR.

These data show that endogenous *Pdgfb* expression is not differentially regulated by increasing VEGF doses and that, in all cases, pericyte recruitment takes place only in the initial stages of angiogenesis and is completed between 1 and 2 weeks. Interestingly, these data also suggest that the induction of aberrant angiogenesis by high VEGF levels may depend on an imbalance between excessive VEGF stimulation and insufficient pericyte recruitment by endogenous *Pdgfb* levels, that are not correspondingly up-regulated.

TGF- β and Sema3A expression is differentially regulated by increasing VEGF doses and PDGF-BB co-expression

As the differences in stabilization kinetics of vessels induced by increasing doses of VEGF alone or with PDGF-BB did not correlate with differences in the quantity or timing of pericyte recruitment, we investigated whether the expression of maturation factors

known to mediate pericyte-endothelium cross-talk might be differentially regulated. Therefore we analyzed the expression of Ang-1, TGF- β1 and semaphorin 3A (Sema3A), an endothelium-associated factor that has been recently associated with vascular normalization and stabilization (Maione 2009).

Two weeks after myoblast injection, expression of all investigated genes was not changed compared with the control condition any VEGF level (data not shown), confirming that the critical signaling between pericytes and endothelium takes place in the initial stages.

After 7 days, expression of Ang-1 was almost 2-fold higher than control, but did not vary between different VEGF levels. On the other hand, both TGF-β and Sema3A expression displayed a trend consistent with that of vascular stabilization, with a 5-fold and 3.5-fold up-regulation, respectively, at low VEGF levels, which was reduced with increasing VEGF dose (Fig.7).

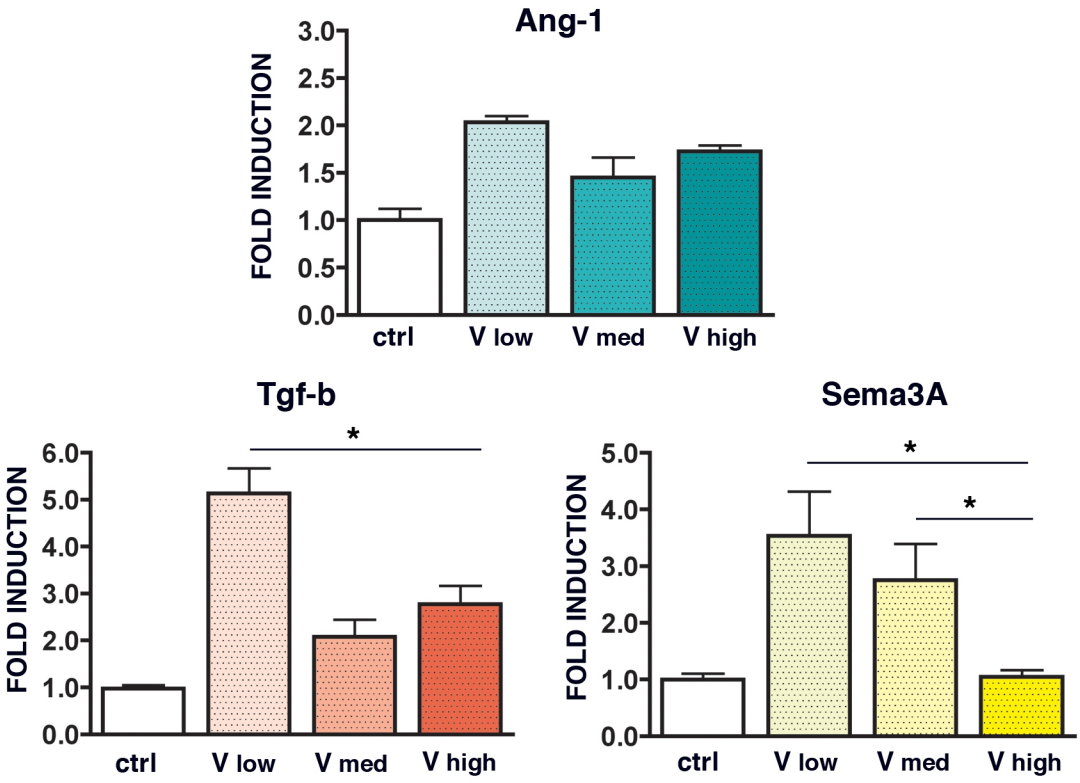

Figure 7. **TGF-β and Sema3A, but not Ang-1, are differentially expressed at different VEGF levels**. Muscles were harvested 7 days after implantation of V-low, V-med and V-high clones or control cells (ctrl). Relative mRNA expression of Ang-1, TGF-β and Sema3A was quantified by qRT-PCR. $p<0.05$

To evaluate the effect of exogenous PDGF-BB co-expression on the regulation of the same maturation-associated genes, we performed qRT-PCR from muscles implanted with VIP-low, VIP-med and VIP-high myoblasts, harvested 7 days after injections.

Again, Ang-1 expression was not regulated by different doses of VEGF and PDGF-BB. Interestingly, the expression trends of TGF-β and Sema3A were completely reversed by PDGF-BB co-expression compared to VEGF alone, showing increasing up-regulation with increasing levels of VEGF and PDGF-BB (Fig. 8).

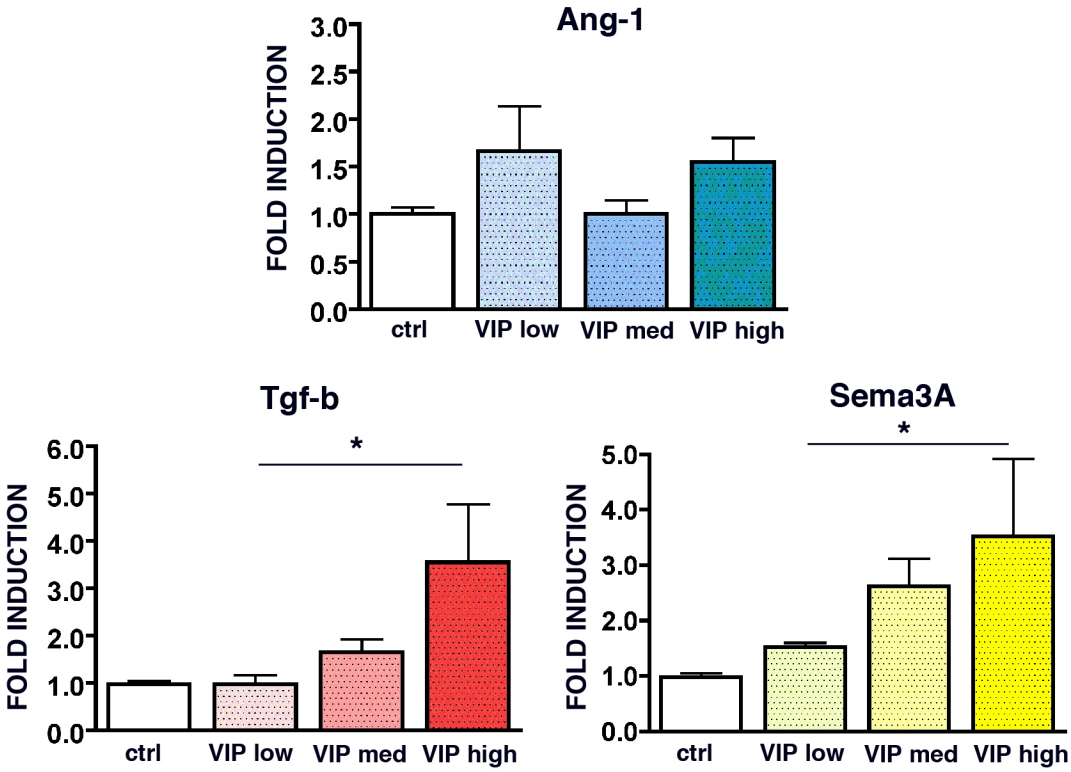

Figure 8. Exogenous PDGF-BB co-expression reverts the VEGF dose-dependent expression of maturation-associated genes. Muscles were harvested 7 after implantation of low-VIP, med-VIP, high-VIP clones or control cells (ctrl). Relative mRNA expression of exogenous PDGF-BB, Ang-1, Ang-2, TGF-β and Sema3A was quantified by qRT-PCR. $p<0.05$

The expression trends of TGF-β and Sema3A with increasing VEGF doses and with PDGF-BB co-expression correlate well with the observed trends of vascular stabilization in the same conditions. Therefore, these results suggest that differential regulation of TGF-β and Sema3A signaling may represent the molecular mechanism underlying the VEGF-dose dependency of vascular stabilization despite similar amounts and timing of pericyte recruitment. Further experiments will be necessary to validate this hypothesis and elucidate the signaling network between different VEGF and PDGF-BB doses, TGF-β and Sema3A.

4.4 Discussion

VEGF over-expression in skeletal muscle provides a potential strategy for treating ischemia associated with peripheral artery disease. However, uncontrolled VEGF delivery either by retrovirally transduced myoblasts (Springer 1998), plasmid DNA (Schwarz 2000), adenoviral vectors (Petterson 2000) or adeno-associated vectors (Zacchigna 2007) (Karvinen 2011) induces the formation of aberrant angioma-like vascular structures. Furthermore, short VEGF expression (less than 4 weeks) leads to unstable vessels that promptly regress after stimulus cessation (Dor 2002) (Tafuro 2009).

Here we have shown that PDGF-BB co-delivery can significantly accelerate vascular stabilization in presence of uncontrolled VEGF levels, making more than 90% of induced vessels independent of VEGF signaling already after three weeks (Fig 1). Furthermore, using clonal myoblast populations expressing specific low, medium ang high homogeneous VEGF levels, we found that VEGF impairs stabilization in a dose-dependent fashion, while PDGF-BB selectively accelerates stabilization at high VEGF levels (Fig 3).

As previously described, high VEGF alone induced aberrant vessels wrapped by α-SMA$^+$ cells, whereas PDGF-BB co-expression restored NG2$^+$ pericyte coverage and yielded normal capillary networks instead (Fig 5 e and f) (Ozawa 2004). It has been recently found that VEGF negatively regulates pericyte function by inhibiting PDGFR-β phosphorylation through the formation of a non-functional VEGFR2/ PDGFR-β complex (Greenberg J 2008). Therefore, increasing VEGF doses might interfere with endogenous PDGF-BB signaling and prevent pericyte recruitment and PDGF-BB co-expression could overcome the anti-pericyte effect of high VEGF doses, re-establishing formation of the functional PDGFR-β. This provides a likely mechanism for the observed switch between

normal and aberrant angiogenesis. In fact, our previous data (Banfi, manucript submitted) show that this transition is not a fixed property of VEGF dose, but depends on the balance between VEGF and PDGF-BB signaling *in vivo*. However, it is unlikely that the competition between VEGF and PDGF-BB for PDGFR-β may explain the negative effect of increasing VEGF doses on vascular stabilization. In fact, such a mechanism would regulate stabilization through differential pericyte recruitment. However, all normal capillaries induced by both low and medium VEGF doses, as well as by all VEGF and PDGF-BB doses, showed no differences in either quantity or timing of pericyte recruitment, despite clearly different stabilization rates (Fig 5).

The role of pericytes in protecting from vascular regression is probably complex. Studies on vascular regression in the retina, as a result of hyperoxia, and in tumors, as a result of VEGF withdrawal, demonstrated a protecting role for pericytes against regression (Helfrich 2010) (Benjamin 1998). However, in retina and tumors, vessel regression has been described also for pericyte-covered vessels (Inai 2004) (von Tell 2006). Our data show that all normal capillaries induced by different doses of VEGF alone or with PDGF-BB were fully covered by pericytes by 2 weeks (Fig.5), but stabilization was still incomplete and further increased by 3 weeks. Therefore, pericyte recruitment is necessary, but further steps are required, which can take a variable amount of time.

We speculated that VEGF dose might influence vascular stabilization by modulating the nature of pericyte-endothelial interaction, rather than pericyte coverage per se. In particular, the trends of expression of both TGF-β and Sema3A correlated well with the obsedved trends of vascular stabilization with increasing VEGF doses and PDGF-BB co-expression. This is consistent with the roles described for these molecules in vascular maturation. TGF-β has been shown to promote the establishment of basement

membrane around new vessels and to favour endothelial cell quiescence (Holderfield 2008).

Semaphorin 3A (Sema3A) has been recently described to cooperate with $VEGF_{165}$ in regulating angiogenesis through the shared receptor neuropilin-1 (Np-1) (Narazaki 2006). $VEGF_{165}$ and Sema3A are integrated via a reciprocal negative feedback pathway and have opposite effects on endothelial cell proliferation and migration. In normal angiogenesis the balance between the two provides a mechanism to limit vessel formation, while in tumors high VEGF expression overcomes the inhibitory effect of Sema3A (Vacca 2006). In an *in vivo* model of tumor progression, expression of exogenous Sema3A improved pericyte coverage of tumor blood vessels (Maione 2009). Further, Sema3A is a strong attractant for a subset of NP-1-positive bone marrow-derived cells, recently described to favour vascular stabilization (Zacchigna 2008). Since Sema 3A can act both directly on endothelial cells and indirectly through recruitment of circulating accessory cells, it will be necessary to determine whether the molecular mechanism involves the cross-talk between endothelium and pericytes or recruited myeloid cells. Quantification of the amount of NP-1 positive myeloid cells in muscles implanted with the different clones is currently ongoing.

Considering the specific coincidental up-regulation of both TGF-β and Sema3A expressions, and the fact that TGF-β has been shown to regulate Sema3A expression during tooth development (Kettunen P. 2004), it is tempting to consider the possibility that may start a signal cascade in which one factor is upstream of the other. To elucidate the signaling network between VEGF, TGF-β and Sema3A, as well as ist modulation by PDGF-BB co-expression, further in vitro experiments are needed to study the expression of these genes in endothelial and pericyte cells after stimulation with different doses of VEGF and PDGF-BB.

Our findings have important implications to improve therapeutic angiogenesis by VEGF-gene therapy

Although low level of VEGF alone succeded in providing stable angiogenesis at short time points, PDGF-BB co-expression adds a clear contribution in accelerating vascular stabilization; indeed high VIP-induced vessels stabilization rate increased from around 50% at 2 weeks up to 80% at 3 weeks, while low-V associated stabilization increased only of 10% in the same time-frame.

In fact, in the presence of uncontrolled VEGF expression, the dual effect of PDGF-BB to both normalize aberrant vasculature induced by high VEGF levels and to significantly accelerate vascular stabilization, ensured that all induced vessels were normal capillaries and that 50% were VEGF-independent already within 2 weeks, which is a time compatible wuth adenoviral expression, whereas none of the vessels induced by VEGF alone were stable after the same time (Fig 1).

Taken together, these results suggest that coordinated co-expression of VEGF and PDGF-BB could overcome the need to both control the distribution of VEGF doses in vivo and to maintain sustained expression, that have substantially limited the usefulness of VEGF-based gene therapy until now.

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Chapter 5

Adenoviral co-delivery of $VEGF_{164}$ and PDGF-BB induces safe and persistent angiogenesis

5.1 Introduction

Recombinant adenoviruses are the most frequently used vectors in gene therapy clinical trials (www.wiley.co.uk/genetherapy/clinical) due to several attractive features. In fact, they can efficiently infect quiescent, differentiated cells *in vivo*, deliver transgenes up to 30 Kb of size and they do not integrate into the host genome (Volpers 2004).

In immunocompetent animals, adenoviral vectors have a short duration of expression of around 10-14 days (Korpisalo 2007). While this is a disadvantage if a permanent replacement of a defective gene is needed, a limited duration of expression is actually desirable in therapeutic angiogenesis applications, since the production of angiogenic growth factors beyond the period necessary to induce new and stable vessels can increase the risk of toxic effects (Korpisalo 2010).

However, when VEGF is the delivered gene, the duration of expression provided by adenoviruses is too short to allow the efficient stabilization of the initial angiogenic response (Ozawa 2004)(Dor 2002)(Rissanen 2007). Furthermore, the necessity to control VEGF dose *in vivo* to avoid the formation of aberrant structures, as already described in section 3.2, makes adenoviral VEGF gene delivery even more problematic.

We have previously found that PDGF-BB co-expression can induce the growth of homogeneous normal capillary networks despite heterogeneous and high VEGF levels by using a transduced myoblast-based gene delivery platform (Banfi, manuscript submitted). Furthermore, in chapter 4 we have shown that PDGF-BB significantly accelerates stabilization of VEGF-induced vessels. Therefore, we hypothesized that VEGF and PDGF-BB co-expression might induce both homogeneously normal and persistent angiogenesis

despite the heterogeneous levels and short duration of expression allowed by adenoviral vectors.

To test this hypothesis, we analyzed the phenotype and the persistence of vessels induced after intramuscular injection of adenoviral vectors expressing VEGF alone or in combination with PDGF-BB, either in immunocompromised mice, which allow indefinite expression, or in immunocompetent animals, which clear the virus within 2 weeks.

We found that, when VEGF is co-expressed together with PDGF-BB, only normal capillaries were induced, a portion of which persisted even after viral expression ceased, while VEGF alone caused the growth of aberrant vascular structures that were still unstable after 2 weeks.

Based on this evidence we conclude that PDGF-BB co-expression could allow the formation of homogeneously normal and stable new vessels within the short duration of transgene expression, allowed by adenoviral vectors, thereby overcoming two major limitations of VEGF gene therapy in a clinically relevant setting.

5.2 Material and methods

Adenovirual production

Recombinant adenoviruses have been produced using Adeno-X™ Expression System (Clontech) according to manufacturer's recommendations. Briefly, target gene was cloned into pShuttle vector, subcloned into Adeno-X viral DNA and used for transfection of HEK293 cells with Fugene HD reagent (Roche). After 1 week, viral particles were collected from transfected cells by repeated freezing–thawing and used for infection of fresh HEK293. After 4–5 lysis-infection cycles, viral particles were collected and purified by a double Cesium chloride gradient. All adenoviral constructs expressed a truncated version of CD8 as marker gene. Viral titer (ifu= infectious unit) was determined after infection of HEK293 cells by calculating the number of infected cells after infections at different MOI (Gueret 2002).

Adenoviral vectors were diluted in physiological solution and injected in ear and hindlimb muscles of SCID or C57BL6 mice (Charles River) at titer of $1\text{-}3x10^8$ ifu.

Tissue staining

The entire vascular network of the ear could be visualized following intravascular staining with a biotinylated *Lycopersicon esculentum* lectin (Vector Laboratories) that binds the luminal surface of all blood vessels, as previously described (Ozawa 2004). Mice were anesthetized, lectin was injected intravenously and 4 minutes later the tissues were fixed by vascular perfusion of 1% paraformaldehyde and 0.5% glutaraldehyde in PBS pH 7.4. Ears were then removed, bisected in the plane of the cartilage, and stained with X-gal

staining buffer (1 mg/ml 5-bromo-4-chloro-3-indoyl-β-D-galactoside, 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 0.02% Nonidet P-40, 0.01% sodium deoxycholate, 1 mM $MgCl_2$ in PBS pH 7.4). Tissues were stained using avidin-biotin complex-diaminobenzidine histochemistry (Vector Laboratories), dehydrated through an alcohol series, cleared with toluene and whole-mounted on glass slides with Permount embedding medium (Fisher Scientific). Vascular morphology was analyzed at 1, 2 and 4 weeks post-injection.

For tissue sections, mice were anesthetized and fixed by vascular perfusion of 1% paraformaldehyde in PBS pH 7.4. *Tibialis anterior* and *gastrocnemious* muscles were harvested, embedded in OCT compound (Sakura Finetek), frozen in freezing isopentane and cryosectioned. Immunofluorescence was performed on cryosection of 10 μm in thickness; section were permeablized by incubation with 0.3% Triton (Sigma-aldrich) and 2% goat serum (Invitrogen) in PBS for 1 hour at room temperature. The following primary antibodies and dilutions were used: rat monoclonal anti-mouse PECAM-1 (BD-Pharmingen) at 1:100; mouse monoclonal anti-mouse α-SMA (clone 1A4, MP Biomedicals) at 1:400; rabbit polyclonal anti-NG2 (Millipore) at 1:200. Fluorescently labeled secondary antibodies (Molecular Probes, Invitrogen) were used at 1:200. Antibodies incubation were performed at room temperature for 1 hour.

5.3 Results

Adenoviral co-delivery of VEGF and PDGF-BB prevents the formation of aberrant vessels and induces only normal capillaries

We recently found that co-delivery of PDGF-BB can prevent the formation of aberrant vessels induced by uncontrolled VEGF levels in a cell-mediated gene transfer system (Banfi submitted) suggesting that coordinated co-expression of VEGF and PDGF-BB might allow the induction of controlled normal angiogenesis despite high and uncontrolled VEGF levels, generated by the direct delivery of a gene therapy vector *in vivo*. To test this hypothesis, we produced 4 different adenoviral vectors: a control virus expressing only the truncated form of CD8 used as a marker in all other vectors (Ad-CD8), a virus expressing only PDGF-BB (Ad-PDGF), a virus expressing only VEGF (Ad-VEGF) and finally a virus expressing both VEGF and PDGF-BB linked through an Internal Ribosome Entry Site (IRES) sequence (Ad-VIP for VEGF-IRES-PDGF) (Fig.1).

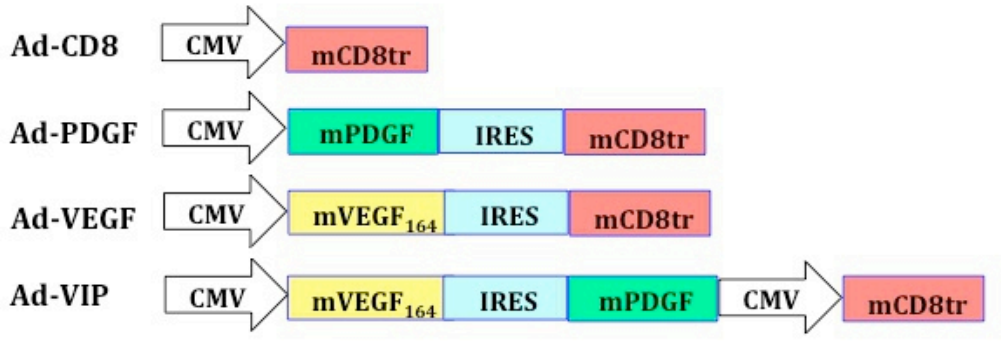

Figure 1. **Adenoviral vectors.** We produced four viral vectors, expressing either $VEGF_{164}$ alone, PDGF-BB alone or the two factors linked through an IRES sequence. The control vector expressed only a truncated form of mouse-CD8a as a marker.

Effective growth factor production from the recombinant adenoviruses was confirmed by ELISA after *in vitro* infection of HEK293 cells.

We first addressed the question whether PDGF-BB co-expression could normalize VEGF-induced aberrant angiogenesis after adenoviral delivery. To allow full development of all vascular phenotypes, we used immunocompromised SCID mice to avoid clearance of the viral vectors and ensure sustained transgene expression.

$1*10^8$ ifu (infectious unit) of each virus were injected in the posterior auricular muscle of SCID mice and the morphology of induced vessels was visualized by intravascular lectin staining 2 and 4 weeks later.

Both Ad-CD8 and Ad-PDGF-BB did not affect the pre-existing capillary network either at 1 (Fig 2a and 2b), 2 (Fig 2e and 2f) and 4 weeks (Fig 2i and 2j).

On the other hand, after 1 week Ad-VEGF induced a massive enlargement of pre-existing vessels (Fig 2c), which gave rise to aberrant bulbous structures of heterogeneous sizes and irregular shapes by 2 weeks (Fig 2g). VEGF-induced angiogenesis was still diffusely aberrant 4 weeks after vector delivery (Fig 2k). The Ad-VIP induced a clear increase in vessel density in the injection areas. However, Ad-VIP induced vessels were always homogeneous normal capillaries with regular shape and slightly increased diameter compared to pre-existing vessels, and were stable over all 4 weeks (Fig 2d, 2h and 2l).

Therefore, adenoviral co-delivery of VEGF and PDGF-BB completely prevented the formation of angioma-like vascular structures and yielded only normal capillaries despite uncontrolled and sustained gene expression.

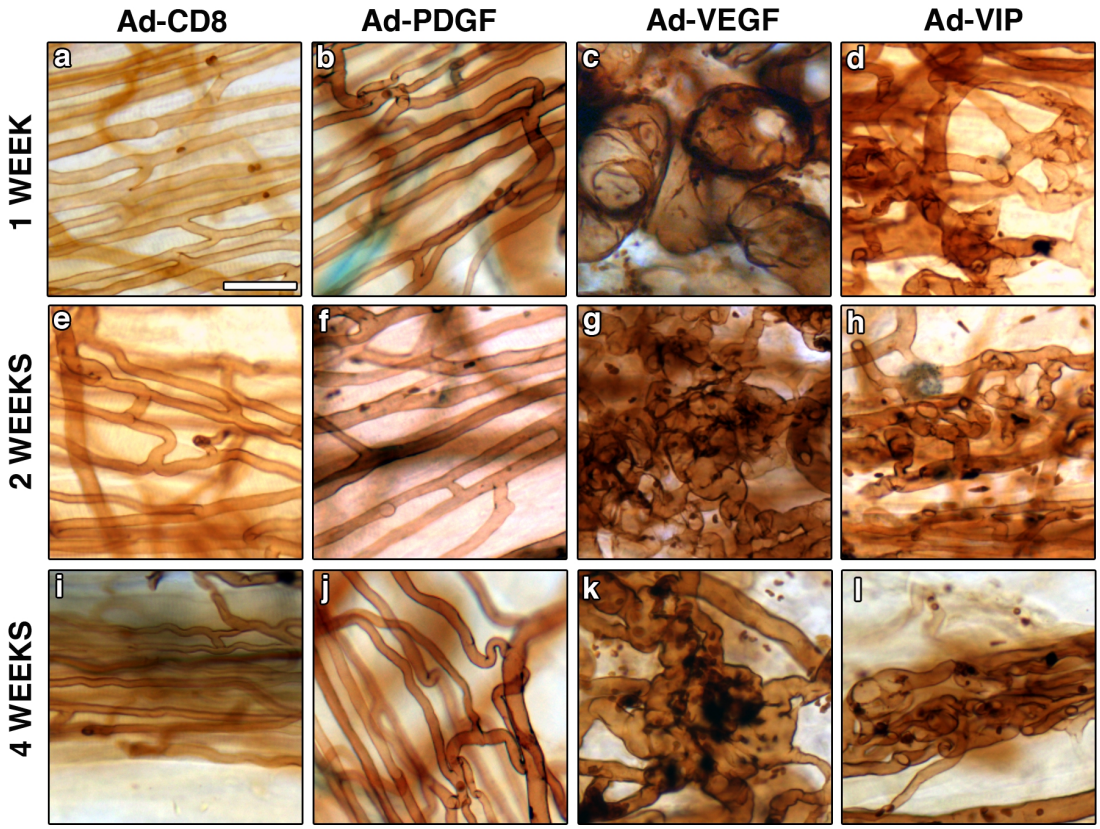

Figure 2. **Adenoviral co-delivery of VEGF and PDGF-BB prevents the formation of aberrant vasculature**. Adenoviruses expressing VEGF, PDGF-BB, or both (Ad-VIP) were injected in the ear muscles of immunocompromised mice. Adenovirus expressing only CD8 was used as a control. Blood vessels were visualized in whole-mount preparations after intravascular lectin perfusion (in brown) at 1week (a-d), 2 weeks (e-h) and 4 weeks (i-l) post-injections. Size bar=40 μm.

Adenoviral co-delivery of VEGF and PDGF-BB induces persistent vessels

In order to verify whether PDGF-BB co-expression could accelerate the stabilization of VEGF-induced angiogenesis in a clinically relevant model, we injected $1*10^8$ ifu of the adenoviruses described in Fig 1 above in the posterior auricular muscle of immunocompetent C57BL6 mice. It has been previously shown that, in this animal model, adenoviruses are cleared by the host immune system within 14 days (Korpisalo 2007).

Therefore, we analyzed the induction of new vessels 1 week after injection and their persistence 2 and 4 weeks later by intravascular lectin staining.

After one week of expression, Ad-VEGF induced the formation of enlarged, angioma-like vascular structures, similar to those induced in SCID mice (Fig 3b). Two weeks after injection aberrant angiogenesis was still visible although less extensive (Fig 3d), but completely disappeared after 4 weeks, consistently with the adenoviral clearance timing. Only very few enlarged but homogeneously shaped were still detectable (Fig 3f).

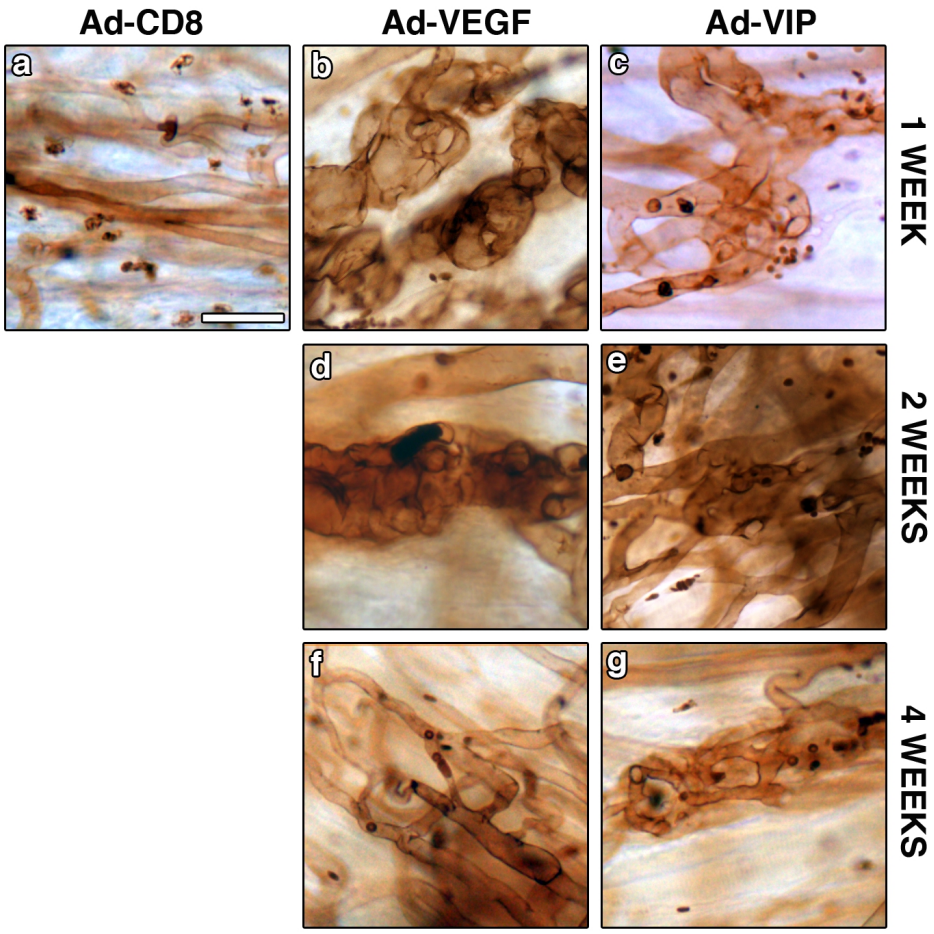

Figure 3. **Adenoviral co-delivery of VEGF and PDGF-BB induces normal and persistent vessels**. Adenoviruses expressing VEGF alone (Ad-VEGF) or together with PDGF-BB (Ad-VIP) were injected in the ear muscle of immunocompetent mice. Adenovirus expressing only CD8 was used as a control. Blood vessels were visualized in whole-mount preparations after intravascular lectin perfusion (in brown) 1 (b, c), 2 (d, e) and 4 weeks (f, g) after injection. Size bar=40 μm.

Similarly to the results in immunocompromised mice, Ad-VIP induced the formation of only normal capillaries after 1 week of expression (Fig 3c) which did not appear modified after 2 weeks (Fig 3e). Interestingly, a portion of VIP-induced network of uniformely enlarged vessels was still present after 4 weeks (Fig 3g).

To analyze the maturation status $3*10^8$ ifu of each adenovirus were injected in the tibialis anterior skeletal muscles of C57BL6 mice. Samples were harvested after 1,2 and 4 weeks and vascular structures were visualized in frozen section by immunostaining for CD31 (endothelial cells), α-SMA (smooth muscle cells) and NG-2 (pericytes).

Viral transduction was confirmed both by CD8 staining of infected fibers and by infiltration of immune cells. Both at 1 and 2 weeks, we observed some CD8-positive fibers and a massive infiltrate of $CD8^+$ T lymphocytes. After 4 weeks, however, no sign of viral presence was detectable, confirming the clearance by the immune system (data not shown).

Confirming the results obtained by myoblast-based gene delivery, aberrant vascular structured induced by Ad-VEGF skeletal muscle were not surrounded by $NG2^+$ pericytes but were covered by SMA^+ cells (Fig 4b). These structures were detectable 1 and 2 weeks after injection (Fig 4b and 4d). However, after 4 weeks aberrant vessels regressed completely and only few endothelial structures were still visible (Fig 4f).

PDGF-BB co-expression prevented the formation of angioma-like structures and caused the formation of only normal capillaries surrounded by $NG2^+$ pericytes that were clearly visible both 1 and 2 weeks after injection (Fig 4 c and e).

A portion of Ad-VIP-induced angiogenesis became stable and was still present after 4 weeks (Fig 3g). Interestingly, the stabilized capillaries were associated with morphologically normal pericytes that, however, were partly $NG2^+$ and partly SMA^+.

Taken together, these results confirmed the potential of PDGF-BB co-expression to induce normal vessels that stabilize more rapidly and persist after the cessation of the VEGF stimulus.

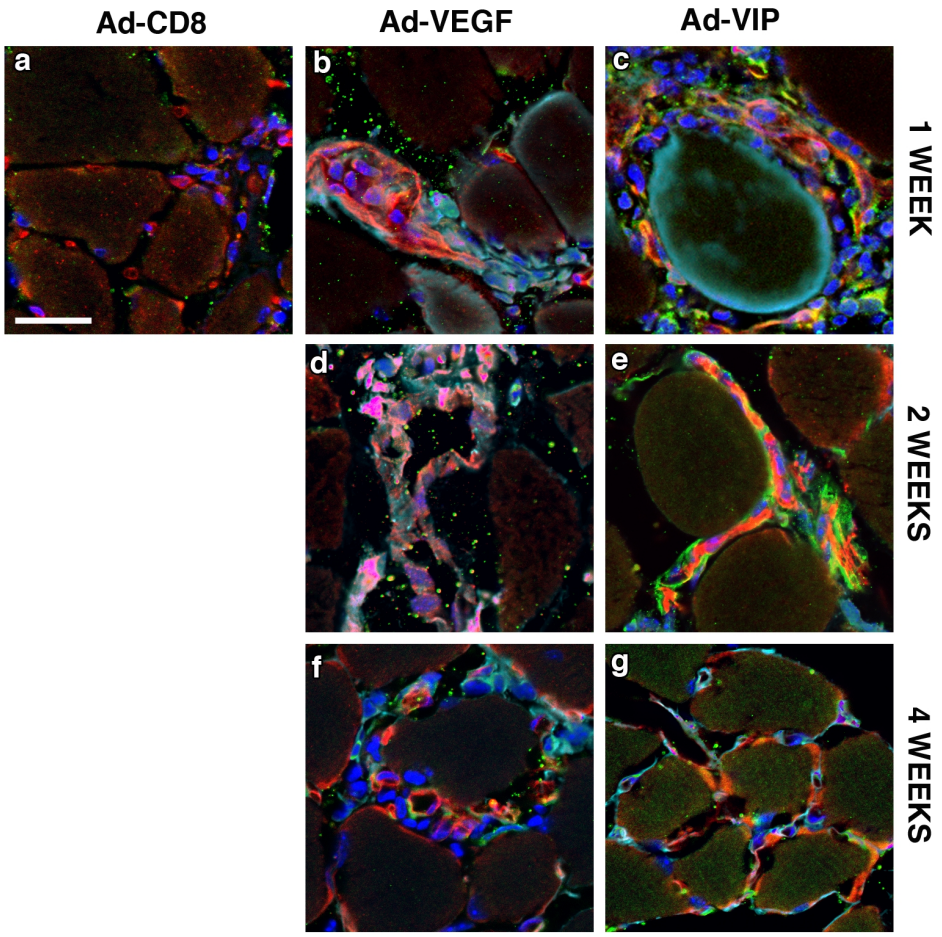

Figure 4. **Adenoviral co-expression of VEGF and PDGF-BB induces normal and persistent vessels in skeletal muscle**. Adenoviruses expressing VEGF alone (Ad-VEGF) or together with PDGF-BB (Ad-VIP) were injected in tibialis anterior muscles of immunocompetent mice. Adenovirus expressing only CD8 was used as a control. Vascular structures were visualized by immunofluorescent staining of endothelium (CD31, in red), pericytes (NG2, in green) and smooth muscle actin (cyan) 1 (b, c), 2 (d, e) and 4 weeks (f, g) after injection. Nuclei are stained with DAPI (blue). Size bar=25 μm.

5.4 Discussion

Angiogenesis induction by adenoviral delivery of VEGF which failed to show convincing efficacy in clinical trials, suffers from two main limitations:

1) VEGF dose can be controlled only at the level of total dose, by changing the titer of viral particles delivered, but not at the microenvironmental level, leading unavoidably to the generation of heterogeneous expression levels in tissues. This makes it difficult to identify a therapeutic window for VEGF gene delivery (Banfi 2005).

2) Adenoviral expression typically declines to basal level during the second week after injection (Korpisalo P. 2007). This prevents the stabilization of new vessels which normally require about 4 weeks of sustained VEGF expression (Dor 2002).

We found that PDGF-BB co-expression can overcome both limitations, inducing the formation exclusively of normal angiogenesis, which stabilizes rapidly and persists after the vessation of adenoviral vector expression.

The inability of VEGF-expressing adenoviruses to induce safe and stable vessels in skeletal muscle has been extensively reported. Vajanto et al. described an excessive vascular growth associated with edema and muscle necrosis 8 days after intramuscular injection of VEGF-expressing adenoviruses in rabbits. However, abnormal vascular growth had disappeared after 30 days (Vajanto 2002). Petterson et al. reported the formation of vascular malformations, defined as glomeruloid bodies, 18 hours after intramuscular Ad-VEGF injection and visible only up to 3 weeks (Petterson 2000). Moreover, an increased vascular density has been associated only with the transient period of VEGF expression allowed by adenoviruses in ischemic rabbit muscle (Gounis 2005). In agreement with these findings, we showed that Ad-VEGF, both in ear and skeletal muscle, caused the formation of

aberrant vasculature that regressed after the cessation of viral expression. However, few normal capillaries were still visible after 1 month (Fig 3f and 4f), consistently with the ability of low levels of VEGF alone, which are likely represented among the heterogeneous levels induced by Ad-VEGF, to induce normal capillaries that partly stabilize in 2 weeks, as described in chapter 4.

Our data suggest that PDGF-BB co-expression represents a convenient and effective strategy to overcome the side-effects of adenoviral-driven VEGF expression. In fact, the expression of both VEGF and PDGF-BB from a single bicistonic virus completely abolished the formation of aberrant vessels and generated the formation of only normal capillaries at all time points. Furthermore, in an immunocompetent mouse model, a portion of vessels induced by Ad-VIP persisted after 4 weeks, in agreement with the accelerated stabilization rate observed with myoblasts expressing heterogeneous VEGF and PDGF-BB levels, described in Chapter 4.

The ability of VEGF and PDGF-BB adenoviral delivery to induce stable vessels and to reduce VEGF-associated edema formation has been recently tested. Korpisalo et al. expressed the two growth factors from two separate vectors into rabbit hindlimb skeletal muscle (Korpisalo 2008). However, they found that, at 6 days, the combination of VEGF and PDGF-BB did not significantly improve angiogenesis and failed to reduce VEGF-induced acute edema. In addition, vascular structures induced by the combination of Ad-VEGF and Ad-PDGF showed an impaired pericyte coverage, since pericytes were recruited away from vascular structures, and did not stabilize after 28 days. These results emphasize the importance of a proper co-localization of VEGF and PDGF-BB gradients in target tissue in order to induce safe and stable angiogenesis. In fact, when the two factors were co-

expressed from a single bicistronic vectors, ensuring a coincidential production from the same infected cells in a fixed relative amount, we found that aberrant VEGF-induced angiogenesis was completely abrogated and switched to normal peritcyte-covered capillaries.

Interestingly, Korpisalo et al. observed a prolongation of the duration of muscle perfusion after the injection of Ad-VEGF and Ad-PDGF compared to Ad-VEGF alone, despite an impaired pericyte recruitment. This has been ascribed to a paracrine effect of PDGF-recruited cells of the monocyte/macrophage lineage that expressed further endogenous (Korpisalo 2008). We cannot exclude a role for recruited inflammatory cells also in our system. However, our data clearly show that when VEGF and PDGF-BB are expressed by the same cells, creating a coincidental gradient of the two factors, PDGF-BB can normalize VEGF-induced aberrant vascular growth and accelerates vessels stabilization by restoring a proper pericyte coverage.

Taken together, these results suggest that PDGF-BB co-expression overcomes some of the intrinsic limitations of VEGF gene therapy with adenoviral vectors, leading to safe and stable angiogenesis despite short-term, heterogeneous expression of VEGF.

Further experiments will be necessary to determine whether the normalization and stabilization of vascular growth induced by viral co-delivery of VEGF and PDGF-BB can translate in functional improvement in a hindlimb ischemia model. Furthermore, the fact that homogeneous normal angiogenesis is achievable despite heterogeneous and uncontrolled growth factor expression suggests that higher and more effective dosed of viral vectors may be safe compared to delivery of VEGF alone. A rigorous determination of the therapeutic window of viral co-delivery of VEGF and PDGF-BB will need to be

determined in clinically reflective animal models.

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Summary and future perspectives

Blood vessel growth is a complex process that requires the coordinated interactions of many signaling pathways and different cell types. However, despite this molecular complexity, the whole cascade of events can be set in motion by a single factor, VEGF, that is the most important regulator of both physiological and pathological angiogenesis and its over-expressed has been investigated in several ischemic tissues with the aim to restore blood flow supply. However, VEGF gene delivery presents two main limitations that make it challenging to achieve therapeutic angiogenesis in a clinical setting:

1) VEGF can induce normal or aberrant angiogenesis depending strictly on its expression level in the microenvironment around each producing cell *in vivo* and not on its total dose. Therefore, it's not sufficient to control the total amount of delivered VEGF-expressing vector, but it is necessary to control the distribution of expression levels from each transduced cell *in vivo* (Ozawa 2004).

2) In addition, new induced vessels require at least 4 weeks of expression in order to become stable and independent from further VEGF signaling.

The use of short-term expressing gene therapy vectors, such as adenoviruses, is desirable both for the intrinsic safety of limited duration of expression and for the convenience of an off-the-shelf treatment. However, traditional short-term expressing vectors cannot fulfill the requirements of either dose control and or sufficient duration of expression necessary in order to induce safe and efficient angiogenesis

Myoblast-mediated gene transfer provides a highly controlled delivery system that allows the selection and the *in vitro* characterization of myoblasts expressing specific VEGF levels and ensures a sustained expression over time.

Using this tool, we previously found that co-expression of PDGF-BB, which controls pericyte recruitment and vascular maturation, ensures the induction of only normal and functional capillaries despite the expression of high and heterogeneous VEGF levels (Banfi, manuscript submitted). Here, we aimed to establish whether PDGF-BB co-expression could also accelerate the stabilization of VEGF-induced vessels and, therefore, provide a safe and efficient strategy to achieve therapeutic angiogenesis within the short duration of expression afforded by adenoviruses.

In Chapter 4, we first assesed the stabilization time-course of vessels induced by myoblasts expressing heterogeneous VEGF levels, in the absence or presence of PDGF-BB, after 2 or 3 weeks of expression. We found that 50% of capillaries induced by VEGF and PDGF-BB co-expression were VEGF-independent already after 2 weeks and 90% after 3 weeks, compared to none after 2 weeks and only 30% after 3 weeks with VEGF alone. These data suggest that VEGF and PDGF co-expression from a single bicistronic construct represents a convenient strategy to induce persistent vessels within a short time of expression, with clear implications for therapeutic applications.

Next we defined, for the first time, whether VEGF dose regulates the time-course of vascular stabilization and how PDGF-BB affects stabilization at different VEGF doses. Interestingly, we found that VEGF negatively regulates vascular stabilization in a dose-dependent fashion. In fact, normal capillaries induced by low VEGF levels stabilized faster than similarly normal vessels induced by higher doses at every time point considered. Aberrant vascular structures induced by very high VEGF levels never stabilized. Surprisingly, we found that PDGF-BB accelerated vascular stability only at medium and high VEGF levels, while it did not have significant effects on vessels induced by low VEGF doses.

Mechanistically, we found that vascular stabilization is not simply dependent on the presence of pericytes, because normal capillaries induced by different doses of VEGF alone or with PDGF-BB were equally pericyte-covered but stabilized with different time-courses. Therefore, we investigated the regulation of several factors involved in vascular maturation in the different conditions. Intriguingly, we observed a correlation between the trend of expression of TGF-β1 and Sema3A and the trend of vascular stabilization at different VEGF and PDGF-BB doses.

Sema3A has been recently described to promote vascular stabilization indirectly through the recruitment of a subset of neuropilin-1^{+} (NP1^{+})-CD11b^{+} bone marrow-derived cells (Zacchigna 2008). These cells are thought to exert their stabilizing effect by expressing pro-maturation factors, such as TGF-β1 and PDGF-BB. Therefore, as a next step, it will be interesting to determine whether Sema3A and TGF-β1 are involved in vascular maturation through molecular mechanisms involving only the cross-talk between endothelium and pericyte or whether their action is mediated through the recruitment of bone-marrow derived myeloid cells. In order to clarify these interactions, we will test whether different VEGF doses, that promote different stabilization kinetics and are associated with a differential regulation of Sema3A and TGF-β1, induce the recruitment of different amount of CD11b^{+}-NP1^{+}positive cells in the injected muscle from the circulation. Furthermore, the flow cytometry isolation of endothelial cells, pericytes and myeloid cells from muscles injected with myoblast expressing different VEGF doses, alone or with PDGF-BB, and the subsequent analysis of their expression of each molecule by qRT-PCR, will allow us to define quantitatively and qualitatively the role of the different cell types in VEGF-dose dependent vascular stabilization.

On the other hand, nothing is known regarding a possible direct regulation of Sema3A expression in vascular cells by VEGF, nor its possible dose dependency. Therefore, it will be necessary to investigate the signaling network between VEGF, TGF-β1 and Sema3A, as well as its modulation by PDGF-BB, *in vitro.* In particular, endothelial cells will be stimulated with different doses of VEGF, PDGF-BB or both and the expression of Sema3A and TGF-β1 will be monitored.

The combination of in vivo, ex vivo and in vitro approaches described above should help elucidating the molecular mechanisms by which VEGF controls the maturation of induced vessels and also clarifying the role of bone marrow-derived cells in the stabilization of VEGF-induced vessels in skeletal muscle.

On the basis of results obtained in the previous chapter, in Chapter 5 we aimed to test whether VEGF and PDGF-BB co-expression from an adenoviral vector might induce safe and persistent angiogenesis in the short frame of expression allowed by this vector. Indeed, we found that PDFG-BB co-expression prevented the formation of aberrant vessels induced by Ad-VEGF and yielded only normal capillaries, when the two factors were expressed from a single bicistronic vector. Furthermore, in an immunocompetent model, in which adenoviral vectors are cleared within 10-14 days, we found that a proportion of vessels induced by VEGF and PDGF-BB co-expression had successfully stabilized and persisted after the cessation of VEGF expression. Based on this evidence, we conclude that VEGF and PDGF-BB co-expression provides a clinically relevant gene therapy approach for therapeutic angiogenesis.

However, although AV can infect a wide range of cells and tissues, adult muscle fibers are significantly less transducible compared to immature or regenerating myofibers (van

Deutekom 1998). Therefore, adenovirus transduction of normal adult muscle is limited to the damaged and regenerating myofibers around the needle tracks, resulting in rather limited areas of. Therefore, a more effective strategy, could rely on adeno-associated viruses (AAVs) as the delivery vector. AAVs are small vectors that can transduce mature skeletal fibers much more efficiently than adenoviruses, but lead to persistent gene expression due to their low immunogenicity. The use of this vector would allow the testing of the hypothesis that co-expression of VEGF and PDGF-BB, that yield homogeneous, normal and stable angiogenesis despite heterogeneous levels of expression, can be safe even after sustained expression. In fact, continuously growing angioma-like structures are completely prevented and the induced normal capillary networks have been found to be stable over at least several months (Gianni-Barrera, manuscript submitted).

In order to translate the proof-of-principle established in this thesis into a clinical application, further work will be necessary to: 1) determine the safety and efficacy of coordinated co-expression of VEGF and PDGF-BB in models of hindlimb or cardiac ischemia, where upregulation of endogenous factors may affect the response to the delivered molecules; and 2) carefully establish the therapeutic window of different vector doses in clinically reflective large animal models.

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…My family, always.

…Antonio, sometimes ;-).

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Oral presentation Advances in the cellular and molecular biology of angiogenesis (27-29 June 2011, Birmingham)

Publications

"Coordinated co-expression of PDGF-BB accelerates stabilization of $VEGF_{164}$-induced vessels in a dose dependent fashion" *Reginato S., Gianni-Barrera R., Heberer M., Banfi A.* (Manuscript in preparation)

"Adenoviral co-delivery of $VEGF_{164}$ and PDGF-BB induces safe and persistent angiogenesis" *Reginato S., Gianni-Barrera R., Heberer M., Banfi A.* (Manuscript in preparation)

"Taming of the wild vessel: promoting vessel stabilization for safe therapeutic angiogenesis" *Reginato S., Gianni-Barrera R., Heberer M.; Banfi A.* (Manuscript in preparation)