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Controlled Protein Delivery in the Generation of Microvascular Networks

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Abstract

Rapid induction and stabilization of new microvascular networks is essential for the proper functioning of engineered tissues. Many efforts to achieve this goal have used proangiogenic proteins—such as vascular endothelial growth factors—to induce the formation of new microvessels. These proteins have demonstrated promise in improving vascularization, but it is also clear that the spatial and temporal presentation of these signals is important for achieving proper vascular function. Delivery systems that present proteins in a localized and sustained manner, can promote the formation and stabilization of microvascular networks by precisely presenting proangiogenic proteins at desired locations, and for specified durations. Further, these systems allow for some control over the sequence of release of multiple proteins, and it has become clear that such coordination is critical for the development of fully functional and mature vascular structures. This review focuses on the actions of proangiogenic proteins and the innovations in controlled release technologies that precisely deliver these to stimulate microvascular network formation and stabilization.

Keywords

protein delivery; microvascular network; angiogenesis; vascularization; stabilization; tissue engineering

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1. INTRODUCTION

In the campaign to design a viable 3D engineered tissue, the formation of a functional microvascular network is arguably the greatest current hurdle. In most tissues the cell-to-capillary distance is 200 μm at maximum; when this distance is exceeded, new vasculature is required. Successes in tissue engineering have almost exclusively been in the development of tissues normally avascular or thin walled tissues that do not require an extensive vascular network. These notable successes include articular cartilage [1], large caliber blood vessels [2–4], tracheas [5, 6] and bladders [7].

The body does, however, have the extraordinary capacity to generate new vascular networks in many situations. During development, the first vascular structures to be formed are created through a process called vasculogenesis in which vascular progenitor cells migrate to peripheral sites, differentiate in situ and self-assemble to form arteries, veins, and capillaries. Later in the course of development as well as during responses to injury or ischemia, new vessels develop through the process called angiogenesis in which new vessels are created by sprouting from existing blood vessels. Knowledge of these processes and the molecules that regulate them may allow for the capitalization of the body's innate capabilities to form vessels within engineered tissues.

After decades of effort, it is now very clear that the creation of a microvascular network within an engineered tissue is a complex process. Many attempts have been made to coax the formation of such a network, with varying success. Such efforts include the delivery of vascular cells [8–12] and macroporous hydrogels with conduits for vascular formation [13]. The delivery of various pro-vasculogenic/angiogenic proteins has been a notable area of intense focus. Both vasculogenesis and angiogenesis are highly regulated processes that are controlled by proteins delivering paracrine and contact-dependent signals. Many key proteins have been identified (Table 1), and the spatiotemporal pattern of delivery that optimizes their effectiveness are starting to be elucidated. This knowledge provides a basis for the recapitulation of these signals using specialized delivery systems, and such efforts are continually improved as the understanding of these signals advances.

A particular challenge is that the creation of new vasculature requires two phases: efforts must first be made to promote formation within or the invasion of the engineered tissue by new endothelial cell (EC)-lined vascular structures. In the second phase, these primitive tubes must be stabilized and matured by recruitment of and investment by contractile cells such as pericytes (PCs) or vascular smooth muscle cells (vSMCs). While these two processes are interconnected, they can be influenced by different proteins, and studies are revealing that the spatial and temporal patterning of factors affect the success of mature vessel formation [14–18]. Technologies developed in the field of controlled drug delivery can be exploited to tune the presentation of these proteins. In this way, the chosen protein may be made available at a specific place and time. This paper presents a comprehensive review of the proteins that appear to be most important in engineering the formation of new, mature vessels in the context of tissue engineering, and discusses the potential for specialized delivery methods that will be essential in translating these proteins into clinically relevant products.

2. WHAT CAN BE DELIVERED TO STIMULATE THE FORMATION OF VASCULAR NETWORKS IN NEWLY FORMED ENGINEERED TISSUES? -- LESSONS FROM THE FIELD OF THERAPEUTIC REVASCULARIZATION

Over the past 25 years, over 25 pro-angiogenic proteins and their targets have been identified (Table 1). We are slowly beginning to elucidate the importance of intricate spatial and temporal patterns of protein expression that are required during natural vascular development. These advances in the understanding of the molecular regulators of vascular development form the basis for many of the diverse delivery strategies aimed at driving microvascular network formation; these methods will likely become more sophisticated with future discoveries. As discussed in more detail below, it is now understood that the successful completion of the angiogenic and vasculogenic processes require the complex yet coordinated presentation of many different growth factors, some contributing to the initial formation of vascular structures and others to vessel stabilization and maturation. However, the line between formation and stabilization is often blurred, and several known angiogenic proteins are capable of acting in a context-dependent manner contributing to both processes. Much of the information about the function of these proteins as therapeutics has come from attempts to use them to stimulate the revascularization of ischemic tissues, rather than from inducing neovascularization of tissue-engineered constructs, which is our primary interest here. These processes are likely to differ in some ways because the microenvironments in which the blood vessels are being coaxed to form are different, but they are likely to share key features.

2.1 Formation

Vascular endothelial growth factor (VEGF)—The VEGF family is one of the most widely studied groups of proteins in the regulation of vascular development. This family includes VEGF-A, -B, -C, -D, -E, -F and placenta-derived growth factor (PlGF). VEGF-A is the most extensively researched and utilized of this family of proteins. It exists as 5 different isoforms, a result of alternative splicing, each with varied activity and matrix binding properties. These isoforms are classified by number of amino acids making up the protein: 121, 145, 165, 189 and 206; VEGF-A₁₆₅ is the most prevalent and effective stimulator of angiogenesis [19, 20].

VEGF-A was discovered to be a potent mitogen for micro- and macrovascular ECs by 1989 [21] and many studies have since been performed to evaluate its therapeutic value. Animal work showed that delivery of VEGF promotes modest angiogenesis in ischemic tissue, but the response was not optimal as improvements did not continue past 10 days [22]. Interestingly, overexpression of VEGF in myoblasts implanted in mouse skeletal tissue resulted in local arteriole formation, an essential process for the formation of a complete vascular bed, but also led to endothelial overgrowth and hemangiomas around the implanted cells [23]. Similar overgrowth occurred when murine myoblasts overexpressing VEGF were implanted into the mouse myocardium [24]. When studied further, a microenvironmental threshold was discovered under which normal vessel formation occurred. The threshold existed for local concentrations, as each transduced cell had to release a concentration over

the threshold to cause an effect; increasing the number of cells alone, and not the expression levels, did not cause the same effect [25].

Large-scale clinical trials using VEGF to promote vessel development in humans have not been successful, notably the VIVA study, with no benefits observed over placebo in patients with angina when VEGF was delivered by intracoronary and intravenous infusion [26]. It is likely that these shortcomings are due to the short half-life of VEGF in the bloodstream (90 min) [27], and the fact that VEGF increases vascular permeability when delivered alone [19]. It has also been suggested that the different isoforms of VEGF-A play different roles during angiogenesis. When the whole VEGF-A family was induced with a synthetic zinc-finger transcription factor in mice, the neovasculature that resulted was not hyperpermeable as were the vessels created by expression of VEGF- A₁₆₅ alone [28]. The general conclusion to be drawn from these efforts is that delivery of one VEGF-A isoform can promote the growth of new vessels, but is not sufficient to mature and stabilize these vessels. Its delivery must be tightly regulated with presentation at the right time with the right microenvironmental dose and in combination either with other growth factors that promote stabilization [16–18, 29], or other isoforms. Many of these studies involving VEGF have been carried out with the goal of therapeutic revascularization. The optimal protein cocktail for creating microvessels in an engineered tissue may be different, and is yet to be determined.

Fibroblast growth factor (FGF)—Twenty-two members of the FGF family have been identified. FGF1 and FGF2 have been shown to stimulate angiogenesis *in vivo*, but the exact roles of these became unclear after complex results from knockout studies [30]. Some of the known pro-angiogenic actions of the FGFs include promotion of the proliferation, migration and differentiation of vascular cells [31] and effects on EC protease production, cell-matrix interaction, and cell-cell communication [32]. FGF2 stimulation has been connected to the upregulation of several other angiogenic molecules including VEGF, hepatocyte growth factor (HGF), and monocyte chemotactic protein-1 (MCP-1), in endothelial and stromal cells [31, 32]. Additionally, another member of this family, FGF9, is capable of activating Hedgehog signaling, leading to the expression of VEGF-A, -B and -C, and angiopoietin-2, all involved in the development of the coronary vascular plexus [31, 32]. In particular, FGF has been strongly linked to the actions of VEGF. When FGF signaling in ECs is blocked, VEGF receptor 2 (VEGFR2) is downregulated and the cells are no longer responsive to VEGF [33].

While initially thought only to stimulate formation of new vessels, FGF2 has also been linked to stabilization. FGF2 was shown to induce modest vascular stabilization alone, but when delivered together, FGF2 and platelet-derived growth factor (PDGF) act synergistically to promote long-lasting maturation. Experiments suggest this is caused by the upregulation of platelet-derived growth factor receptor (PDGFR) in vSMCs when exposed to FGF2[34].

These widespread actions of FGF in regulating various angiogenic proteins, suggest that these molecules play a role in orchestrating the angiogenic process, making them excellent candidates for therapies. The FGFs, in particular FGF2, have been the focus of several

animal and clinical studies with varying success. FGF2 has been delivered by intravenous injections, bolus injection and in slow-release capsules implanted in the heart of patients with coronary artery disease or peripheral arterial disease. All three delivery schemes resulted in modest improvements in neovascularization and function, but an optimal dosing scheme remains to be determined (reviewed in [32]).

Hepatocyte growth factor (HGF)—Upon its discovery, HGF was determined to be a potent mitogen for hepatocytes, acting on its receptor, c-Met [35]. Since then, a number of other cell types have also been shown to respond to this protein. Importantly, HGF acts on ECs, PCs and vSMCs, preventing apoptosis and promoting proliferation and migration [36]. HGF stimulates tube formation of human umbilical vein ECs (HUVEC), and induces mobilization of endothelial progenitor cells and vascular outgrowth from aortic explants [37]. Recent evidence suggests that PCs secrete high levels of HGF that can enhance EC sprouting *in vitro* (Chang et al., unpublished data). While the angiogenic properties of HGF are still being investigated, it has become clear that it is an interesting candidate for vascular therapies.

Chemokines—First discovered as chemoattractants for leukocytes during inflammation, some members of this superfamily of proteins are now known to have angiogenic properties. The members of one chemokine family contain the sequence cysteine-X-cysteine, in which the “X” represents an unconserved amino acid, and are therefore labeled “CXC” chemokine ligands [38]. This family is further separated into ELR-positive and ELR-negative CXC chemokines. ELR refers to another 3 amino acid sequence: glutamic acid-leucine-arginine [38]. All ELR-positive CXC chemokines – including CXCL1, 2, 3, 5, 6, 7, 8 and interleukin-8 (IL-8) – promote angiogenesis through interaction with two receptors, CXCR1 and CXCR2. CXCR2 is more clearly linked to angiogenesis, particularly in the chemotactic response of ECs [39, 40] and the homing of circulating endothelial progenitor cells [41, 42]. An ELR-negative CXC chemokine, CXCL12 (or stromal-cell derived factor-1, SDF-1), is linked to the recruitment of CD34(+) cells and mesenchymal stem cells (MSCs), both capable of contributing to neovascularization [43].

The CC chemokine family also has been shown to contribute to neovascularization, particularly CCL2, CCL11 and CCL16 [38]. CCL2, also known as MCP1, is the most well-studied of these, and has been implicated both in the formation and maturation of new vessels. Through the CCR2 receptor, CCL2 promotes EC chemotaxis, tube formation and mural cell recruitment *in vitro* [44–47] and neovascularization and maturation *in vivo* [48–51]. Its actions appear to be dependent on membrane type-1 matrix metalloproteinase [45], but may be independent of its effects on leukocytes, binding directly to CCR2 on ECs [50]. A possible mechanism is suggested by the fact that CCL2 has been associated with upregulation of VEGF-A gene expression [52]. CCL11 stimulates migration of ECs and promotes angiogenesis upon binding to the CCR3 receptor [53]. CCL16 is primarily found in the liver, suggesting that it is important in physiological and pathogenic hepatic angiogenesis [54]. Through CCR1, CCL16 promotes EC migration and angiogenesis [54].

2.2 Stabilization

A fully mature vascular network is one that is long-lasting, invested by appropriate mural cells, selectively permeable, located and specialized appropriately for the tissue in which it resides and properly differentiated into arteries, arterioles, capillaries, venules and veins. The mural cells – vSMCs and PCs – significantly contribute to the stabilization of vascular structures; they stabilize via mechanisms that depend on both cell-cell contact and the release of soluble factors. With the goal of recapitulating these cellular effects, a variety of delivery systems have been developed for release of chemoattractants to recruit mural cells (e.g., platelet-derived growth factor) [16–18, 29], or for release of the proteins known to be secreted by mural cells (e.g., angiopoietin-1) [55].

Interestingly, many of the proteins that have classically been thought of as stabilization factors also contribute to formation of vascular structures. This blurring of the line between formation and stabilization is exemplified in the actions of PCs; they support and stabilize the microvasculature, but they have also been shown to promote initial formation of microvascular networks [56]. These observations highlight the complexity of signals involved in the stimulation of neovascularization.

Platelet-derived growth factor (PDGF)—PDGF was originally discovered as a heterodimeric factor released by platelets, consisting of an A and B chain, but AA and BB homodimers are secreted by many different cell types, including fibroblasts, keratinocytes, and ECs [36]. Knockout of PDGF-BB or its receptor (PDGFR β) leads to perinatal death in mice from edema and hemorrhage from unstable microvessel lacking PC coverage, suggesting that PDGF is essential for vascular maturation [57]. PDGF is known to be a potent chemoattractant for both vSMCs and PCs [58], and, for this reason, it is one of the most widely studied factors for vascular stabilization. PDGF also contributes to formation of vascular networks, as ECs can sometimes express PDGF- β receptors [59]: PDGF induces proliferation of ECs, tube formation, and sprouting *in vitro* [60]. In chorioallantoic membranes and ischemic hindlimbs, PDGF contributes to angiogenesis [57].

Angiopoietins—The angiopoietin family of proteins consists of angiopoietin-1, -2, -3 and -4. The functions of angiopoietin-1 (Ang-1) are the most studied. This protein is secreted by perivascular cells such as PCs and vSMCs and acts on its receptor, Tie2, which is expressed almost exclusively on ECs. Ang-1 is essential for the proper development and maturation of vascular networks [61–66]. Knockout of Ang-1 leads to embryonic lethality, even though early stages of vascular development occur normally. Remodeling and stabilization are impaired, and ECs appear rounded and fail to interact and adhere properly to mural cells and basement membrane proteins [65]. Further, Ang-1 is constitutively expressed in adults, acting to mediate survival signals for ECs and regulate the recruitment of mural cells; and its receptor, Tie2 is highly expressed during adult angiogenesis [62]. It appears that Ang-1 inconsistently plays a role in two seemingly distinct processes, angiogenesis and vascular stabilization. While angiogenesis requires increased permeability as well as proliferation and migration of ECs, vascular stabilization requires reinforcement of EC-EC contacts, increased barrier function and support of EC survival. This paradox can be resolved by the fact that Ang-1 can act through distinct pathways determined by context [62–64]. In the

absence of cell-cell contacts, Ang-1 acts to promote angiogenesis. Ang-1 mediates contact between the cell and ECM molecules through Tie2, and migration is promoted. Although still controversial, several groups have observed a mitogenic effect of Ang-1, leading to EC proliferation through ERK activation [62–66]. In the presence of EC-EC contacts, Ang-1 acts to quiesce and stabilize ECs, promote survival of ECs and inhibit inflammation and angiogenesis. Endothelial barrier function is also strengthened by Ang-1 in this context via the improvement of EC-EC adhesion through vascular endothelial protein tyrosine phosphatase (VE-PTP), VE-cadherin and PECAM-1 [62–66].

Transforming growth factor- β 1 (TGF- β 1)—The TGF- β super family consists of three isoforms, arising from different genes [67]. These proteins are synthesized as an inactive precursor that must be proteolytically processed on the cell surface or extracellularly to release the active protein [68, 69] and both the latent and active forms may be stored extracellularly bound to extracellular matrix [68]. For example, TGF- β can be proteolytically activated by matrix metalloproteinase-9 (MMP-9) when bound to the hyaluronan receptor CD44 [69]. TGF- β 1 is the member of this family that has been most extensively studied for its role in angiogenesis. It acts in a context dependent manner, exhibiting both pro- and anti-angiogenic properties when different signaling pathways are stimulated within the cell [70, 71]. Through the TGF- β 1-ALK1 pathway, Id1, a protein required for migration, is upregulated in ECs and fibroblasts *in vitro*. Through the TGF- β 1-ALK5 pathway, ECs upregulate plasminogen activator inhibitor, preventing matrix degradation and promoting vessel maturation [72]. Further contributing to maturation, mesenchymal cells differentiate into mural cells upon exposure to TGF- β 1 [70, 73]. TGF- β 1 is an inhibitor of EC proliferation and PCs interact with ECs to activate the latent cytokine, an effect proposed to contribute to vessel stabilization by PCs [74].

2.3 Others

There are many other known angiogenic proteins, and although these are less well studied and incompletely understood, they may contribute to future advances in stimulating vascular network development in engineered tissues. These include, but are not limited to ephrin-B2, interleukin-1 and -6 (IL-1, -6), epidermal growth factor (EGF), nerve growth factor (NGF), erythropoietin, insulin-like growth factor (IGF), and hedgehog proteins.

3. PRESENTING ANGIOGENIC PROTEINS: DELIVERY METHODS

During neovascularization angiogenic proteins are presented in a regulated manner, and must be provided in a specific temporal and spatial pattern to direct the formation of functional vascular structures. To achieve this in the context of tissue engineering, specialized delivery platforms have been developed (Fig. 1). Although these systems vary, all must meet key requirements. First, and most importantly, the drug delivery system must be compatible with the formation of a microvascular network; it must support cell growth and must not physically impede neovascularization by blocking the growth of new vessels. Further, it must not produce any byproducts that adversely affect the cells involved. Second, since the overall goal is the formation of a new tissue, the system must allow for the co-implantation of parenchymal cells, such as hepatocytes. Thirdly, since protein function is

dependent on structure, fabrication conditions must be gentle. To prevent denaturation, temperatures, pH and chemical compositions must be considered.

3.1 Bulk scaffolds

Free incorporation—The most straightforward approach to delivery is the inclusion of proteins in a bulk scaffold (Fig. 1a). The protein is suspended in a liquid solution of scaffold material, and trapped inside upon gelation, crosslinking or solidification. Because the process is simple – and does not require chemistry that is specific to the protein – multiple proteins, and even cells, can be incorporated if desired. Protein molecules diffuse within the scaffold and are released into surrounding tissues. This approach is limited in its ability to control spatial and temporal presentation, particularly with proteins that do not bind to the scaffold. Scaffolds can be composed of purified natural materials, such as fibrin and collagen, and synthetic polymers, including poly(lactic-*co*-glycolic acid) (PLGA).

Fibrin is an attractive candidate for tissue engineering as it is a natural material that can be broken down by serine proteases during tissue repair and it is already widely used in biomedical applications. It is often fabricated by combining fibrinogen and thrombin, and proteins and cells can easily be incorporated during preparation. Fibrin contains several cell binding domains, including RGD, and, therefore is conducive to cell engraftment. Fibrin has been successfully used in varied systems for the delivery of FGF2. A fibrin gel system was used to locally deliver FGF2 to transplanted cord blood-derived mesenchymal stem cells (CBMSCs). The fibrin gels containing FGF2 and cells were implanted in murine ischemic hindlimbs and resulted in enhanced survival of the CBMSCs, increased EC homing and PC recruitment [75]. Alternatively, when immobilized in fibrin scaffolds and codelivered with granulocyte-colony stimulating factor (G-CSF), FGF2 lead to increased capillary formation when delivered with unfractionated bone marrow cells in hindlimb ischemia [76].

Collagen type I is the major structural constituent of many tissues, is compatible with cell engraftment, and forms a hydrogel from collagen monomers upon adjustment of pH and temperature. Collagen type I gels are widely used for a variety of *in vitro* assays. These assays include the evaluation of contraction abilities of fibroblasts, vSMCs, and other contractile cells, and the evaluation of the angiogenic potential of ECs and other vascular cells as these form vessel-like cords in this setting. Collagen gels are therefore readily translated to use in tissue engineering, as proteins and cells can easily be added during fabrication. Collagen and gelatin – denatured collagen – often have poor encapsulation efficiency [20], but release profiles can be extended by manipulating the collagen matrix. For example, by crosslinking collagen scaffolds, NGF and glial cell line-derived neurotrophic factor (GDNF) were delivered for up to 30 days, a technique that could be applied to angiogenic factors [77]. Additionally, collagen gels do support cell growth, and are used *in vivo* as a scaffold for implantation of cells, including ECs, vSMCs and PCs [10, 11]. Moreover, collagen serves as a good base scaffold for suspension of protein-releasing microparticles made of other materials, discussed further below [51, 78].

Alginate, a natural polysaccharide derived from seaweed, has been well established as a biocompatible material as it is used in many food products and has become one of the most used materials for cell encapsulation. Alginate is ionically crosslinked in the presence of

multivalent cations, e.g. calcium, under extremely mild conditions, and proteins can be encapsulated without being denatured during fabrication. Alginate does not contain cell adhesion sites, however, and must be modified to permit cell attachment. Bulk alginate gels have been used in an injectable form for co-delivery of VEGF and PDGF, each with distinct kinetics [29].

Synthetic materials have not been widely used as a bulk scaffold for angiogenic protein delivery, but are more widely used in the formation of micro- and nano-particles, discussed below. This is due, in part, to the fact that they are not as conducive to cell engraftment. Although they can be modified, synthetic materials do not contain cell adhesion sites, and fabrication of these materials sometimes requires harsh conditions.

Immobilization—To improve upon the limitations of free incorporation, proteins that cannot directly bind to the scaffold may be linked to it. Immobilized proteins are those that are covalently linked to the scaffold material, making them available indefinitely to invading or incorporated cells (Fig. 1a). Fibrin gels with immobilized ephrin-B2 led to increased EC binding *in vitro* and stimulating angiogenesis in the chick embryo chorioallantoic membrane [79].

To gain more control over the presentation of growth factors, the link between the protein and the scaffold can be engineered to be degradable so that the protein is released at a specified time. This was accomplished with a variant of VEGF-121, enabling it to bind to fibrin. The link to fibrin was sensitive to proteolysis, allowing for local release of VEGF. At 3 weeks, the subcutaneous fibrin implant with this variant displayed increased vessel density when compared to wild-type VEGF-121 [80].

Coupled proteins—In some cases, angiogenic proteins that do not directly bind to the scaffold are capable of binding to other proteins, such as heparin (Fig. 1a). If heparin can be incorporated into the scaffold, the heparin-binding angiogenic protein can bind to this molecule and retention durations can be extended. This has been accomplished by covalently linking heparin to both fibrin and collagen, with improvement in neovascularization *in vivo* [81, 82]. Also, “designer self-assembling peptides” were created that included heparin-binding domains. The heparin-binding growth factor, VEGF, was released over one month, when added to the scaffold with heparin, and this resulted in a higher microvessel density, increased cell survival, and improved cardiac function when implanted in an infarcted rat heart [83].

Fusion proteins—Advances in protein engineering have allowed for the creation of fusion proteins, which are formed by the splicing of two genes and expression of this fused gene. In this way, the desirable properties of two different proteins can be combined into one (Fig. 1a). For example, the angiogenic properties of VEGF were combined with the fibrin-binding capabilities of Factor XIIIa [81] and FGF2 was fused to the Kringle4 domain, a fibrin-binding peptide from human plasminogen [84], both of which improved retention durations and, therefore, improved the formation of new vascular networks in fibrin implants.

Injectable scaffold—To minimize the invasiveness of implantation procedures, injectable scaffolds that deliver angiogenic proteins have been developed. These are matrices that can be injected while still in liquid form, and solidify inside the body, eliminating the need for an incision. Proteins are incorporated before injection and diffuse throughout the matrix and into the surrounding tissues. Since fibrin gels are made from two distinct components, fibrinogen and thrombin, this system lends itself to the development of an injectable system. Angiogenic factors can be suspended in one of the solutions; then both can be injected, forming a fibrin gel *in vivo*. FGF2 was incorporated into a fibrinogen/thrombin solution before injection with blood-derived endothelial colony-forming cells (ECFCs) and MSCs, forming a fibrin gel *in vivo*. Inclusion of FGF2 significantly shortened the time required for functional anastomoses with host vasculature [85]. Another injectable hydrogel made of alginate has been successfully developed to deliver VEGF resulting in increased vascularization within bone defect sites [86]. A different approach involved the development of an injectable, pro-angiogenic matrix. Rabbit fibroblasts were cultured under hypoxia in one layer of a bilayered collagen gel. Hypoxia induced factors were secreted, including VEGF, FGF, PlGF and IL-8. The acellular portion, containing the angiogenic proteins, was then removed and fragmented. *In vitro*, positive results were observed in tubulogenesis assays, but this system has not yet been tested *in vivo* [87]. Self-assembling peptide nanofibers (NFs) were created to deliver VEGF. When injected into an infarcted myocardium, VEGF was released from the NFs for 14 days and resulted in substantial improvements in neovascularization and cardiac function in both small and large animal models [88].

3.2 Micro- and Nanoparticles

A more controlled approach to protein delivery involves the encapsulation of proteins within micro- and nanoparticles (Fig. 1b). These particles can be tailored to dictate release profiles by varying the composition, crosslinking extent and size of the particles. Further, these particles can be incorporated into a bulk scaffold, allowing specification of the spatial pattern of delivery (Fig. 1a).

Natural polymers—Many of the same natural materials used in bulk scaffolds can be formed into micro- and nano-particles. Due to its mild preparation conditions, described above, alginate has been successively utilized in the form of microparticles and has been utilized by many groups for protein delivery. Among the delivered proteins are VEGF [16, 51, 78], PDGF [16], FGF1 [89], FGF2 [90], SDF-1 [91], CCL2 (aka MCP-1) [51] and HGF [90]. All of these produced positive results, with respect to neovascularization, in various contexts. Interestingly, delivery of VEGF from alginate microparticles was directly compared to delivery from PLGA microparticles in the context of tissue engineering: higher loading and encapsulation efficiencies, and therefore bioactivity, were observed with alginate microparticles, and release profiles could be tuned by varying crosslinking conditions (Fig. 2) [78]. These data show that there are limits to a popular material, PLGA, in the delivery of proteins in the context of tissue engineering. Further, the identification of this limitation allowed the creation of materials that were more appropriate for protein delivery in this setting. Some more general conclusions can be drawn: hydrophobic polymers, such as PLGA, are convenient in drug delivery applications, but may not be

optimal for tissue engineering. On the other hand, hydrophilic polymers, such as alginate, have important limitations in the duration of protein release that can be achieved. Finding materials that allow high protein loading, mild interactions with cells and developing vessels, and sustained release is a key challenge for the future.

Alginate also has a long history in the encapsulation of cells, and therefore, it is easily translated into bifunctional capsules and scaffolds, encapsulating both cells and proteins. For example, a bilayer alginate capsule system was developed, in which FGF1 was encapsulated in an outer layer. Pancreatic islets were encapsulated in the inner layer, and layers were separated with a semipermeable poly-L-ornithine (PLO) membrane. These were transplanted in a rat omentum pouch and explanted after 14 days. An increased vessel density was observed [89]. Additionally, microparticles can be made out of collagen by emulsion and subsequent crosslinking. Using this technique, VEGF was released for 4 weeks [92]. Other natural polymers used to make particles include gelatin [93] and chitosan [94].

Synthetic polymers—Synthetic materials offer more control over the properties of the release system. PLGA is one of the most widely used of these materials as it is biodegradable and FDA approved. The mechanical properties and rate of degradation can be altered by varying the ratio of lactic acid monomers to glycolic acid monomers, the molecular weight of the polymer, and the conditions of fabrication of materials.

PLGA nanoparticles have been used in a wide variety of settings to delivery angiogenic proteins. When delivered to ischemic myocardium, VEGF-releasing PLGA microparticles increased vascularization and function [95]. Release duration of VEGF was increased by embedding PLGA microparticles in alginate hydrogels [96], and FGF2 retention was increased by coupling heparin to PLGA. The heparin-coupled PLGA nanoparticles can then be delivered to an ischemic hindlimb either directly or suspended in a fibrin gel [97]. The basic PLGA microsphere system can be further modified by blending in another polymer, such as polyethylene glycol (PEG) [98], to further tune protein release rates.

Combined delivery—Micro- and nano-particles are particularly well-suited for combined delivery of multiple proteins. A separate particle system can be developed for each protein, in which release profiles are specifically tailored. A protein that promotes formation of vascular structures, such as VEGF, can be delivered first, while a stabilizing protein, such as PDGF, can be delivered for a longer period of time, so that it is available at later time points, when it is needed to recruit mural cells. For example, VEGF and PDGF, each encapsulated in PLGA particles, were incorporated into an alginate bulk scaffold. Each had distinct kinetics, with PDGF being released for a longer period of time [17]. Other methods include the delivery of VEGF and CCL2 (MCP-1) from alginate particles embedded in a collagen fibronectin gel [51], and the delivery of three separate angiogenic factors, VEGF, PDGF and TGF- β 1, from alginate-sulfate scaffolds [18].

3.3 Cells as protein delivery devices

One shortcoming of the protein delivery systems described above is the finite amount of encapsulated protein delivered over a finite period of time. If a longer time course is desired,

the protein source must be replaced. Further, most proteins have a short half-life, and once they are released from the delivery device, they are active for a restricted amount of time. To overcome these limitations, cells can be utilized as protein delivery devices. Either cells that naturally secrete the desired protein constitutively can be chosen, or overexpression of the protein can be achieved through viral transformation or plasmid transfection of cells. The cells can be freely injected into the site of interest or incorporated into a scaffold (Fig. 1a) or encapsulated into a particle before implantation (Fig. 1b, and Fig. 3). For example, overexpression of VEGF in SMCs and in mesenchymal stem cells (MSCs) leads to improved EC function and neovascularization in ischemic myocardium [99, 100], and overexpression of Ang-1 in MSCs stimulated better recover of blood flow in a rat ischemic hindlimb [55]. Interestingly, with the goal of engineered skeletal muscle, the addition of embryonic fibroblasts to a co-culture of myoblasts and endothelial cells on highly porous, biodegradable polymer scaffolds led to an increase in VEGF expression within the system and better vessel formation and stabilization [101]. Additionally, myoblasts overexpressing VEGF were implanted into non-ischemic mouse skeletal muscle, leading to the formation of arterioles adjacent to the site of implantation. While this is important for proper therapeutic revascularization, there was aberrant endothelial overgrowth and hemangioma formation around the implanted myoblasts [23]. When these VEGF-expressing myoblasts were encapsulated and implanted subcutaneously, large masses of ECs and SMCs formed. Intraperitoneal implantation caused significant local inflammation [102].

Alternatively, several studies have employed the delivery of a mural or progenitor cell types to support endothelial cell growth and tube formation. Co-delivery of mural cells, such as SMCs [10] or PCs [11], or progenitor cells, such as the 10T1/2 mesenchymal progenitor cells [103] has been shown to greatly improve maturity, stability and retention of new microvasculature. This produces a more authentic tissue engineering system, but also a more complicated system, as it involves more than protein delivery. Cell-cell contacts, paracrine signaling and crosstalk between the two cell types also plays a role in this observed benefit.

3.4 Smart materials

Perhaps one of the biggest challenges in drug delivery is the control of temporal presentation. While release kinetics can be tuned to some extent by altering the characteristics of polymer scaffolds and nanoparticles, true and exact temporal control requires an on-off switch, in which delivery can be turned on or off by a stimulus. The stimuli might be temperature, pH, ionic strength, ultrasound excitation, or electric currents [104]. Proteins function is dependent of structure, however, and the possibility of denaturation must be considered when designing these systems, so as to preserve proper function.

For tissue engineering, perhaps the most easily translated system is one in which the stimulus can be applied externally, at a specified time after implantation. The application of magnetic fields is a particularly simple technique that generally does not have deleterious side effects. One example of this is the magnetically triggered nanocomposite membrane in which a drug reservoir is surrounded by a composite of nanogels and iron oxide particles in ethylcellulose matrix. When exposed to a magnetic field, the iron oxide particles heat up and

irreversibly shrink the nanogels, leaving space for the drug to escape [105]. Alternatively, a similar principle can be utilized to degrade polymers that are thermally sensitive. If iron oxide particles can be added to a polymer structure that degrades upon an increase of temperature, proteins can be delivered upon application of a magnetic field [106].

The development of smart materials is a relatively new field, and much remains to be investigated, but this will likely be an exciting technique for the future of tissue engineering.

4. APPLICATIONS

The delivery strategies presented in the last section have been applied to a variety of angiogenic proteins (Table 2). While the first goal of these drug delivery platforms is to improve neovascularization, parenchymal cell growth must also be permitted and encouraged if the systems are to be generally useful for tissue engineering. Most notably, angiogenic protein delivery systems have been utilized with the goal of increasing neovascularization in engineered liver and bone. When FGF2 is locally delivered with hepatocytes in poly-L-lactic acid (PLA) discs, microvessel density was improved and hepatocytes engrafted and survived better [107]. Also, VEGF-releasing alginate scaffolds were implanted next to liver tissue, resulting in promotion of neovascularization, vascular stabilization and hepatocyte engraftment [108]. Alginate has been used to deliver VEGF to bone defect sites, increasing vascularization and improving bone repair [86]. Additionally, VEGF that was delivered from subcutaneously implanted heparin crosslinked demineralized bone matrices, improved invasion of blood vessels [109]. To more directly increase bone formation, both VEGF and bone morphogenic protein-2 were delivered, along with human bone marrow stromal cells in a composite scaffold composed of alginate and PLA. Bone repair of critical sized femur defects in mice was enhanced [110]. Another angiogenic molecule, FGF1, is also known to regulate and induce osteogenesis. For this reason, it was locally delivered in a fibrin/hydroxyapatite composite and improved both bone and vascular growth *in vivo* [111].

5. CONCLUSION AND FUTURE PROSPECTS

The development of controlled protein delivery systems has contributed to advances in tissue engineering. Perhaps the most important challenge in this field is in the induction of functional vascular network formation in these new tissues. Many protein delivery platforms have been developed to present various angiogenic molecules in a controlled manner, and these technologies have fueled recent advances in this pursuit. Methods to deliver one or multiple proteins at a specified location, time and sequence have allowed us to stimulate the formation of more numerous and more stabilized vascular structures, and allowed for the support of several types of parenchymal cells. While great strides have been made in this field, more work remains to be done to improve resulting vascular structures to better support engineered tissues. Further, it is likely that requirements for vessel formation depend on the type of tissue, and this also must be taken into account when developing schemes for engineering new tissues. Vessel densities and vascular differentiation and organization are often specific to each tissue type and we must learn to better control angiogenic processes to support the highly complex tissues that make up the human body. We must concurrently

gain a better understanding of the necessary spatiotemporal aspects of angiogenic protein presentation and match these advances with improved delivery strategies.

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Fig. 1. Methods of delivering angiogenic proteins

(a) Delivery from scaffold: *Free incorporation* involves suspension of the protein within the scaffold material before gelation, crosslinking or solidification. *Immobilization* is achieved by covalently linking the protein to the scaffold material. *Coupled proteins* can be incorporated into the scaffold where one protein, such as heparin, can bind to both the scaffold and desired growth factor. *Fusion proteins* are formed by the splicing of two genes and expression of this fused gene. This results in a protein that can both bind to the matrix and perform a desired function. Proteins can also be encapsulated in *micro-/nanoparticles*.

This is a more controlled approach to protein delivery in which release profiles can be tailored. *Cells* can be induced to overexpress a desired protein, and incorporated into the scaffold.

(b) Delivery (no scaffold): *Encapsulated cells* and protein-releasing *micro-/nanoparticles* can also be directly injected into the site of interest

Fig. 2. Comparison of alginate to PLGA for encapsulation and controlled release of VEGF
 Alginate microparticles release bioactive VEGF at high efficiency. *A*) Size distribution of PLGA microparticles (mean=30±18 µm) and alginate microparticles (mean=10±5 µm) encapsulating VEGF. *B*) Loading and encapsulation efficiency of VEGF. *C*) Bioactivity of encapsulated VEGF. VEGF bioactivity, as measured *via* sprout formation, for alginate (solid line) and PLGA (dashed line) formulations compared with native (never encapsulated) VEGF (top thin dotted line) and media without VEGF (bottom thin dotted line), average of 96 microcarriers evaluated per condition. *D*) VEGF release from alginate is tunable *via* modulation of ionic cross-linking agents, as seen in different release profiles for particles cross-linked with 700 mM CaCl₂ (dashed line), 700 mM ZnCl₂ (gray line), or a 1:1 mixed population (black line). For all panels, particles were pooled from 3 separate batches ($n = 3$). Data are mean + SE; * $P < 0.05$; *** $P < 0.001$. Figure reproduced with permission from ref. [78]

Fig. 3.
NIH 3T3s encapsulated in alginate microspheres. Scale bar: 100 μm

Table 1

Angiogenic proteins, their receptors and effects.

	Protein or Protein Family	Receptor(s)	Effects
Formation	VEGF Family: VEGF-A,-B,-C,-D,-E,-F, PlGF	VEGFR1,2,3	<i>VEGF-A₁₆₅</i> -Increase angiogenesis in ischemic tissue [22] -Unsuccessful in clinical trials [26] <i>Complete VEGF-A family of isoforms</i> : Decrease permeability of new vessels compared to delivery of one isoform [28]
	FGF Family: FGF1, FGF2, FGF9	FGFR1,2,3,4	-Proliferation, cell-matrix interaction and cell-cell communication [32] <i>FGF2</i> : -Upregulation of VEGF, HGF, CCL2 in EC and stromal cells [31, 32] -Formation of new vessels, modest stabilization; longer-lasting maturation with co-delivery of PDGF <i>in vivo</i> (mouse?) [34]
	HGF	c-Met	-Increase tube formation, outgrowth from aortic explants [37] -Sprouting from cell spheroids [Chang et al, unpublished]
	Chemokines		
	ELR-positive CXC Family: CXCL1,2,3,5,6, 7,8 IL-8	CXCR1, CXCR2	-Increases chemotaxis of ECs [39, 40] -Homing of circulating endothelial progenitor cells [41, 42]
	ELR-negative CXC Family: CXCL12 (SDF-1)	CXCR4	-Recruitment of CD34(+) cells and MSCs [43]
	CC family: CCL2 (MCP-1), CCL11, CCL16	CCR1, CCR2, CCR3	<i>CCL2</i> : -Formation and maturation of new vessels <i>in vivo</i> [48–51] -EC chemotaxis, tube formation and mural cell recruitment <i>in vitro</i> [44–47]
Stabilization	PDGF	PDGFR	-Chemoattractant for vSMCs and PCs [58] -Proliferation of ECs, tube formation and sprouting <i>in vitro</i> [60] -Angiogenesis in chorioallantoic membranes and ischemic hindlimbs [57]
	Angiopoietins: Ang-1, Ang-2	Tie2	<i>Ang-1</i> : -Survival signals in ECs, recruitment of mural cells [62] -Promote angiogenesis: promote cell-matrix interactions, migration and proliferation in EC <i>in the absence of EC-EC contact</i> [62–66] -Quiesce and stabilize ECs, promote survival and strengthen barrier function <i>in the presence of EC-EC contact</i> [62–66]
	TGF- β 1	TGFBR1,2,3	-Increase in EC migration [72] -Upregulation of plasminogen activator inhibitor, preventing matrix degradation and promoting maturation [72] -Differentiation of mesenchymal cells into mural cells [70, 73]
Others	Ephrin-B2	EPHB3,4	
	IL-1, IL-6	IL-1R, IL-6R (CD126)	
	EGF	EGFR	-Increase EC proliferation -Angiogenesis <i>in vivo</i> [36]
	NGF	P75 LNGFR, TrkA	
	Erythropoietin	EpoR	EC proliferation, angiogenesis <i>in vivo</i> [36]
	IGF	IGF1R (CD221)	EC proliferation, prevent EC apoptosis, induce VEGF, increase plasminogen activators [36]
	Hedgehog proteins	PTCH1	

Table 2

Delivery methods and proteins delivered

	Delivery method	Materials	Protein delivered
Within scaffold	Free incorporation	Fibrin	FGF2 and cells [71], FGF2 and G-CSF [72]
		Type I collagen	NGF and GDNF [73]
		Alginate	VEGF and PDGF [25]
	Immobilization	Fibrin-covalent link	VEGF-165 and FGF2 [102], Ephrin-B2 [75]
		Fibrin-link sensitive to proteolysis	VEGF-121 [76]
	Coupled proteins	Covalently linked heparin, fibrin or collagen	VEGF [77, 78]
		Designer self-assembling peptides	VEGF [79]
	Fusion proteins	Fibrin binding capabilities of Factor XIIa	VEGF [77]
		Fibrin binding Kringle4 domain	FGF2 [80]
	Injectable scaffolds	Fibrin	FGF2 and cells [81]
		Alginate	VEGF [82]
		Collagen gel	Hypoxia induced, cell derived factors including VEGF, FGF, PlGF and IL-8 [83]
		Self-assembling peptide nanofibers	VEGF [84]
	Composite delivery: micro-/nanoparticles	PLGA particles in alginate hydrogels	VEGF[92] VEGF and PDGF [16]
Alginate particles in collagen-fibronectin gel		VEGF and CCL2 [47]	
Alginate-sulfate scaffolds		VEGF, PDGF and TGF- β 1 [17]	
Without scaffold	Micro-/nanoparticles injection	Alginate	VEGF [74, 15], PDGF [15], FGF2 and HGF [86], SDF-1 [87], VEGF and CCL2 [47]
		Alginate with PLO membrane, with pancreatic islets	FGF1 [85]
		Collagen	VEGF [88]
		Gelatin	VEGF [89]
		Chitosan	VEGF [90]
		PLGA	VEGF [91]
		PLGA + heparin	FGF2 [93]
		PLGA + PEG	VEGF [94]
	Cell delivery	SMCs	Overexpression of VEGF [95]
		MSCs	Overexpression of VEGF [96] Overexpression of Ang-1 [51]