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CONCISE REVIEW

STEM CELLS TRANSLATIONAL MEDICINE

Therapeutic vascularization in regenerative medicine

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Abstract

Therapeutic angiogenesis, that is, the generation of new vessels by delivery of specific factors, is required both for rapid vascularization of tissue-engineered constructs and to treat ischemic conditions. Vascular endothelial growth factor (VEGF) is the master regulator of angiogenesis. However, uncontrolled expression can lead to aberrant vascular growth and vascular tumors (angiomas). Major challenges to fully exploit VEGF potency for therapy include the need to precisely control in vivo distribution of growth factor dose and duration of expression. In fact, the therapeutic window of VEGF delivery depends on its amount in the microenvironment around each producing cell rather than on the total dose, since VEGF remains tightly bound to extracellular matrix (ECM). On the other hand, short-term expression of less than about 4 weeks leads to unstable vessels, which promptly regress following cessation of the angiogenic stimulus. Here, we will briefly overview some key aspects of the biology of VEGF and angiogenesis and discuss their therapeutic implications with a particular focus on approaches using gene therapy, genetically modified progenitors, and ECM engineering with recombinant factors. Lastly, we will present recent insights into the mechanisms that regulate vessel stabilization and the switch between normal and aberrant vascular growth after VEGF delivery, to identify novel molecular targets that may improve both safety and efficacy of therapeutic angiogenesis.

KEYWORDS
extracellular matrix, genetic therapy, ischemia, neovascularization, tissue engineering, vascular endothelial growth factor

1 | INTRODUCTION

Blood vessel growth is an integral process in regenerative medicine. Vascular regulation is also key for the repair of naturally avascular tissues such as cartilage, where inhibition of angiogenesis has been shown to favor the spontaneous chondrogenic differentiation of progenitors in vivo.[1] Focusing only on the situations where new vascular induction is required, two main areas of therapeutic interest can be conceptually distinguished: (a) the expansion of pre-existing vascular networks in ischemic tissues to restore blood flow and salvage function (therapeutic angiogenesis); and (b) the rapid de novo vascular invasion of engineered grafts to enable progenitor survival and differentiation.

Roberto Gianni-Barrera and Nunzia Di Maggio contributed equally to the study.

Ischemia is caused by an inadequate blood supply for tissue demand. The most prevalent cause is progressive atherosclerotic stenosis of cardiac or limb arteries (coronary or peripheral artery disease, respectively; CAD and PAD), which can be compounded by microvascular dysfunction in metabolic syndromes such as diabetes. The consequences of CAD range from chest pain upon exertion (*angina*) to myocardial infarction and end-stage heart failure, whereas PAD leads to progressive muscle pain with walking (*claudicatio*), skin ulcerations, and necrosis, necessitating amputation. Impaired wound healing in diabetic patients, especially of the foot, can have dire consequences through infection and necrosis. Together with their high prevalence in western-style societies, cardiovascular ischemic diseases pose a heavy social and economic burden, due to early mortality, impaired quality of life with pain and loss of mobility, and recurrent hospitalization.[2] Current treatments for CAD and PAD mainly include endovascular procedures such as balloon angioplasty/stenting and bypass surgery or possibly exercise training for limb ischemia. However, many patients with end stage CAD or PAD are not candidates for such procedures due to a lack of target vessels. On the other hand, standard treatment of diabetic foot ulcers is purely supportive.[3] Therapeutic angiogenesis, that is, the generation of new vascular networks through delivery of specific growth factors, is an attractive strategy to restore perfusion to ischemic tissues and fill this unmet clinical need.

Another area of regenerative medicine in which vascular growth plays a key role is the vascularization of tissue-engineered grafts.[4] In particular, bone replacement is required in several situations due to trauma, surgery, or idiopathic conditions such as avascular necrosis of small bones, where spontaneous regeneration is insufficient. Bone tissue-engineering holds promise for the generation of osteogenic grafts, combining osteo-progenitors with biocompatible scaffolds.[5,6] However, for defects of clinically relevant size, the lack of rapid vascularization in vivo causes severe ischemia and progenitor death in the graft core deeper than 1 to 2 mm.[7] Similar biological principles apply to the vascularization of both ischemic tissue and engineered grafts, but with different translational considerations.

Significance statement

The promotion of blood vessel growth for therapeutic purposes remains a challenge both for the treatment of ischemic conditions and the generation of functional tissue-engineered grafts. Physiological angiogenesis is a complex and highly concerted process. A fine understanding of the cellular and molecular mechanisms of vascular growth needs to provide the biological basis for the design of rational therapeutic approaches.

2 | BIOLOGICAL BASES OF THERAPEUTIC VASCULARIZATION

After the discovery of vascular endothelial growth factor (VEGF), first as a permeability factor in 1983[8] and then as an endothelial mitogen in 1989,[9,10] intense investigations began into the new concept of therapeutic angiogenesis, aiming at restoring the blood supply in ischemic tissues by growing new blood vessels with VEGF and later other angiogenic factors. Positive preclinical and early clinical evidence seemed to indicate great potential for this strategy. However, a decade of subsequent controlled clinical studies showed that the simple delivery of the VEGF protein or gene to ischemic tissue has no clear efficacy at safe doses.[11] The disappointing clinical results are a stark contrast to the fundamental biological role of VEGF as the master regulator of vascular growth. In fact, VEGF kicks off the complex cascade of cellular and molecular events leading to the orderly assembly of new endothelial structures (morphogenesis), their association with mural cells/pericytes (maturation), and subsequent ability to persist indefinitely in the absence of further growth factor signaling (stabilization), to form fully functional vascular networks.[12] Therefore, a better understanding of the physiological mechanisms of vascular growth is important to exploit its therapeutic potential. Here, we will address some key aspects of the biology of VEGF and angiogenesis, and their therapeutic implications.

2.1 | VEGF and its gradients

The mammalian VEGF family comprises five main ligands (VEGF-A, -B, -C, and -D and placenta-derived growth factor, PlGF) and three receptors (VEGF-R1, -R2, and -R3). Although the principal role of VEGF-C and -D is to stimulate lymphatic angiogenesis through VEGF-R3, blood vessel growth is mostly coordinated by the signaling of VEGF-A and -B and PlGF through R1 and R2 (for a comprehensive review, see reference 13). Despite the multiplicity of players, the molecular target for therapeutic angiogenesis is essentially VEGF-A signaling, as VEGF-B and PlGF play more accessory or tissue-specific roles.[14] We will refer to VEGF-A simply as VEGF throughout the manuscript.

An important feature of VEGF function, with widespread therapeutic implications, is its interaction with extracellular matrix (ECM), which dictates its spatial localization in tissues and regulates the outcome of the angiogenic process. In fact, alternative mRNA splicing of the *Vegfa* transcript gives rise to three major isoforms with different degrees of affinity for the ECM.[15] These comprise 120, 164, and 188 residues in rodents (or 121, 165, and 189 in humans, respectively) due to the presence or absence of heparin-binding domains that interact with ECM proteoglycans, so that matrix affinity is very low in the shortest isoform and increases with molecular size.[16] The isoforms also display differences in their signaling. In fact, $VEGF_{164/165}$ binds the coreceptor Neuropilin-1 (Nrp1), enhancing activation of VEGF-R2 and endothelial proliferation and migration, whereas $VEGF_{120/121}$ does not.[17] Furthermore, a distal splice site in the last exon of the VEGF gene can give rise to a second set of "b" isoforms, which differ only in the sequence of the last six residues and are therefore named $VEGF_{xxxb}$. However, contrary to the classic isoforms, the b variants are antiangiogenic and provide a further layer of regulation to the angiogenic balance in tissues.[18] As a consequence of differential matrix binding, $VEGF_{120/121}$ is highly diffusible in tissues, $VEGF_{188/189}$ remains extremely localized at the site of secretion and $VEGF_{164/165}$ instead generates intermediate gradients of concentration around the producing cells. The importance of differential matrix affinity of VEGF isoforms was shown elegantly in transgenic mice selectively producing only one isoform from the endogenous locus, so that regulation of expression was not altered.[19] Diffusible $VEGF_{120}$ induced malformed vessels, which were aberrantly enlarged and lacked branching, whereas vessels generated by sticky $VEGF_{188}$ showed opposite defects, with very small diameters and hyperbranching. $VEGF_{164}$ was the only isoform capable of inducing physiological vascular networks in the absence of the other ones, thanks to its intermediate matrix affinity. It should be noted that the key requirement for physiological VEGF function is a balance between diffusibility and binding, rather than a specific isoform. In fact, normal vascular morphogenesis also took place in the absence of $VEGF_{164}$, as long as $VEGF_{120}$ and $VEGF_{188}$ were both expressed. The importance of balanced matrix affinity therefore makes $VEGF_{164/165}$ the isoform of choice for therapeutic delivery.

2.2 | Cellular mechanisms: Sprouting and intussusception

Sprouting is the best characterized cellular mechanism of angiogenesis and is the primary process by which new vessels grow out of preexisting ones to invade surrounding tissue, for example, during embryonic development, endochondral ossification, menstrual decidua regeneration, or tumor vascularization. Sprouting entails the specification of endothelium into two functionally distinct phenotypes, that is, tip and stalk cells, and is guided by the formation of VEGF concentration gradients.[20] The first endothelial cell reacting to VEGF becomes a tip, which extends numerous thin filopodia from the basal side into the surrounding matrix to sense the gradient and migrates toward its source (Figure 1A). Each tip cell instructs its neighboring cells to acquire the stalk phenotype and these proliferate to form the new vessel trunk (Figure 1B). Interestingly, while tip cells respond to the gradient of VEGF distribution, stalk cell proliferation is regulated by its absolute concentration.[20] Finally, to form a functional network the tip cells of two vascular sprouts first make filopodia contacts with each other, then fuse and finally form a new continuous perfused lumen through a complex series of cellular rearrangements[21] (Figure 1C). Tip cell fusion can also be promoted by macrophages, which act as chaperones between the contacting filopodia[22] (Figure 1B). The balanced formation of tip and stalk cells is finely regulated by Dll4/Notch1 signaling, whereby the first endothelial cell sensing the VEGF gradient becomes a tip by default and upregulates the ligand Dll4. This activates Notch1 on the neighboring cells, which are so instructed to downregulate both VEGF-R2 and Dll4 expression and acquire a stalk phenotype, through a mechanism of lateral inhibition.[23] Disturbance of Notch1 signaling causes excessive tip cell formation and increased sprouting, which, however, is accompanied by impaired stalk cell generation and therefore leads to nonfunctional endothelial structures that have no lumen and are not perfused.[23]

Vascular network expansion can take place also by the alternative mechanism of intussusception (from the Latin meaning "growth within itself"), also referred to as splitting angiogenesis.[24] Intussusception can be initiated very rapidly by increased blood flow and shear stress in the absence of growth factors,[25] but it can also follow VEGF upregulation.[26] It is still unclear what determines whether VEGF induces sprouting or angiogenesis, but its distribution in matrix is likely to play a role and the absence of a concentration gradient appears to favor intussusception. For example, both outcomes have been described in skeletal muscle: spontaneous upregulation of VEGF by ischemia leads to sprouting,[27] but its over-expression at significantly higher and therapeutic levels, which saturate the little matrix between fibers and abrogate local gradients, causes angiogenesis by intussusception.[26] Contrary to sprouting, no tip cells are formed and activated endothelial cells respond exclusively by proliferation without migration, leading first to circumferential enlargement of the vessel (Figure 1D,E), which then splits longitudinally into new daughter vessels. Splitting requires the formation of tissue pillars across the vascular lumen. These can form through two alternative processes: (a) a vascular wall invagination that creates a contact between the opposite endothelial cells[28] (Figure 1F), or (b) the extension and fusion of intraluminal filopodial-like protrusion from the endothelium[29] (Figure 1G,J,K). Subsequently, the endothelial junctions reorganize and myofibroblast invade the core, stabilizing the structures into mature transluminal tissue pillars (Figure 1H,L,M). Finally, these align along the length of the vessel, fuse together, and divide the affected vascular segment longitudinally (Figure 1I).

In recent years, it has become increasingly clear that intussusception is a therapeutically important mechanism of angiogenesis.[12,26,30] The molecular regulation of intussusception is still poorly understood and likely quite different from that of sprouting. Inhibition of Notch signaling in the absence of growth factor delivery has been described to stimulate intussusceptive vascular expansion in the chicken chorioallantoic membrane[31] and in the mouse liver.[32] On the other hand, recent data show that the outcome of VEGF-induced intussusceptive angiogenesis can be modulated by pericyte recruitment by platelet-derived growth factor-BB (PDGF-BB)[33] and stimulation of endothelial EphB4 signaling by pericyte-expressed ephrinB2.[34]

2.3 | PDGF-BB and the pericyte-endothelium crosstalk

After endothelial assembly, new microvascular networks need to undergo maturation by associating with pericytes. This important cell type has exquisitely regulatory functions and provides signals that switch off endothelial proliferation and permeability and make the new vessels stable, that is, independent of continued VEGF stimulation and able to persist indefinitely after the angiogenic signals subside. Pericytes are recruited by PDGF-BB secreted by activated

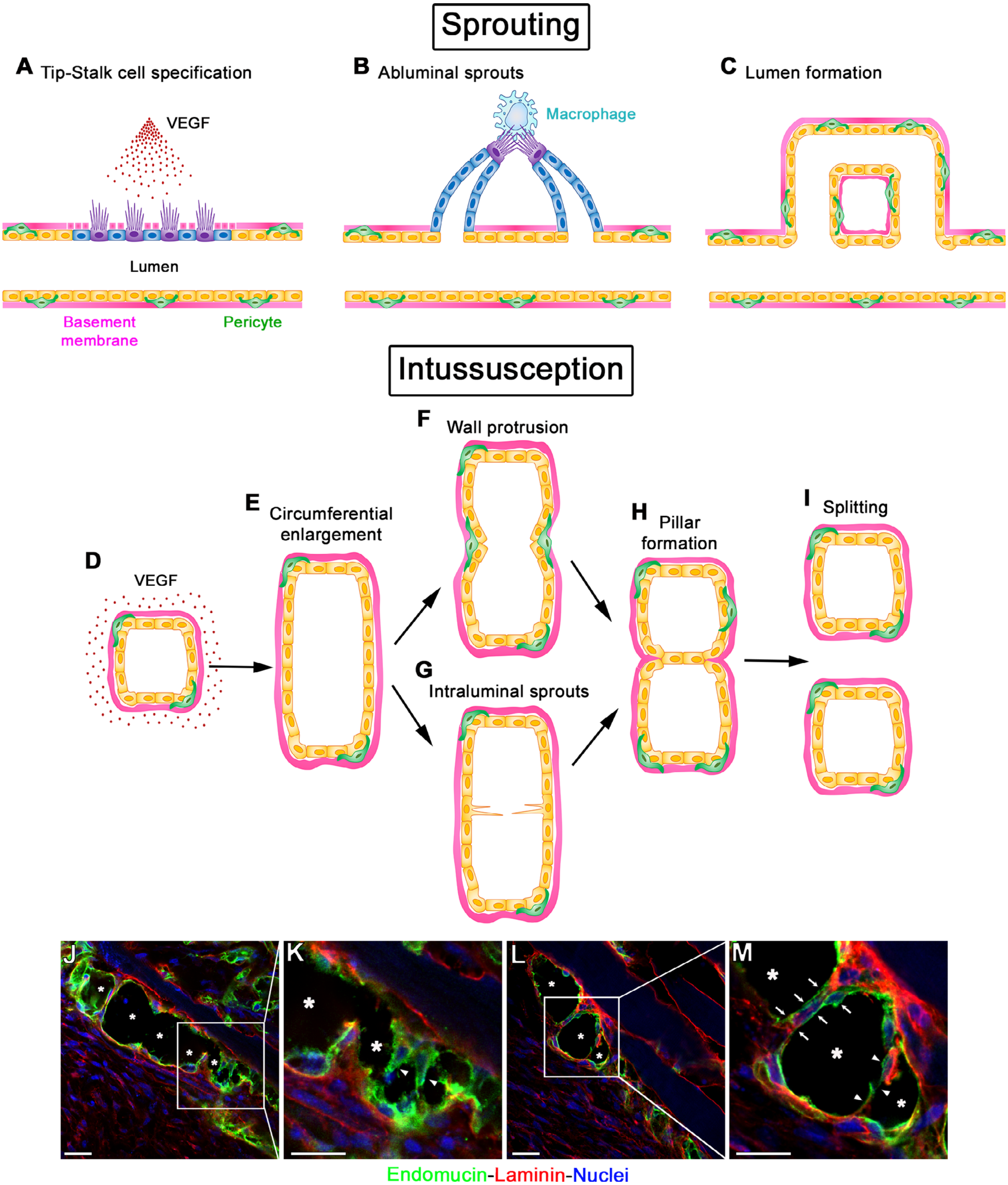

FIGURE 1 Sprouting and intussusception: two alternative modes of angiogenesis. Schematic representation of the processes generating new vascular structures by sprouting (A–C) or by intussusception (vascular splitting; D–I). J–M, Immunofluorescence images of vessels undergoing intussusception after vascular endothelial growth factor delivery in murine skeletal muscle, stained for endomucin (endothelial cells, green), laminin (basal lamina, red), and with DAPI (nuclei, blue). Circumferentially enlarged vessels displayed: (a) no degradation of the basement membrane; (b) intraluminal filopodia-like protrusions from the endothelial layer (white arrowheads in high-magnification panels K and M); and (c) mature intraluminal tissue pillars (white arrows in high-magnification panel M). * represents vascular lumen; scale bars = 20 μm in all panels

endothelium and interference with this process is incompatible with embryonic development, leading to sustained endothelial proliferation, vessel wall fragility, and lethal bleeding.[35,36] PDGF-BB function also critically relies on its interaction with ECM and gradient formation. In fact, removal of its matrix-binding domain leads to pericyte detachment from nascent vessels and severe vascular dysfunction.[37]

Pericytes regulate endothelial function through both paracrine and cell contact-dependent signals. The principal pathways mediating this molecular crosstalk are transforming-growth factor-β1 (TGF-β1), angiopoietins-1 and -2 (Ang-1 and Ang-2), and ephrinB2/EphB4.[38] TGF-β1 is produced in a latent form and its activation requires cell-to-cell contact between endothelial and mural cells through proteolytic cleavage of the latency-associated peptide by plasmin at the vessel wall interface.[39] TGF-β1 regulates both the endothelium and pericytes and can promote contrasting functions, depending on alternative Alk1 and Alk5 receptor stimulation and downstream SMAD pathway activation.[40] In fact, activation of Alk5-SMAD2/3 specifically promotes vascular stabilization by inducing endothelial quiescence and stimulating the production of ECM and basement membrane proteins.[41] In contrast, activation of Alk1-SMAD1/5 promotes endothelial cell proliferation and migration as well as endothelial-to-mesenchymal transition.[41]

Angiopoietins are the ligands of the endothelium-specific tyrosine kinase receptor Tie2. Ang1 is expressed by mural cells and activates Tie2: it facilitates the further recruitment and association of pericytes with newly formed vascular structures, acts as a survival signal for endothelial cells and inhibits VEGF-induced vascular leakage.[42] On the other hand, Ang2 is a context-dependent partial agonist of the Tie2 receptor, mostly acting as an inhibitor of its activation. It is stored preformed in Weibel–Palade bodies of endothelial cells, from which it is rapidly released upon VEGF stimulation. It plays a key role to initiate the angiogenic process by promoting the dissociation of pericytes and endothelial cells and allowing their formation of new vascular structures. However, it also promotes endothelial cell apoptosis and vascular regression if VEGF signaling is disrupted at this crucial stage.[42]

Eph receptors and ephrin ligands are both membrane-bound and, therefore, binding and activation of Eph and ephrins requires cell–cell contact. Characteristically, engagement of the Eph–ephrin system elicits bidirectional signaling, that is, “forward signaling” in the cell expressing the Eph receptor and “reverse signaling” in the cell expressing the ephrin ligand.[43] In tumor angiogenesis, activation of reverse signaling via the EphB4 ligand ephrinB2 was shown to stimulate pericyte association to blood vessels and inhibit their permeability through the activation of the Ang1/Tie2 pathway.[44] Conversely, in a tissue-specific mutant model, ephrinB2-deficient pericytes failed to establish proper cell-to-cell contacts with microvascular endothelium, leading to hemorrhaging and perinatal mortality.[45] EphrinB2 has also been shown to directly modulate VEGF-R2 and VEGF-R3 activation in sprouting tip cells by controlling their internalization, necessary for receptor activation of downstream signaling.[46,47] Recently, we also found that EphB4 activation also regulates splitting angiogenesis independently of VEGF-R2 internalization or phosphorylation, but rather by modulating the activation of ERK1/2 downstream of the receptor and the speed of endothelial proliferation.[34]

2.4 | Circulating monocytes

Besides endothelial and mural cells, different populations of circulating myeloid cells play a crucial role in both induction and maturation of new vessels.[48] CXCR4+ monocytes are recruited by VEGF expression, do not incorporate into new vessels (unlike circulating endothelial progenitors), but are retained in a perivascular position through Stromal Cell-Derived Factor-1 (SDF-1)/CXCR4 signaling and sustain vascular growth through further secretion of pro-angiogenic factors.[49] On the other hand, a similar CXCR4+ population of circulating monocytes has been recently shown to promote intussusceptive vascular growth during liver regeneration by participating in the formation and stabilization of intraluminal pillars.[50] Tissue macrophages also aid the completion of sprouting angiogenesis by promoting tip cell fusion and anastomosis, acting as cellular chaperones.[22] These macrophages expressed both the surface receptors Tie2 and Neuropilin1 (Nrp1) and therefore were phenotypically similar to the family of pro-angiogenic Tie2-expressing macrophages (TEM). TEM have been described as tumor-infiltrating myeloid cells that convey pro-angiogenic signals and can provide a mechanism of resistance to anti-angiogenic drugs, promoting cancer progression.[51] A population of circulating Neuropilin1-expressing monocytes (NEM) has been shown to also stimulate vascular maturation and smooth muscle cell recruitment. These cells coexpress CD11b and Nrp-1, which is a receptor for both VEGF and Semaphorin-3A (Sema3A), are recruited at sites of VEGF expression and promote the formation of arteries by the paracrine actions of several factors such as Ang1, TGF-β, and PBGF-BB.[52] We recently found that NEM are specifically recruited to angiogenic sites by Sema3A produced by activated endothelium and accelerate stabilization of newly induced microvascular networks, that is, their ability to survive independently of VEGF signaling and persist indefinitely.[53]

3 | THERAPEUTIC CONSIDERATIONS FOR VEGF DELIVERY

3.1 | The control of dose distribution

Therapeutic use of VEGF requires careful dose control, as uncontrolled delivery has been shown to cause aberrant vascular growth and angioma-like vascular tumors in muscle, heart and other tissues.[54-57] The physiological affinity of VEGF for ECM has significant therapeutic implications for its dosing and delivery strategies. In fact, upon VEGF gene delivery, it remains tightly localized in the microenvironment around each producing cell and different levels of expression do not average with each other. Therefore, the therapeutic outcome between safety and efficacy of factor delivery is determined by the distribution of microenvironmental concentrations in tissue rather than simply the total dose delivered. This concept is exemplified by experiments in which the average and microenvironmental dose of VEGF could be controlled independently by cell-based gene delivery.[58] Different VEGF doses were expressed in skeletal muscle by populations of genetically modified muscle progenitor cells (myoblasts). Random genomic integration of the viral vector leads to different expression levels in each transduced cell. Implantation of this heterogeneous population always leads to aberrant angioma-like vascular growth, even if the total dose is reduced several-fold by dilution

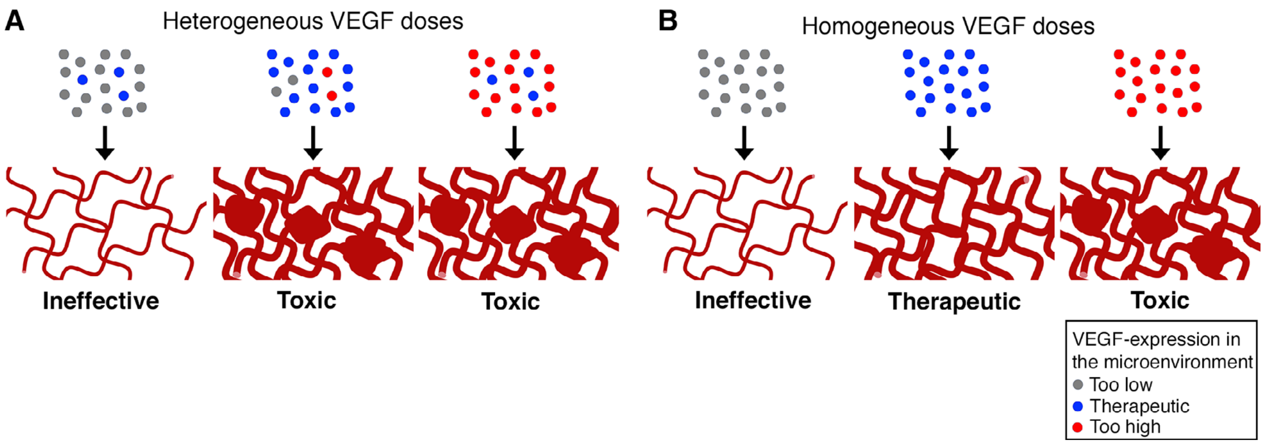

FIGURE 2 Functional outcomes of vascular endothelial growth factor (VEGF) dose distribution in tissue. A, Heterogeneous dose distributions (eg, by gene therapy vectors) lead to hotspots of excessive expression that remains localized in the microenvironment around producing cells (red spots, upper right panel) and lead to toxic effects. Reducing the total dose does not completely avoid toxic hotspots even if therapeutic levels are achieved in some areas (blue spots, upper middle panel), until the total dose is so low that mostly ineffective levels are achieved (gray spots, upper left panel). B, Homogeneous distribution of the total dose allows therapeutic levels (blue spots, upper middle panel) to be achieved and to harness the therapeutic window of VEGF delivery

with nonexpressing cells. On the other hand, by isolating and expanding single myoblasts it is possible to obtain monoclonal populations in which every cell produces the same level. Under these conditions, which ensure a homogeneous microenvironmental distribution of the dose, it is possible to induce only normal and physiological angiogenesis across a wide range of doses, as well as to effectively restore functional blood flow in limb ischemia.[59] In contrast, the same total doses are toxic when delivered by dilutions of the heterogeneous population, because rare hotspots of excessive production cannot be avoided.[58]

The need to control the distribution of microenvironmental doses is key when addressing the therapeutic window of VEGF delivery. In fact, when heterogeneous expression levels are generated in the tissue, such as by direct delivery of gene therapy vectors, effective doses in some microenvironments are accompanied also by ineffective and by toxic levels in other areas. In order to ensure safety, the total vector dose must be limited, but this increases the frequency of ineffective levels, thereby jeopardizing efficacy (Figure 2A). As a consequence, the usable therapeutic window of VEGF gene delivery appears to be very narrow.[55] On the other hand, VEGF does not have an intrinsically very steep dose–response curve and its biological therapeutic window is much larger,[58,59] but it is key to control the distribution of doses in the microenvironment in vivo in order to exploit the therapeutic potential of VEGF delivery, ensuring both safety and efficacy (Figure 2B).

Translating this concept poses a fresh set of challenges. In fact, gene therapy is the preferred approach for therapeutic angiogenesis, thanks to a very robust delivery potential and standardized GMP vector production, but it is extremely difficult to avoid heterogeneous expression levels in vivo with uncontrolled vectors. Several groups are actively developing new generations of vectors, for example, ones that may self-regulate in the tissue,[60] and investigating other improvements in the gene therapy approaches: an excellent coverage of the latest discoveries can be found in a recent review by Ylä-Herttuala and Baker.[61]

3.2 | Cell-based gene delivery

The distribution of expression levels in vivo can be controlled by a cell-based gene therapy approach. As detailed above, transduction of a progenitor population with an integrating viral vector leads to stable but heterogeneous expression levels in different cells. We developed a fluorescence-activated cell sorting (FACS)-based technology to predict the level of transgene expression in single live cells and to purify populations homogeneously expressing specific levels.[62] The transgene of interest (eg, VEGF) is linked in a bicistronic viral vector to a nonfunctional truncated version of the lymphocyte-specific marker CD8a, which acts simply as a membrane-localized reporter protein. In this way, changes in the level of expression of VEGF are reflected by a parallel change in cell-surface expression of CD8a, which can be detected and quantified on live cells by FACS, enabling the purification of specific populations homogeneously expressing different VEGF levels (Figure 3). This technology can be used with different progenitor classes[63] for different applications. FACS purification of VEGF-expressing myoblasts was shown to yield only normal and functional angiogenesis in skeletal muscle,[62,64] while purification of VEGF-expressing adipose-derived stromal cells ensured controlled vascular growth in the normal myocardium[65] and prevented deterioration of cardiac function in a model of myocardial infarction by limiting fibrotic scarring.[66]

Cell-based VEGF gene delivery has also been investigated to promote the rapid vascularization of tissue-engineered grafts, for example, for cardiac patches or bone regeneration. Differently from the ischemic conditions described above, in these strategies, suitable progenitors are seeded on appropriate scaffolds and need to rapidly attract a vascular supply after in vivo implantation in order to differentiate and function. It is therefore desirable to link the angiogenic signal to the presence of the seeded cells by genetically engineering them to produce VEGF. Cardiac patches engineered with FACS-purified

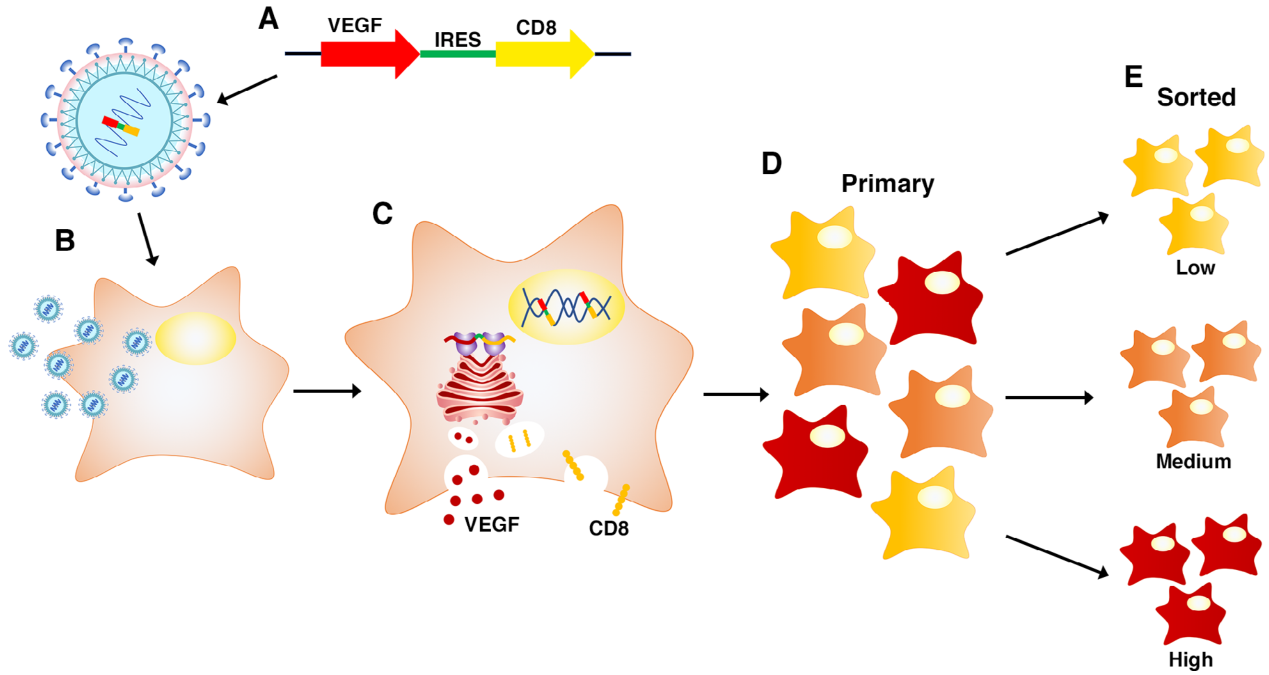

FIGURE 3 Fluorescence-activated cell sorting (FACS)-purification of genetically modified progenitor populations expressing homogeneous vascular endothelial growth factor (VEGF) levels. A, Retroviral vector carrying a bicistronic cassette, in which the sequence of VEGF is linked to that of the membrane-bound reporter CD8 through an Internal Ribosome Entry Site (IRES) sequence. B, Progenitors of interest are transduced with this retroviral vector. C, After integration in the cell chromatin, the IRES sequence enables cotranslation of both proteins from the same mRNA molecule. Therefore, the amount of CD8 on the cell membrane reflects that of secreted VEGF regardless of their absolute level of expression. D, Transduced cells in the primary population express heterogeneous VEGF levels depending on their viral copy number and the transcriptional activity of the chromatin integration sites. E, These can be FACS-sorted into homogeneous subpopulations stably expressing specific VEGF levels based on the intensity of membrane CD8 staining

VEGF-expressing myoblasts[67] or adipose-derived stromal cells[68] could drive efficient vascularization of the patch itself and also provided controlled VEGF release to induce extrinsic angiogenesis in the underlying myocardium.

VEGF-expressing human bone marrow osteoprogenitors could effectively increase the vascularization of engineered bone grafts by threefold, inducing physiologically structured vascular networks with both conductance vessels and capillaries.[69] However, sustained and uncontrolled VEGF expression unexpectedly caused a global reduction in the quantity of bone, posing a challenge to its clinical application. Progenitor engraftment or differentiation was not impaired, but rather the recruitment of osteoclasts was strongly increased, thereby disrupting bone homoeostasis toward excessive resorption. Current research is aiming at decoding the crosstalk between angiogenesis and osteogenesis, as well as ensuring that the two processes are therapeutically coupled.

3.3 | Recombinant factor engineering for matrix decoration

It is desirable to independently control the dose and duration of angiogenic factor delivery in grafts. This is technically challenging with gene transfer, while protein delivery suffers from short half-life in vivo. Extensive efforts have generated many strategies to ensure sustained release of recombinant growth factors from natural and synthetic biomaterials. These approaches have been recently reviewed by Browne and Pandit.[70] However, it has also been possible to create modified versions of growth factors by protein engineering, so that they can be used to decorate natural matrices and be presented to cells in their physiological context during tissue regeneration.[71] The most ubiquitous and abundant ECM protein is collagen, which is also employed as a basis for a variety of biomaterials. Therefore, recombinant growth factors have been engineered with specific collagen-binding domains, allowing their use to decorate collagen-based biomaterials and prolonging their bioavailability and efficacy (reviewed by Addi et al[72]). On the other hand, tissue regeneration after damage starts in all cases with the deposition of a fibrin-based matrix rich in growth factors,[73] which provides ideal conditions for cell migration, vascular invasion and progenitor differentiation. Therefore, significant efforts have been directed at mimicking ECM decoration to ensure physiological presentation of morphogens.

Growth factors have been incorporated into fibrin matrix exploiting the coagulation process itself (Figure 4A, top). For example, murine $VEGF_{164}$ was fused to the octapeptide NQEQVSPL, which is the substrate of the transglutaminase coagulation factor XIIIa, allowing its covalent crosslinking into fibrin hydrogels and release only by enzymatic cleavage.[74] Further addition of the fibrinolysis inhibitor aprotinin, also engineered with the same technology, could finely tune the hydrogel degradation rate and therefore independently control

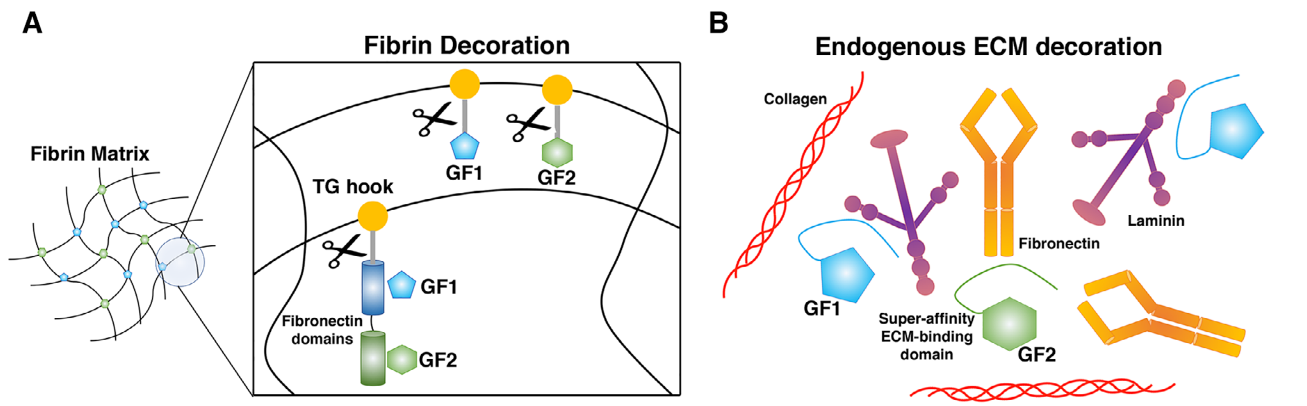

FIGURE 4 Strategies for matrix decoration with engineered recombinant factors. A, Fibrin matrices can be decorated with engineered growth factors to mimic extracellular matrix (ECM) functions. Taking advantage of the coagulation cascade, an octapeptide substrate of the TransGlutaminase factor XIII (TG hook) can be fused to growth factors (GFs), enabling their covalent crosslinking to fibrin. Specific domains of ECM proteins (eg, fibronectin) can also be incorporated through a TG hook to exploit their natural affinity for different GFs. B, Endogenous ECM can be decorated with therapeutic GFs engineered to exhibit super-affinity to a broad range of ECM components

the duration of factor release. Controlled VEGF delivery to skeletal muscle through this optimized platform was shown to yield exclusively normal, stable, and functional angiogenesis, over a wide range of easily controllable doses, and restored blood flow to ischemic tissues.[75]

The natural affinity of different ECM proteins for growth factors can also be exploited (Figure 4A, bottom). For example, fibronectin has binding sites for fibrin, integrins, and growth factors. By engineering a recombinant fibronectin fragment comprising all these sites, it has been possible to codeliver the morphogens bone morphogenetic protein-2 and PDGF-BB within a fibrin matrix to promote bone regeneration.[76] Interestingly, presentation of the factors in their physiological matrix context greatly increased their functional efficacy, ensuring robust bone regeneration at very low and otherwise ineffective doses. Taking a reverse strategy, a short domain found in placenta-derived growth factor-2 was found to mediate broad binding to a wide variety of ECM proteins. Engineering of any growth factor with this peptide endowed them with super-affinity for ECM, thereby enabling the in situ decoration of endogenous matrix with exogenously provided therapeutic proteins[77] (Figure 4B). The increased efficacy of the modified factors avoided the need to deliver supra-physiological doses of VEGF, thereby increasing safety.

3.4 | Modulation of dose-dependent outcomes

An alternative class of approaches may help reduce the need to precisely control the distribution of VEGF doses in vivo. VEGF-A is a very powerful activator of VEGF-R2 signaling, but alternative ligands have been found to have milder activation profiles and therefore may require less stringent dose control. The properties of VEGF-B might particularly benefit applications to the cardiac muscle. In fact, VEGF-B is specifically active in the myocardium, while it is poorly angiogenic in other tissues.[78] Furthermore, VEGF-B does not bind to VEGF-R2, but rather activates it indirectly, by displacing inactive VEGF-A from reservoir sites. Therefore, even robust over-expression of VEGF-B does not easily lead to excessive VEGF-R2 signaling, because it is limited to the endogenous levels of VEGF-A for its action.[79,80] Another example is VEGF-D: in its native form it stimulates lymphatic angiogenesis through VEGF-R3, but it can also generate a shorter form by proteolytic cleavage of both the N- and C-termini, therefore named VEGF-D$^{\Delta N\Delta C}$. VEGF-D$^{\Delta N\Delta C}$ shifts its affinity to VEGF-R2 and stimulates blood angiogenesis instead. However, because it has no heparin-binding domain, it leads to a more diffuse distribution through ECM and induces more physiological vascular growth than VEGF-A over-expression.[81] Clinical trials of therapeutic angiogenesis by adenoviral delivery of VEGF-D$^{\Delta N\Delta C}$ are ongoing.[82,83]

The outcome of VEGF over-expression may also be significantly modified by promoting pericyte recruitment. Studies in skeletal muscle showed that the transition between normal and aberrant angiogenesis is not determined exclusively by VEGF dose, but rather by the balance between endothelial activation by VEGF and pericyte recruitment by PDGF-BB.[84] Codelivery of VEGF$_{164}$ and PDGF-BB at a fixed relative ratio, achieved through coexpression from a single bicistronic vector, ensured the generation and long-term stability of exclusively normal and functional microvascular networks regardless of absolute VEGF dose,[84,85] by limiting endothelial proliferation.[33] Although PDGF-BB alone does not induce vascular growth either in normal or ischemic skeletal muscle,[84,86] the beneficial effects of the VEGF/PDGF-BB combination have been described in a variety of preclinical settings, including gene delivery with adenoviral[86] or adeno-associated viral vectors,[87] sustained release of the recombinant factors from polymeric biomaterials[88] or treatment with modified proteins engineered for super-affinity to the ECM.[77]

As described above, pericytes exchange a complex molecular crosstalk with endothelium and these signaling pathways can offer more specific targets to regulate VEGF effects. For example, stimulation of the Tie2 receptor by Ang1 has been shown to significantly reduce the detrimental hyperpermeability and vascular leakage that accompany VEGF stimulation.[89] This therapeutic benefit can be further compounded by the contemporaneous inhibition of Ang2.[90] On the other hand, activation of endothelial EphB4 by ephrinB2 finely tunes ERK1/2 phosphorylation downstream of VEGF-R2, thereby limiting the rate of endothelial proliferation induced by VEGF.[34] Therefore, systemic treatment with recombinant ephrinB2-Fc was shown to prevent aberrant angiogenesis by robust and uncontrolled VEGF expression, without interfering with efficient normal microvascular network formation.[34]

Although VEGF is the principal target for therapeutic angiogenesis, the delivery of other factors has shown promising preclinical results, such as the fibroblast growth factor (FGF) family (FGF-1, FGF-2, and FGF-4) or hepatocyte growth factor, and clinical studies are currently underway to evaluate their potential in coronary or peripheral artery disease (CAD or PAD).[61]

3.5 | Duration of delivery and vessel stabilization

Another important consideration for the therapeutic effectiveness of newly induced vasculature is its long-term persistence. In fact, while new vascular networks are rapidly formed in a matter of few days after VEGF delivery, they are initially unstable and will regress if VEGF stimulation is withdrawn too early. Several lines of evidence in models of inducible transgene expression[91,92] or pharmacologic blockade[58] support a need to sustain VEGF stimulation for about 4 weeks before new vessels are stabilized and persist independently. This requirement impairs the efficacy of transient VEGF gene delivery, for example, by adenoviral vectors that have been widely used in clinical trials and afford robust expression, but that are also cleared by the immune system in about 10 days.[93]

More recently, we found that VEGF dose-dependently impairs the kinetics of vascular stabilization, that is, vessels induced by lower levels of VEGF stabilize faster. However, pericyte recruitment is not affected. Rather, VEGF negatively regulates the production of Sema3A by activated endothelium, which in turn impairs the recruitment of Nrp1-expressing monocytes, activation of TGF-β1-SMAD2/3 signaling and the induction of endothelial quiescence.[53] In a therapeutic perspective, treatment with recombinant Sema3A was shown to accelerate the stabilization of newly induced angiogenesis and allow its persistence despite transient VEGF expression.[53] Interestingly, the stabilization of newly induced vasculature can also be promoted by the mechanical properties (low stiffness) of a biomaterial environment, through the recruitment of a novel population of mechano-sensitive Piezo1+ monocytes.[94]

4 | CONCLUSIONS AND PERSPECTIVES

The biology of angiogenesis is complex and a better understanding of its cellular and molecular mechanisms is necessary for the design of rational and more effective therapeutic strategies. Therapeutic angiogenesis in ischemic tissues and de novo vascularization of engineered grafts present rather different requirements and therefore might be best achieved by different approaches.

Tissue-engineered grafts are avascular upon implantation and need to attract vascular in-growth. In order to rapidly guide sprouting of new vessels and their migration toward the graft core, it is desirable that the graft matrix presents an optimized microenvironment of angiogenic cues. This can be achieved by predecorating a suitable material (such as fibrin or collagen) with optimized doses and combinations of engineered factors, as described above, or also by employing decellularized ECM, enriched in morphogens by suitable progenitor cell lines.[95] The process can also be accelerated by prevascularization, that is, preseeding with endothelial cells to generate self-assembling vascular structures inside the construct that can anastomose with the penetrating host vessels.

On the other hand, ischemic tissues are already vascularized and proangiogenic therapy aims at expanding the microvascular networks to promote collateral artery remodeling and restore physiological blood flow.[13] For this, it is key to treat large volumes of target tissue and gene delivery by viral vectors has a clearly superior efficacy compared with both protein and cell-based approaches. For example, adenoviral vectors combine two clinically desirable features, namely robustness of expression and transient duration, limited by the immune response. However, these same features compromise safety (by uncontrolled distribution of high levels) and efficacy (by regression of unstable vessels). Based on recent advances in elucidating the molecular regulation of the switch between normal and aberrant angiogenesis and of vascular stabilization, as described above, it can be envisioned that local delivery of VEGF-expressing adenoviral vectors might be complemented by systemic treatment with drugs targeting ancillary pathways (eg, EphB4 or Sema3A signaling), to ensure a safe and effective outcome while exploiting the clinically attractive features of VEGF gene therapy.

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CONFLICT OF INTEREST

The authors declare no potential conflicts of interest.

AUTHOR CONTRIBUTIONS

R. G.-B., N.D.M.: manuscript writing, figure preparation, final approval of manuscript; L.M., M.G.B., E.M.: manuscript writing, final approval of manuscript; L.G., D.J.S.: manuscript writing, financial support, final approval of manuscript; A.B.: conception and design, financial support, manuscript writing, final approval of manuscript.

DATA AVAILABILITY STATEMENT

Data sharing is not applicable to this article as no new data were created or analyzed in this study.

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