Tissue Engineering of the Microvasculature

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ABSTRACT

The ability to generate new microvessels in desired numbers and at desired locations has been a long-sought goal in vascular medicine, engineering, and biology. Historically, the need to revascularize ischemic tissues nonsurgically (so-called therapeutic vascularization) served as the main driving force for the development of new methods of vascular growth. More recently, vascularization of engineered tissues and the generation of vascularized microphysiological systems have provided additional targets for these methods, and have required adaptation of therapeutic vascularization to biomaterial scaffolds and to microscale devices. Three complementary strategies have been investigated to engineer microvasculature: angiogenesis (the sprouting of existing vessels), vasculogenesis (the coalescence of adult or progenitor cells into vessels), and microfluidics (the vascularization of scaffolds that possess the open geometry of microvascular networks). Over the past several decades, vascularization techniques have grown tremendously in sophistication, from the crude implantation of arteries into myocardial tunnels by Vineberg in the 1940s, to the current use of micropatterning techniques to control the exact shape and placement of vessels within a scaffold. This review provides a broad historical view of methods to engineer the microvasculature, and offers a common framework for organizing and analyzing the numerous studies in this area of tissue engineering and regenerative medicine. © 2019 American Physiological Society. *Compr Physiol* 9:1155-1212, 2019.

Didactic Synopsis

Major teaching points

- The ability to engineer microvasculature is crucial to the success of therapeutic vascularization and tissue engineering.
- Currently, the main strategies for engineering microvasculature are based on angiogenesis, vasculogenesis, and microfluidics.
- Engineered vasculature can be formed in an intrinsic or extrinsic manner.
- Local and sustained delivery of vascularizing signals usually leads to more robust formation of vascular networks, compared to systemic and transient delivery of the same signals.
- Biomaterial scaffolds can provide a means for local, controlled release of vascularizing signals and a space for vascular ingrowth.
- Widespread success of vascularization methods in laboratory and animal studies has yet to lead to clinically relevant therapies in humans.

Introduction

Before considering *how* to engineer microvasculature, it is useful to consider *what* microvessels are and *why* one would want to create them. Surprisingly, a universally accepted definition of "microvessel" does not exist. To the anatomist, a microvessel is any structure that lacks the well-organized elastic lamellae of arteries and the valves of veins; these constraints limit microvessels to diameters of less than ~100 μm (629,630). In the lymphatic system, the initial capillaries and small collecting lymphatics are considered to be microvascular (496).

To the physiologist, a microvessel is defined by its primary function of regulating exchange of fluid, solutes, and cells between the lumen and the surrounding tissue (690). Arteries, veins, and collecting lymphatics serve mainly to conduct blood and lymph from one part of the vascular tree to another. In contrast, microvessels provide a large surface area-to-volume ratio that enables rapid exchange across the endothelial layer. With this focus on functionality, microvessels again refer to diameters less than ~100 μm, depending on the material to be exchanged.

To the engineer, the definition of a microvessel is quite broad. A microvessel can be much wider than 100 μm in diameter and does not even need to be lined by endothelial cells (ECs) (107,578). In fact, it appears that the only requirement for a microvessel is that it is able to conduct fluid.

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To the surgeon, no strict definition of microvessel is noted. A practical requirement is that a microvessel lies beyond the ability of the surgeon to suture, which means that arteries narrower than ~500 μm in diameter qualify (45,63). As microsurgical instruments and techniques become more refined (300), which structures are considered microvascular will become correspondingly more restricted. These perspectives from the fields of anatomy, physiology, engineering, and surgery should be kept in mind when assessing and comparing published results.

In this review, a microvessel will be defined by three main criteria: first, it should be able to conduct fluid; studies that show clear evidence of fluid flow within de novo tubes will take priority over studies that examine solid "cords" of ECs that lack a lumen. Second, it should be lined by ECs; immature endothelial tubes that lack a proper mural coat of pericytes or smooth muscle cells (SMCs) will also be considered. Third, it should generally have a diameter of at most ~100 μm; this requirement will be relaxed when discussing results primarily from the engineering community. These three criteria appear to be the bare minimum needed to define structures that are useful for applications in therapeutic vascularization and tissue engineering.

Objectives of vascularization

In contrast to the large vessels of the blood and lymphatic circulation, which exist to conduct fluid, the microvessels exist primarily to provide exchange of substances between the vascular lumen and the surrounding tissue. For solutes of small molecular weight, such as oxygen and glucose, the exchange is primarily diffusive. For larger solutes, advection across endothelial intercellular junctions and shuttling by caveolae play the more important role. For leukocytes, active mechanisms that involve adhesion to the endothelial surface and translocation across the endothelium guide the transport.

The motivation for engineering microvasculature stems mainly from three envisioned applications. First, new microvessels can be used to provide *therapeutic vascularization* (99). In patients with tissue ischemia, such as in the heart or limbs, revascularization through bypass vascular grafts may not be possible, often because the underlying occlusive disease is too diffuse. For these patients, one may be able to induce the formation of new microvessels that can provide an equivalent bypass. Therapeutic vascularization can also potentially provide perfusion to ischemic wounds and tissue flaps. In patients with lymphedema, a similar approach may be used to form lymphatic microvessels to help drain the edematous region.

Second, the ability to engineer microvasculature can provide a means to *vascularize engineered tissues*. In this application, a scaffold is typically used to contain the cells that will eventually form the volume of the engineered tissue. Without early perfusion in the scaffold, a densely seeded implant will develop a necrotic core, because the vast bulk of the cells will not be sufficiently nourished, aside from a thin shell at the surface of the implant. If one wishes to develop highly cellularized tissues, then methods to form microvessels in a scaffold are needed.

Third, engineered microvasculature can be used to create *microphysiological systems in vitro* that serve as models of both the vessels themselves and vascularized tissues. These systems are not intended to treat any patient's condition, but rather to understand the physiology of living tissues in the presence of perfusion and in the well-controlled *in vitro* environment. This objective is often coupled with traditional engineering approaches that use microlithography and other patterning techniques to create miniaturized "vessel-on-a-chip" or "organ-on-a-chip" devices that connect to external pumps, actuators, and sensors.

Vascularization strategies

These three applications—therapeutic vascularization, engineering vascularized tissues, and creating vascularized microphysiological systems—have guided much of the efforts in engineering microvasculature for the last several decades. These efforts have focused on three complementary strategies for vascularization: those based on angiogenesis, vasculogenesis, or microfluidics (Fig. 1).

Angiogenesis is microvascular growth by sprouting, and is a normal process of vascular development in the developing human. When tissues grow in the adult, such as in a healing wound or a tumor, angiogenesis is the main mechanism for vascular expansion. The main issues in angiogenic vascularization concern which angiogenic molecules, cells, scaffold, or signals to use, how to deliver them, and in what doses to achieve results that are comparable to those that form naturally by angiogenesis (Fig. 1A).

Vasculogenesis is microvascular growth by the coalescence of individual vascular progenitor cells. Like angiogenesis, it is a normal developmental process. The earliest vascular networks in the embryo form by vasculogenesis, in which angioblasts align to form networked cords of cells that subsequently canalize to form lumens that can accept pressure-driven flow. It remains controversial whether vasculogenesis contributes to vascular growth in the adult; bona fide angioblasts almost certainly do not exist in the adult, but other cells may substitute for angioblasts in performing a vasculogenic function. For a vasculogenic approach, the main questions are which cell populations to use and how to coax them to form open networks in the adult (Fig. 1B).

Microfluidic vascularization has no natural biological analog and draws its inspiration from surgical approaches to tissue reconstruction. It refers to an artificial form of vascularization in which scaffolds are designed to contain perfusable channels that can subsequently support the growth of microvessels. The underlying motivation is to avoid the tubulogenesis that is needed in angiogenic and vasculogenic approaches. As an unnatural mode of vascularization, it relies heavily on engineering techniques for patterning biomaterials (Fig. 1C).

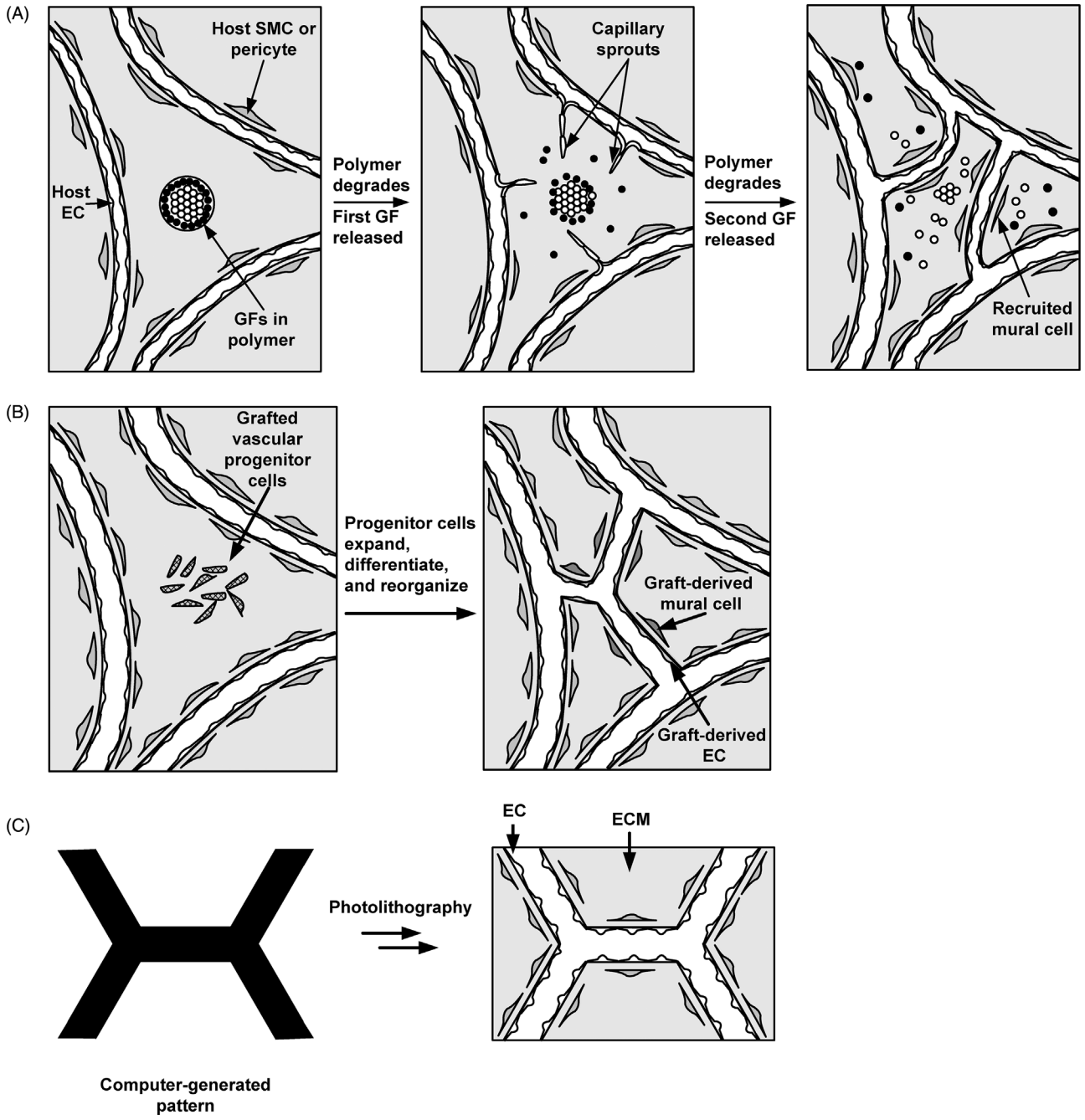

Figure 1 Vascularization strategies based on (A) angiogenesis into a graft, (B) vasculogenesis within a graft, and (C) direct seeding and perfusion of a microfluidic scaffold. GF, growth factor; ECM, extracellular matrix. Adapted, with permission, from (584).

Although this review attempts to organize studies by application and vascularization approach, it is important to note that the distinctions are not so clear-cut. For instance, using injections of bone marrow to promote vascular growth undoubtedly has an angiogenic component from the elaboration of growth factors by the injected cells, but vasculogenesis may also contribute. Likewise, scaffolds are often used to deliver angiogenic factors and vasculogenic cells in the same volume. In these cases, the studies in question are discussed in the section that corresponds to the primary application and vascularization approach; to some extent, these choices are arbitrary.

Scope of review

This review is designed to provide an up-to-date (as of 2018) overview of efforts to engineer microvasculature *in vivo* and *in vitro*. It is intended to provide both newcomers and current researchers with a broad historical view of the fundamental issues in this field, how these issues were successfully

addressed or not, and how other issues emerged. It is not intended to be topical, and the reader who is interested primarily in the most recent studies is pointed to further literature at the end of this article. As noted earlier, this review prioritizes the discussion of studies that create tubular structures that contain a complete endothelial lining and that sustain fluid flow; studies that result mainly in the formation of solid endothelial cords or that examine tubular structures that allow perfusion but lack endothelium will only be discussed briefly. The three sections on angiogenic, vasculogenic, and microfluidic vascularization are largely self-contained and can be understood out of sequence. Only studies that intend to create new vasculature are considered; although one might argue that the controlled regression or inhibition of vasculature [e.g., during tumor growth (214, 419)] is another form of microvascular "engineering," such strategies are already reviewed thoroughly elsewhere (146, 240, 589) and are not discussed here.

Characterization of Engineered Microvessels

Engineered microvessels are evaluated by their structure and function. Analysis of vascular structure uses well-described morphological and histological methods. Early studies simply counted the number of histologically identifiable vessels per tissue section. More refined approaches rely on staining the vasculature for EC-specific markers, such as CD31, VE-cadherin, Prox1 (for lymphatics), and LYVE-1 (for lymphatics), and using confocal microscopy to assess the three-dimensional distribution of these markers. To label vessels that are actually perfused, one can inject contrast agents, lectins, or fluorescent lipids into the circulation (342).

Functional assessments have focused on the transport properties of the engineered microvasculature. Many of these tests are qualitative and have relied on gross anatomy or histological images. For instance, to assess whether the microvessels assist in the delivery of oxygen, one can determine the fraction of tissue that is necrotic on histological sections. To assess whether lymphatics aid in resolving edema, one can visually determine whether the relevant tissue is swollen.

Quantitative tests of microvascular transport seek to provide normalized physiological measures that can be used to compare the transport properties in different vessels, organs, and subjects (108, 378, 665). These metrics are based on modeling the vascular wall as a uniformly permeable film that can be described by a hydraulic conductivity L_P for the bulk flow of aqueous solution, and by a diffusional permeability coefficient P_d and osmotic reflection coefficient σ for each solute species. More complex models treat the vessel wall as an arrangement of pores that allow passage of small solutes but retard passage of large ones (465), or as a fibrous matrix that is tethered to the underlying endothelial monolayer (110). Organ-level measurements of hydraulic conductivity and reflection coefficients use Pappenheimer's classic technique that plots filtration rates as a function of intravascular pressure (377, 428). Similarly, organ-level measurements of vascular permeability rely on the accumulation of a labeled solute in tissues (102, 379). Blood flow rates per tissue volume can be measured with magnetic resonance imaging and other noninvasive techniques, by tracking tissue signal as a function of time after bolus intravascular injection of a tracer (370, 686). Intravital microscopy with window chambers enables long-term observation of blood flow (93, 487, 488) and lymphatic drainage (94).

Physiological assays of individual vessels have also been developed (Fig. 2). Measurements of hydraulic conductivity

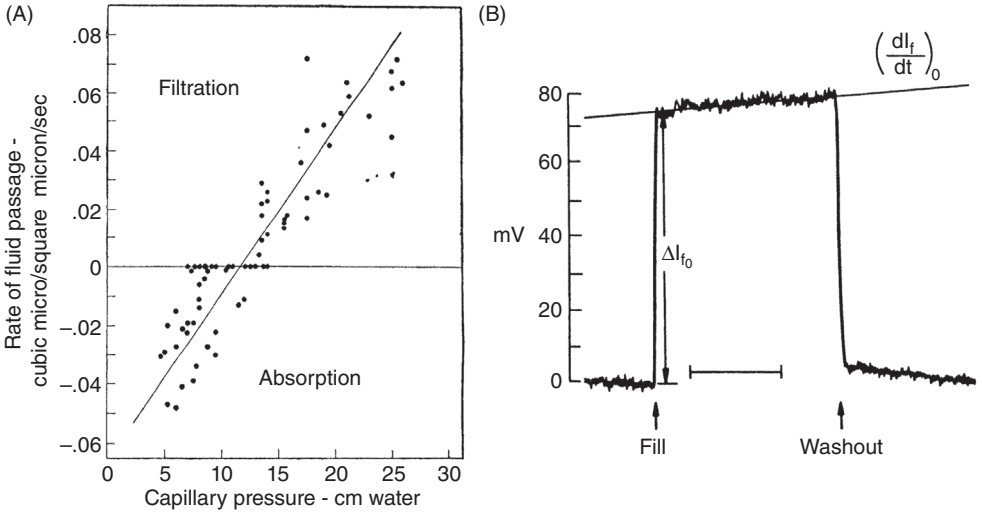

Figure 2 Quantitative metrics of microvascular physiology. (A) Calculation of endothelial hydraulic conductivity relies on measurement of filtration speed as a function of vascular pressure. Reprinted with permission from (313). (B) Calculation of solute permeability relies on measurement of solute accumulation over time. Reproduced, with permission, from (227).

apply Landis's technique, in which a distal portion of the vessel is occluded and slow movement of tracer particles (such as red blood cells) in the proximal section is tracked (Fig. 2A) (313). Measurements of solute permeability coefficients in individual vessels require cannulation of the vessel and sudden introduction of labeled solute (Fig. 2B) (109, 227). Such methods are technically more demanding than organ-level assays, which may explain why they are less commonly seen in the microvascular engineering literature.

Assessment of the ability of engineered lymphatics to enhance solute drainage has relied on the injection of a tracer solute into the tissue (481). The amount of solute that remains typically decays exponentially with time, and the rate constant or half-life of this decay provides a measure of drainage (386). Measurement of drainage constants through individual lymphatics is possible (581). Individual collecting lymphatics can be cannulated for measurement of their contraction amplitudes and frequencies (161, 667).

Although many of the quantitative assays of microvascular physiologists have been known for a long time, functional tests of engineered vessels remain less frequently used than structural characterization. Thus, it can be unclear to what degree engineered microvessels perform their intended physiological function, especially when compared with native microvessels.

Angiogenic Vascularization

Angiogenic vascularization refers to the formation of capillaries by sprouting from existing ones. It is an "extrinsic" form of vascularization, in which the source of vessels resides outside of the original graft or injection site. Sprouting in the blood circulatory system occurs mainly from postcapillary venules, where the absence of a well-organized, layered vascular media facilitates the migration of ECs into the tissue space. In the lymphatic system, sprouting occurs from the initial lymphatic capillaries, which comprise the terminal ends of the lymphatic tree. In the adult, angiogenesis is primarily observed in healing wounds, inflamed tissues, and tumors. As a result, the materials that are used to engineer microvasculature by angiogenesis have consisted mainly of wound cells and tissue leukocytes, along with the soluble and insoluble molecules that they and tumor cells produce.

Angiogenic and lymphangiogenic factors

Blood vessel angiogenesis

In the 1960s and 1970s, the realization that tumors required a vascular supply before they could grow beyond a diffusion-limited size led to the isolation of tumor-derived angiogenic factors [reviewed in (148)]. These studies benefited from the development of *in vivo* angiogenesis assays that provided a clear view of growing capillary networks in the cornea and anterior eye chamber, in the hamster cheek pouch, and on the chick chorioallantoic membrane. Such assays provided a gold standard for testing angiogenic potency *in vivo*, in which the vascular growth could be direct (i.e., from effects of the added factor on ECs) or indirect (i.e., mediated by release of EC-specific factors by leukocytes and other nonendothelial cells) or both (30, 367). Careful attention to minimize tissue disruption was required so that direct angiogenic effects were not masked by inflammation. Later, the isolation of ECs from large vessels and from capillaries enabled the angiogenic process to be studied *in vitro* (147).

Blood vessel angiogenesis is driven mainly by a need for increased delivery of oxygen to a growing tissue. Insufficient oxygen delivery results in tissue hypoxia, which stabilizes hypoxia-inducible factors, particularly HIF1α, that act as transcription factors to stimulate the expression of target genes such as vascular endothelial growth factor-A (VEGF-A) and erythropoietin (501). Angiogenesis can also be stimulated by tissue injury, and here the fibroblast growth factors (FGFs) play a major role (96).

Overall, the regulation of angiogenesis is extremely complex. Even within a single family of angiogenic factors—the VEGFs—receptor binding is promiscuous (Fig. 3). For instance, VEGF-A binds VEGFR-1 and -2 and neuropilin-1 (Nrp-1); VEGF-B and placental growth factor (PlGF) bind VEGFR-1 and Nrp-1; VEGF-C and -D bind VEGFR-3, and when fully processed, VEGFR-2 as well; VEGF-E binds VEGFR-2 (247). Splice variants also differ in their

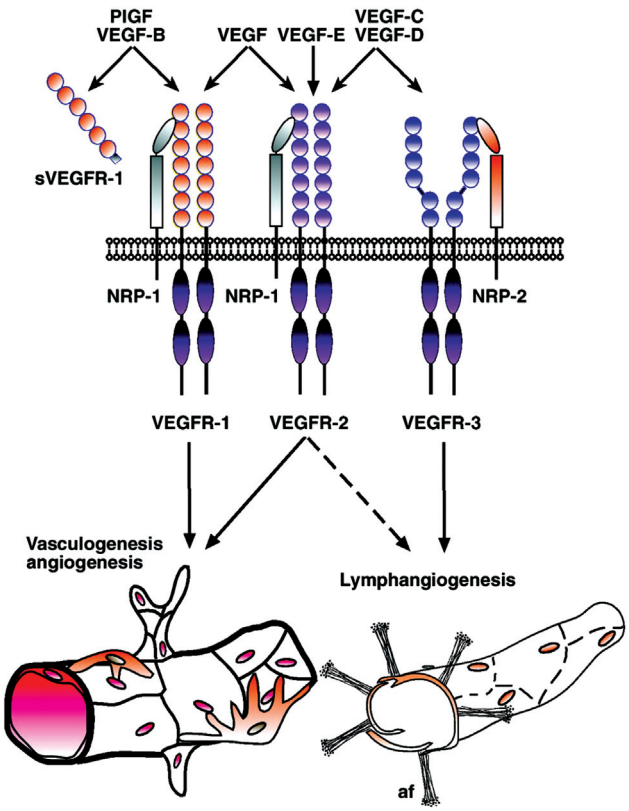

Figure 3 Binding of VEGFs to their receptors, the VEGFRs and neuropilins. Reproduced, with permission, from (247).

receptor binding (537). Part of the challenge in harnessing angiogenesis to produce functional microvessels is to control the delivery of the appropriate factor(s) at the appropriate times and doses without inadvertently inducing bystander effects.

Whether mediated by VEGFs, FGFs, or some other signal, angiogenesis invariably requires degradation of the endothelial basement membrane, loosening of EC cell-cell contacts, loss of pericyte coverage, and migration of ECs into the tissue space. Endothelial proliferation is often observed, but not strictly required, for sprouting (519). Migrating cells tend to orient toward increasing concentrations of the angiogenic signal. Encounters between two capillary sprouts can lead to anastomosis, tubulogenesis, and establishment of flow, which delivers the blood cells that can relieve the hypoxia. Subsequent stabilization of immature capillaries requires pericyte coverage, which is mediated by perivascular gradients of platelet-derived growth factor (PDGF) (345).

The extracellular matrix also plays an important role in angiogenesis, in part by providing binding sites for growth factors and serving as a reservoir that can be mobilized by local proteolysis (231). Other substances, such as lipids, steroid derivatives, and divalent transition metal ions, can induce angiogenesis as well (683).

Lymphangiogenesis

Lymphangiogenesis follows a similar course of sprouting and migration, but the initiating signals are not as clear (10). Given that lymphatics drain tissues, one would expect tissue edema to result in increased lymphangiogenic signaling. Edema is accompanied by increased interstitial fluid pressure and accumulation of VEGF-C in the interstitial space (169). VEGF-C is the classic lymphangiogenic signal, which promotes proliferation and migration of lymphatic ECs (356). In transgenic mice, tissue-specific overexpression of VEGF-C causes persistent increases in lymphatic vessel density and diameter (242, 348), but can also lead to malformations such as lymphangiectasia that do not resolve even when the VEGF-C overexpression is transient (655). Little is known about how lymphatic ECs undergo tubulogenesis, which is required to establish drainage capacity to relieve the fluid congestion. To what extent increased interstitial pressure or changes in interstitial flow alter the response to VEGF-C, or whether these physical changes can provoke lymphangiogenesis independent of changes in VEGF-C levels, is still unclear.

Therapeutic angiogenesis and lymphangiogenesis

One of the earliest attempts at therapeutic angiogenesis was the development by Vineberg in the 1940s of a surgical procedure to revascularize the ischemic myocardium (614, 615). In contrast to arterial bypass grafting (which was to be developed later), the Vineberg procedure consisted of implanting the ligated end of a cut artery (typically, the internal mammary artery) into a tunnel within the myocardium (Fig. 4). The hope

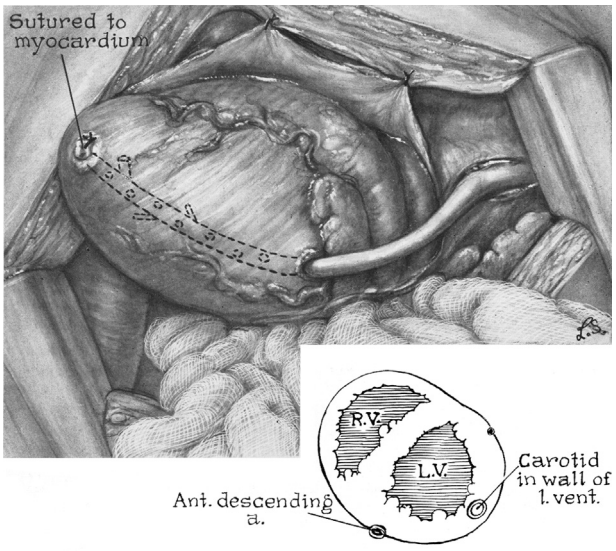

Figure 4 Diagram of the Vineberg procedure for vascularizing ischemic myocardium, using a carotid artery implant. RV, right ventricle; LV, left ventricle. Reproduced, with permission, from (479).

was that signals from the implanted artery would somehow initiate angiogenesis and ultimately grow new microvessels to bridge the gap between the implant and existing myocardial vessels within the ischemic region. Initial claims of revascularization were met with skepticism, and only after the invention of angiography was the presence of collateral bridges between the implanted artery and myocardial vessels definitively established [reviewed in (520)]. Of note, only the implantation of cut arteries resulted in anastomoses; placement of an intact artery with preserved blood flow in a myocardial tunnel did not allow anastomoses to form. Collaterals originated primarily from the distal third of the implant, and large ones formed only when the artery was implanted in ischemic tissue (48). Allowing intercostal branches to bleed freely into the myocardial tunnel appeared to help promote vascularization. If the implanted artery slipped out of the tunnel and adhered to the chest wall, then vessels were generated instead between the artery and the wall (615). In the dog heart, collaterals from the implant provided ~30% of the total flow conductance and ~10% of the total collateral flow to the ischemic zone (602, 603).

Later, Vineberg modified this procedure to include a free graft of omentum, which provided an additional source of ECs and angiogenic factors (616). Adipose tissue, particularly omental fat, can promote angiogenesis, as shown in the rabbit corneal pocket assay (527). Among the released angiogenic signals that have been attributed to intact fat are VEGF-A (674), prostaglandins (527), and a lipid factor, possibly 1-butyryl glycerol (118, 175).

Other arteries, such as the carotid, have also been successfully used to revascularize the heart. Attempts to generate connections between arterial implants and the vascular networks in other organs, such as kidney, skeletal muscle,

and spleen, were unsuccessful (479); arteriovenous implants, which include venous return, successfully vascularized bone (215). Although the precise mechanisms that mediate angiogenesis in the Vineberg procedure still remain unclear, it has been suggested that blood flow that results from pressure oscillations between the implant lumen and myocardium may play a role (34).

Arteriovenous shunts have been shown to generate collateral vessels (180, 556). Although these shunts primarily act as large-bore vessels to revascularize distal ischemic tissues, they also result in the proximal formation of collaterals. Ligation of the shunt reduces blood flow to the most distal tissues but not to tissues near the shunt (180).

These early studies of surgically induced vascularization suggested that local tissue injury (induced by cutting a tissue or by creating an arteriovenous shunt) was needed for successful revascularization. In fact, epicardial injury by abrasion was sufficient to induce vascular growth at the site of the pericardial adhesions that subsequently developed (47). Possible initiators of angiogenesis include the local release of angiogenic factors from the cut surfaces of the tissue and the local deposition of blood cells and a provisional fibrin-based matrix. Similar principles underlie vascular growth from the cut ends of aortic and lymphatic ductal explants *in vitro* (412, 413). Subsequent studies thus focused on the precise angiogenic factors and cell types within these grafts that could promote angiogenesis.

Animal models

Studies of therapeutic angiogenesis for treatment of ischemic myocardium first used occlusion of coronary arteries in large animals, particularly dogs and pigs, since the surgical techniques in these species were already mature. Findings in the dog were often critiqued because the dog myocardium appears to have a rich collateral circulation (599). The collateral circulation of pig myocardium, on the other hand, is less well developed and thereby resembles that of the human. Techniques to reproducibly induce myocardial infarct and ischemia in smaller animals were more challenging to develop; local cryogenic injury to the myocardial wall, which generates a well-demarcated scar, served as an alternative to these techniques.

Models of limb ischemia have focused on occlusion or removal of hindlimb arteries and veins in small animals, particularly mice, rats, and rabbits. Rats and rabbits have the advantage of larger size, while mice provide the opportunity to use transgenic technologies to study genetic effects on angiogenesis. Similarly, models of ischemic wounds and surgical flaps have relied on small animals.

Direct injection of angiogenic factors

With the isolation of angiogenic growth factors and the cloning of their genes in the 1980s, it became feasible to deliver these factors to the ischemic regions directly, either as recombinant proteins or through gene therapy. In principle, this approach could afford better control over the angiogenic process, since the dose, timing, and identity of the added factors are not left to chance, as they would be in a Vineberg-type tissue implant (211). Moreover, combinations of angiogenic factors could optimize the functionality of the microvasculature that forms. For these reasons, by far the most effort in therapeutic angiogenesis has focused on the delivery of recombinant growth factors and plasmids that encode for them (143, 351, 530, 671). This section focuses on studies that use soluble growth factors to induce angiogenesis and lymphangiogenesis. Unless otherwise noted, all growth factors are for human isoforms.

FGFs As the first family of EC growth factors to be isolated and purified, the FGFs were the first ones to be applied toward therapeutic angiogenesis; of these, FGF2 (basic FGF) has been the main focus. The earliest study used soluble FGF2 to treat the infarcted dog myocardium (652). This work provided a template that was reapplied in numerous subsequent studies, in which one or a combination of growth factors (FGFs, VEGFs, etc.) was delivered with a variety of methods (bolus injection, constant delivery, etc.) at a variety of sites (intravascular, intramuscular, etc.) to treat a model of tissue ischemia (myocardial, limb, etc.), as assessed by a variety of readouts (tissue blood flow, capillary density, etc.). Here, intracoronary delivery of FGF2 increased microvascular density in the infarct. FGF2 also increased myocardial blood flow and caused acute vasodilation, although the role of vasodilation on the chronic increase in blood flow is unclear (490, 601).

Because repeated intracoronary infusion of growth factors is unlikely to be clinically practical, systemic delivery of FGF2 was studied (318). Injected FGF2 was removed from the circulation with a half-life of ~50 min, and injections were performed daily. FGF2-treated animals had higher blood flows than controls (no FGF) did, but only when treatment was initiated early (e.g., 3 days after occlusion); the flow-enhancing effects of FGF2 could be maintained for over 2 months and persisted even after discontinuation of therapy for 4 weeks. Side effects of chronic FGF2 administration consisted of anemia, thrombocytopenia, hypotension, and possibly intimal proliferation. Intravenous delivery of bovine FGF2 to ischemic pig heart increased angiographic scores, but did not change blood flow or cardiac function (490).

Pericardial delivery has been used to prolong the retention of FGF2 near ischemic tissue. In the dog, vascular and arteriolar densities in the outer half of at-risk myocardium increased to a greater extent compared to those in the inner half (596). Coadministration with heparan sulfate increased the effects of FGF2. In the pig, pericardial delivery of FGF (along with heparin) resulted in increased collateral indices and vascular densities compared to delivery of heparin or saline, and was accompanied by greater flow and myocardial function (310).

Intramuscular delivery of FGF2 rescued gross limb function and increased capillary density and capillaries per

muscle fiber in the ischemic rabbit hindlimb, in a dose-dependent manner (33). High doses, however, led to muscle injury.

Topical application of FGF2 at the time of wounding increased vascular density by 30% to 50% one week later in rabbit ear skin wounds (404). The increase in vascular density was accompanied by increased epithelialization only in nonischemic wounds (402, 404). In a hyperoxic chamber, treatment of ischemic wounds with FGF2 enhanced epithelialization and formation of granulation tissue (646). These findings suggest that, in ischemic wounds, the growth of new vessels is not the rate-limiting step in wound closure. Sensitivity to ischemia appears to be unique to FGF2 treatment, since FGF4 treatment was effective in both normal and ischemic wounds (646).

In bone grafts to the rabbit mandibular ramus, continuous intra-graft infusion with FGF2 accelerated vascularization by day 10, but did not increase the long-term steady-state vascular density over that obtained with infusion of saline (132). Vascular growth mainly occurred from the surrounding muscle rather than from bone.

Intracoronary administration of FGF1 (acidic FGF, or ECGF), the other major member of the FGF family, in the ischemic dog heart did not lead to greater blood flow than in control (600). The authors rationalized their result in several ways: first, both experimental and control infusions were performed with heparin, which helps to stabilize the FGFs (178) but is itself mildly angiogenic (603), and it is possible that the amount of added heparin already saturated the angiogenic response. Second, the infusion of FGF1 began more than a month after balloon constriction of the left circumflex coronary artery (LCCA) began; this late start of the angiogenic therapy, while perhaps more relevant to the setting of chronic human myocardial ischemia, may have weakened the responsiveness of the ischemic region to FGF. Third, the growth factor was delivered via the left main coronary artery, instead of the distal LCCA; as a result, it may not have been optimally delivered to the ischemic region that was originally perfused from the LCCA. As found in the ischemic hindlimb with FGF2, high doses of FGF1 to the myocardium led to muscle necrosis.

In the rabbit ischemic hindlimb, intramuscular bovine FGF1 (with heparin) led to growth of collaterals and improved perfusion (451). The effect of FGF depended on dose and route of administration (450). Even at the highest dose (4 mg), no inflammation accompanied the vascular growth. Systemic delivery of FGF by injection into the ipsilateral nonischemic forelimb did not induce vascular growth in ischemic or nonischemic muscle.

Other FGFs have been less commonly studied for therapeutic angiogenesis. FGF9 did not promote vascular growth when infused near the ischemic muscle of mouse hindlimb, but instead increased the fraction of vessels that were positive for smooth muscle actin (SMA) (150). This muscularization of microvessels was accompanied by improvement in limb function in 9 days and by full restoration of blood flow in the ischemic limb in 1 month.

VEGFs and PlGF Because FGFs induce proliferation of many other cell types besides ECs, it is possible that FGF-based therapies can induce undesired sequelae that are unrelated to the angiogenic effects. Indeed, they can accelerate the progression of atherosclerosis (127). The discovery of the VEGFs, a family of EC-specific growth factors, led to the hope that these side effects could be avoided with VEGF-based therapies. In the ischemic dog heart, intracoronary VEGF-A (presumably the 165 kDa isoform) led to increased blood flows 2 weeks after growth factor infusion and a near-doubling of arteriolar density, but no increase in capillary density, similar to the results for FGF2 by the same research group (36).

When administered systemically via the left atrium and for only 1 week after initiation of coronary arterial constriction, VEGF-A did not improve relative blood flows or collateral flow resistance over control (319); in contrast, 1-week administration of FGF2 led to increased blood flows and decreased collateral flow resistances. VEGF-A treatment led to acute hypotension, which resolved over a time-scale similar to the circulation half-life of ~50 min.

Sustained peri-myocardial delivery of VEGF-A_{165} (along with heparin) to the ischemic pig heart reduced the time delay for delivery of contrast agent to the ischemic region, compared to heparin-only controls (430); the time delay was confirmed to be inversely correlated with local blood flows based on microsphere deposition. VEGF treatment increased left ventricular ejection fractions and decreased the volume of infarcted and collateral-dependent regions. Peri-myocardial delivery also caused neovascularization primarily near the site of vascular occlusion (191); the induced collaterals were thin-walled with large lumens, a finding that resembles the enlarged, pericyte-poor "mother" vessels that have been found with VEGF treatment in other models (438).

In the ischemic rabbit hindlimb, intra-arterial VEGF-A_{165} led to increased blood pressure ratios, angiographic scores, and capillary densities (570). It resulted in a modest increase in resting blood flow to the ischemic limb, but a large increase in papaverine-induced maximum flow. The 30-day response of maximum flow rates to VEGF treatment was highest in animals that had the lowest maximum flow rates on day 0, which suggests that greater ischemia increases sensitivity to VEGF, most likely through an increase in expression of VEGFRs (195); a second VEGF injection or coinjection of heparin did not further increase the flow (43). VEGF-induced proliferation of ECs and SMCs localized largely in the "midzone" between the proximal and distal arteries by day 5 after injection (568), even though VEGF-A is not a direct growth factor for SMCs. The responses to VEGF depended on the administered dose, without any apparent limit in the increases in blood pressure ratios and angiographic scores; a large total dose of 1 mg did not cause muscle atrophy or skin necrosis (567).

Similar results have been found when VEGF was delivered intravenously (44).

VEGF-A has also shown therapeutic vascularization potential in other settings. In skin wounds in diabetic (*db*/*db*) mice, topical VEGF-A_{165} greatly increased the number of ECs in the wound, increased blood flow, and hastened epithelialization (159). Topical VEGF-A_{121} and VEGF-A_{165} enhanced formation of granulation tissue in normal and ischemic wounds in the rabbit ear (100); in contrast, FGF2 was unable to improve ischemic wound healing (402, 404). Injection of VEGF-A_{165} beneath an ischemic skin wound caused greater vascularization of the wound skin, compared to saline controls and to nonischemic wounds; it also hastened the recovery of tensile strength across the incision (670). Intra-arterial injection of VEGF-A to an ischemic rat skin flap increased capillary density, blood flow, and flap survival over saline control (427). In a model of bronchopulmonary dysplasia, intramuscular injections of VEGF-A_{165} in neonatal rats after exposure to hyperoxia increased lung capillary densities beyond those of untreated normoxic lungs; these changes were accompanied by preservation of normal lung alveolar size and number (307).

A systematic comparison of different forms of VEGF administration in the setting of a rat skin flap showed that all surviving regions of VEGF-treated flaps had similar degrees of vascularization that were greater than that of control flaps (302). Of the VEGF treatments, repeated intravenous dosing resulted in the highest flap survival, and topical application had the lowest. In a rat myocutaneous composite flap, preoperative subcutaneous injection of VEGF protected the flap from ischemic necrosis; once a flap had been raised, however, neither systemic nor local intra-arterial injection of VEGF improved flap survival (669). Preoperative VEGF caused vascular growth in the flap, compared to saline controls.

As with FGFs, VEGF-A can increase blood flow through mechanisms that are independent of angiogenesis, such as vasodilation. Studies to distinguish the contributions of angiogenesis and these other mechanisms toward improved tissue function are rare, and caution is warranted before claiming a causal link between vascular growth and improved tissue function.

VEGF-C, the other member of the VEGF family that has been extensively studied for therapeutic vascularization, induces lymphangiogenesis (242,421). For instance, intradermal VEGF-C caused the growth of lymphatics and increased tissue drainage in the edematous rabbit ear (557).

Although VEGF-C is often viewed as a mainly lymphangiogenic factor, it also promotes therapeutically relevant angiogenesis. In the ischemic rabbit hindlimb, intra-arterial VEGF-C had similar effects as VEGF-A_{165} did (639). Intradermal injection of VEGF-A or VEGF-C both led to local increases in vascular permeability that depended on nitric oxide production; the authors hinted that development of vascular permeability and angiogenesis may be linked.

Besides VEGF-A and -C, other members of the VEGF family have also been studied for their ability to promote therapeutic vascularization. Subcutaneous delivery of VEGF-B_{167} increased vascular density in mouse myocardial infarct (339). The VEGF-related proteins PlGF1 and PlGF2 both induced vascular growth in the ischemic mouse heart without causing edema or hypotension; PlGF2 induced arteriogenesis (the generation of collateral vessels) in the ischemic mouse hindlimb (354).

Other direct angiogenic factors EC migration and proliferation are early steps in angiogenesis, and the eventual maturation of newly formed, leaky capillaries into nonleaky microvessels requires interaction of mural cell-derived angiopoietin (specifically, Ang1) with the Tie2 receptor on ECs (114, 554). Because native Ang1 tends to oligomerize in solution and is difficult to purify in an active state, studies of recombinant Ang1 have typically modified the parent protein to promote stability. Modification of the amino terminus and mutation of Cys^{245} yields the variant Ang1* (developed by Regeneron Pharmaceuticals); replacement of the N-terminal portion with a short coiled-coil domain from cartilage oligomeric matrix protein yields the variant COMP-Ang1 (84).

Daily intravenous COMP-Ang1 for 1 week in the adult mouse resulted in reversible enlargement of tracheal microvessels, but not in angiogenesis (85). This finding is consistent with the developmental role of Ang1 in promoting vascular maturation rather than sprouting. Nevertheless, Ang1 derivatives (particularly, COMP-Ang1) have shown potential in generating new vessels in other settings. In a corneal pocket assay, COMP-Ang1 induced angiogenesis at a level similar to that achievable with VEGF, whereas native Ang1 had little effect (84). Compared to VEGF-induced vessels, the vessels that resulted from COMP-Ang1 were not leaky. When applied topically to healing wounds in diabetic (*db*/*db*) mice, COMP-Ang1 increased both blood and lymphatic vessel densities, increased blood flow to the wounds, and accelerated wound coverage (86).

The other major member of the angiopoietin family, Ang2, is believed to act primarily as a competitive inhibitor of Ang1-Tie2 binding, thereby delaying vascular maturation (157). As observed with Ang1, Ang2 did not promote angiogenesis in a corneal pocket assay (21). It is unclear whether intravenous or topical Ang2 (or its modified derivative COMP-Ang2) promote vascular growth, although results with adenoviral expression suggest that it would (271). Ang3 and Ang4 (mouse and human orthologs, respectively) promoted angiogenesis in a corneal pocket assay (324).

Intra-arterial hepatocyte growth factor (HGF), followed by intravenous HGF, in the ischemic rabbit hindlimb led to even greater increases in vascularization and blood flow than an equivalent dose of VEGF-A_{165} did (608). Since HGF induces the secretion of VEGF-A by SMCs *in vitro*, the observed angiogenic effects *in vivo* may result from

synergistic direct and indirect stimulation of ECs by HGF and HGF-induced VEGF, respectively.

Indirect factors In contrast to the FGFs and VEGFs, which act directly (but not necessarily solely, in the case of the FGFs) on ECs, a large number of angiogenic factors stimulate vascular growth mainly or only through paracrine mechanisms. Transforming growth factor β (TGFβ), tumor necrosis factor α (TNFα), and PDGF-BB are angiogenic when injected subcutaneously (61, 442, 470), but they do not stimulate EC proliferation directly. Instead, these growth factors recruit leukocytes and cause stromal fibroblasts to secrete extracellular matrix, which can result in formation of a neo-tissue that is similar to granulation tissue. Indeed, these growth factors are primarily known to enhance wound healing (280, 403), and at least one (PDGF-BB, as a topical gel) is already clinically approved for this application (632). Neo-tissue growth is invariably accompanied by vascular growth that is most likely stimulated by VEGF and other angiogenic factors that are elaborated by the recruited and/or activated nonendothelial cells. Along with indirect stimulation of angiogenesis, PDGF-BB can affect mural cell coverage of immature vessels (49). In contrast to FGF2, PDGF-BB increased granulation tissue in ischemic skin wounds (404). The less-studied PDGF-AB and -CC appear to act as indirect angiogenic factors in the aged myocardium and ischemic hindlimb, respectively (125, 338).

Combinations Whether direct or indirect, each angiogenic factor yields a distinct combination of vascular effects. It is natural to expect combinations of such factors to provide complementary improvements. Largely as a result of *in vitro* studies that provided evidence of synergy (434), initial application of a combinatorial strategy for therapeutic angiogenesis *in vivo* used two direct angiogenic factors, such as VEGF and FGF. Intra-arterial VEGF and/or FGF2 for rabbit hindlimb ischemia showed synergistic improvements in capillary density, arterial diameter, and blood pressure ratio (20). As mechanistic understanding of the angiogenic cascade improved, growth factor combinations were chosen to promote distinct steps in angiogenesis. In general, a direct angiogenic factor (typically, VEGF) is used to induce endothelial sprouting, while the second factor (e.g., PDGF) is intended to modify the vascular growth. For instance, the combination of VEGF-A_{165} and the maturation factor Ang1* yielded increased vascular densities and diameters over VEGF alone in a corneal pocket assay (21). In the ischemic rat and rabbit hindlimbs, coadministration of FGF2 and PDGF-BB led to synergistic increases in vascular density and blood flow (69). Combinatorial strategies have also shown that ligands that do not promote angiogenesis by themselves can show angiogenic activity when coadministered with direct angiogenic factors. For instance, Ang2 has no observable angiogenic effect by itself in a corneal pocket assay, but in combination with VEGF-A_{165}, it increased vessel lengths and densities (21).

A less common strategy to obtain complementary effects relies on the engineering of chimeric proteins, in which domains from two distinct ligands are combined to activate both of their respective receptors. Intramuscular injection of soluble VEGF-A/Ang1 chimera increased blood flow in the ischemic mouse hindlimb (15). Although such chimeras have shown promise and are perhaps more convenient for *in vivo* application (only one protein needs to be purified), they do not allow the relative levels of receptor activation to be tuned.

Hypoxia mimics Since hypoxia is the main physiological inducer of VEGF-A expression, pharmacological mimicry of hypoxia has been investigated for therapeutic angiogenesis. As a transcription factor, the hypoxia-inducible protein HIF1α would be difficult to deliver in a functional soluble form to an ischemic tissue. Instead, endogenous HIF1α is readily activated by inhibitors of prolyl hydroxylase domain (PHD) enzymes, such as cobalt ion and desferrioxamine (165, 167). Hypoxia mimics have been studied as potential inducers of vascular growth in the preterm neonatal lung, since normal vascular development in the lung occurs under hypoxic conditions. Intravenous delivery of a selective PHD inhibitor to preterm primates partially rescued the lung vascularization and VEGF deficit that is common in this population (27); similar results were found in mouse lung explants that were suffused with the less-specific inhibitor dimethyloxalylglycine (182). Nevertheless, these substances have not always led to expected results. For instance, cobalt and desferrioxamine both caused an increase in VEGF expression, but a *decrease* in vascularization, in developing mouse lungs (182). These discordant effects on vascularization, along with the ability of some hypoxia mimics to inhibit epithelial branching (182), warrants caution in the use of HIF-focused angiogenic strategies to treat bronchopulmonary dysplasia.

In the healing bone, local delivery of desferrioxamine increased vascular density and bone volume (623). In a model of progressive renal disease, cobalt administration preserved the density of glomerular capillaries and decreased the rarefaction of peritubular capillaries, in part through increased proliferation and decreased apoptosis of ECs; these vascular effects were accompanied by less tubulointerstitial injury (572). In the ischemic muscle, dimethyloxalylglycine increased vascular density (381).

Gene transfer

Injection of recombinant angiogenic growth factors has several disadvantages, including high cost and undesirable side effects, particularly when the factors are administered systemically. Moreover, plasma concentrations of VEGFs and FGFs spike immediately after injection and decay over a time-scale of ∼1 h to nearly background levels (319, 320); these large fluctuations in angiogenic signal are unlikely to be optimal for inducing the consistent and controlled growth of functional microvasculature. For these reasons, gene therapy of the same proteins has been envisioned as an alternative. In

effect, the release of proteins by transduced cells can provide a therapy equivalent to slow constant infusion of protein, thereby addressing the issues of cost and dose. Plasmids can be introduced as virus or as naked DNA; the route of administration can be systemic (e.g., intravenous) or local [e.g., intramuscular (640)]. All plasmids described below encode for human protein, unless otherwise noted.

FGFs Intramuscular viral delivery of mouse FGF2 in the ischemic mouse hindlimb led to increased blood flow ratios and limb salvage (366). The beneficial effects of FGF2 were mediated via induction of endogenous VEGF, even though overexpression of exogenous VEGF did not provide the same improvement in limb function. Angiogenesis was accompanied by arteriogenesis, which relied on a VEGF-independent pathway that required the chemokine MCP-1 (154). VEGF and MCP-1 were required for limb salvage, and both are secreted by SMCs and fibroblasts upon addition of FGF2.

Subcutaneous injection of plasmid DNA for FGF2 with a type I collagen carrier increased vascularity in ischemic rat myocutaneous composite flaps (204); this increase, however, was not matched by an increase in blood flow nor by increased flap survival. This result mirrors those observed with topical soluble FGF2 on ischemic skin wounds, in which enhanced vascularization was not accompanied by improved wound healing (402, 404). Electroporation of FGF2 plasmid into rat subcutaneous muscle, followed by elevation of an overlying skin flap, resulted in higher levels of distal vascularization, larger vascular densities, and greater flap viability (152).

Intramuscular delivery of adenoviral FGF4 into the ischemic rabbit hindlimb resulted in vascularization that was accompanied by edema, likely because FGF4 induces expression of VEGF (467). In a pig model of myocardial ischemia, intracoronary delivery of adenoviral FGF5 led to improved myocardial function and blood flow (164). Capillary-to-fiber ratios also increased with FGF5, albeit modestly (an increase of ~10% in the ischemic region, compared with control adenovirus). The difference in physiological and anatomical effects suggests that angiogenesis-independent effects of FGF5 may also be important.

VEGFs and PlGF The most thoroughly studied growth factors for angiogenic gene therapy are the VEGFs, particularly VEGF-A and -C. Balloon delivery of naked plasmid VEGF-A_{121}, VEGF-A_{165}, VEGF-A_{189}, or VEGF-C to rabbit iliac artery helped to revascularize ischemic hindlimb (569,639); intramuscular delivery was effective as well (595). In incisional skin wounds in normal and diabetic mice, intradermal injection of viral VEGF-A_{165} resulted in increased vascularity, thicker granulation tissue, and stronger wounds (158). In the hyperoxic lung, intratracheal delivery of adenoviral VEGF-A_{145} increased lung capillarity and ameliorated alveolar loss and enlargement (577).

The vascular effects of VEGF-B gene therapy have been less clear. Electroporated intramuscular plasmid VEGF-B_{167} and VEGF-B_{186} appeared to improve vascularization in mouse ischemic hindlimb through a VEGFR-1 and nitric oxide-dependent mechanism (528). A later comparison of intramuscular adenoviral VEGF-A_{165}, VEGF-B_{186}, VEGF-B_{167}, and PlGF2 showed benefit with VEGF-B_{186} (and to a lesser extent, VEGF-B_{167}) in pig myocardial infarct but not in rabbit ischemic hindlimb, possibly in part via indirect effects on cardiomyocytes and mural cells (312). VEGF-A_{165} was effective in both settings, while PlGF was effective in ischemic hindlimb only. Intravenous delivery of adenoviral VEGF-B_{167} increased vascular density in mouse myocardial infarct and border zone, but not in ischemic limb (339). Overall, it appears that VEGF-B promotes vascular growth selectively in the myocardium.

Naked plasmid VEGF-C induced lymphangiogenesis and reduced lymphedema severity in rabbit ear and mouse tail models; it did not increase angiogenesis (663). In contrast, VEGF-A_{165} induced angiogenesis but not lymphangiogenesis. This study found that it is important to introduce the VEGF therapy after an injury induces upregulation of VEGFRs, or the plasmid may not have much therapeutic effect.

A systematic comparison of adenoviral VEGFs (VEGF-A_{165}, VEGF-B_{167}, VEGF-C, the exclusively VEGFR-3 binding variant VEGF-C^{156S}, VEGF-D, and the mature form VEGF-$D^{\Delta N\Delta C}$) in rabbit ischemic hindlimb showed VEGF-A_{165} mainly induced angiogenesis (with a bit of lymphangiogenesis), VEGF-B_{167} had little effect, VEGF-C and -D induced lymphangiogenesis (with a bit of angiogenesis), VEGF-C^{156S} induced lymphangiogenesis only, and VEGF-$D^{\Delta N\Delta C}$ was equally good at both (468). Strongest angiogenesis and lymphangiogenesis were with VEGF-$D^{\Delta N\Delta C}$ and VEGF-D, respectively. Angiogenesis, but not lymphangiogenesis, depended on nitric oxide. VEGF-A_{165} and VEGF-$D^{\Delta N\Delta C}$ caused edema and induced arteriogenesis. In normal mouse tissues, adenoviral VEGF-A induced angiogenesis, arteriogenesis, and lymphangiogenesis (405, 406, 438). Although the newly formed lymphatics persisted, they did not clear interstitial carbon particles well (405, 406). VEGF-C or VEGF-$D^{\Delta N\Delta C}$ produced lymphatics that improved drainage to a transplanted lymph node (571), while adenoviral VEGF-C reestablished drainage across surgical wounds (476).

Since lymphatic vessels are critical to removal and presentation of antigen, induction of lymphangiogenesis may worsen chronic inflammation. Alternatively, lymphangiogenesis may alleviate inflammation, in part by reducing edema. Intra-articular injection of viral plasmid VEGF-C in a mouse model of chronic rheumatoid arthritis showed that the latter possibility was observed (680). Compared to controls, mice that were treated with VEGF-C plasmid demonstrated improved solute drainage, increased lymphatic vessel area, reduced synovial volume and cartilage erosion, and improved joint movement; no changes in blood vessel area were observed.

Intradermal injection of adenoviral mPlGF2 in the normal mouse ear led to long-lived (> 1 year) vascular growth without edema or hemorrhage (354).

Although gene delivery of VEGF-A showed promising results in initial studies, several potential issues have arisen (71, 134, 329). First, VEGF-A promotes hyperpermeability, which can lead to edema and compromise of the ischemic tissue. In the ischemic mouse hindlimb, intramuscular injection of viral VEGF-A_{165} plasmid led to decreased blood flow ratios and eventual autoamputation of the limb, compared with injection of control plasmid (366). Second, the dose of VEGF appears to play an important role in the organization of the resulting microvessels. High doses can lead to vascular malformations, such as glomeruloid bodies that resemble vascular growth in tumors (438). Third, even though VEGF-A gene therapy induces angiogenesis, the higher vessel densities have not always translated into higher blood flows and can paradoxically lead to lower flows, and the neovessels often have lower pericyte coverage, compared to those induced by FGFs (366). These drawbacks have led to a reappraisal of therapies that are based purely on VEGF-A, and suggest that perturbations that focus on angiogenic "master switches" may yield more consistent benefits.

In contrast to VEGF-A, VEGF-E binds selectively to VEGFR-2, which indicates that it may be suited for avoiding deleterious inflammatory side-effects of VEGF-A therapy that are mediated by VEGFR-1 on leukocytes. Because VEGF-E is derived from the Orf virus and hence immunogenic, studies of its effect have used plasmids that encode for chimeric proteins of VEGF-E and human PlGF (233). Intramuscular injection of plasmid VEGF-A and two plasmids for viral VEGF-E/human PlGF showed that all three enhanced blood flow ratios in the ischemic rat hindlimb, but only the VEGF-E plasmids did not recruit tissue macrophages. Moreover, only VEGF-A treatment increased the expression of inflammatory cytokines IL-8 and TNFα.

Other direct angiogenic factors As observed with topical delivery of the recombinant protein, intravenous delivery of adenoviral COMP-Ang1 improved wound healing in the normal (274) and diabetic mouse and increased blood and lymphatic vessel densities (86). Sustained expression over weeks resulted in permanent microvascular enlargement in the normal adult mouse trachea, in contrast to the reversible change that occurred with short-term administration of recombinant protein (85). In healing wounds, the increases in blood vessel density were sustained for at least 6 weeks after injection of vector; in contrast, lymphatic densities were only transiently elevated (274).

Although the variant Ang1* showed no angiogenic effect when applied in soluble form in the corneal pocket (21), its expression via intramuscular delivery of naked plasmid into the ischemic rabbit hindlimb resulted in increased vascularization and blood flow (521). Because soluble Ang1* did increase vascular densities when coadministered with soluble VEGF in the corneal pocket (21), the efficacy of Ang1* gene therapy in ischemic tissue can be rationalized to result from induction of endogenous VEGF by ischemia.

Gene therapy of the other angiopoietins has been tried with some success. Intramuscular delivery of naked Ang2 plasmid did not result in any vascular changes in the ischemic rabbit hindlimb (521). In mouse skin wounds, adenoviral Ang2, Ang3, and Ang4 all resulted in sustained modest increases in blood vessel densities and transient increases in lymphatic densities (274). COMP-Ang2, an oligomeric variant of Ang2, showed indistinguishable angiogenic effects from COMP-Ang1 in healing mouse skin (271). This last result suggests that activation of the Tie2 receptor through oligomerization is sufficient to promote vascular growth.

Liposomal delivery of HGF plasmid to infarcted rat heart resulted in increased vascularization and blood flow (16).

Indirect factors Subcutaneous injection of plasmid DNA for PDGF-BB with a type I collagen carrier increased vascularity and blood flow in ischemic rat myocutaneous composite flap and led to a greater area of surviving tissue (204).

Combinations Angiogenic gene therapy has also benefited from combinatorial approaches. Intramuscular codelivery of adenoviral VEGF-A_{165} and PDGF-B to rabbit ischemic hindlimb led to longer lasting improvements in perfusion, compared to delivery of VEGF or PDGF alone (299). Surprisingly, VEGF-A by itself led to increases in SMA-positive cells, while PDGF-B by itself had a modest effect; combination therapy resulted in loose pericyte coverage and more edema than VEGF-A alone. Codelivery of plasmid VEGF-A_{165} and Ang1 resulted in capillary densities that were higher than those achieved with delivery of plasmid VEGF-A alone (75). Codelivery of adenoviral COMP-Ang1 and Ang2 in mouse skin wounds resulted in an initially greater increase in blood vessel density (compared to COMP-Ang1 alone) but a smaller sustained increase (274). In the hyperoxic lung, codelivery of adenoviral VEGF-A_{145} and Ang1* resulted in capillary growth, but not lung edema; in contrast, VEGF therapy by itself yielded fenestrated, leaky capillaries (577).

Expression of chimeric proteins has shown that a single vector can be used to obtain a combination of vascular effects. Intramuscular delivery of viral VEGF-A/Ang1 chimera in normal mouse skeletal muscle resulted in increased vascular coverage and blood flow that were similar to those achieved with VEGF-A alone (15); expression of the chimera did not yield the vascular malformations that were found with VEGF-A. In the ischemic hindlimb, the chimera led to lower vascular permeability and less tissue necrosis than VEGF-A did.

Hypoxia mimics Naked plasmid delivery of constitutively active HIF1α in the ischemic rabbit hindlimb resulted in increases in vascularization and blood flow that were at least as large as those induced by VEGF-A_{165} gene therapy (613).

Clinical trials

Phase I trials of intramuscular or intra-arterial naked plasmid VEGF-A_{165} gene therapy in patients with critical limb

ischemia showed that the therapy was well tolerated, with mild edema in the ischemic limb as the main side effect (42, 234). As observed in animal studies, plasma concentrations of VEGF peaked 2 to 3 weeks after plasmid injection and then decreased to baseline. Ankle- and toe-brachial indices improved by 8 weeks, and limb salvage occurred in some patients. Results with soluble recombinant VEGF were suggestive of a dose-dependent effect (199). Similarly, promising results were found in patients with myocardial ischemia (352), and using other angiogenic factors (498, 500). Nevertheless, it has been challenging to obtain definitive proof of clinical efficacy versus placebo [reviewed in (187)].

No single cause of this discrepancy is apparent, and several possibilities have been suggested (311). First, some species (such as the dog) may be particularly well suited for angiogenic therapies because they have well-developed collateral circulation. Second, even with species (such as the pig) whose collateral architectures more closely resemble that of humans, animal studies of therapeutic angiogenesis almost always apply an ischemic insult to young, otherwise healthy animals. In the clinic, however, the patient population is typically elderly, with substantial comorbidities (e.g., diabetes, obesity, and hypercholesterolemia) that affect vascular response. Indeed, a comparison of topical TGFβ1 in rabbits of different ages showed that healing of ischemic wounds was negligible in old animals, even with application of growth factor (647); although this study did not analyze the effect of age on vascular growth, it suggests that responsiveness to growth factors may depend on patient age. In the ischemic hindlimbs of old rabbits, treatment with intra-arterial VEGF was roughly equivalent to sham treatment in young rabbits; endogenous expression of VEGF was lower in older mice, but VEGFR-2 expression did not depend on age (469). Third, the endpoints in clinical trials are different, and often more stringent, than those in preclinical studies. Clinical trials of critical limb ischemia typically seek a reduction in amputation rate; in contrast, preclinical studies generally accept an increase in vascular density and/or blood flow as proof of efficacy. While the clinical endpoints are more relevant to the patient, they can depend on factors that are independent of vascular growth *per se*. For instance, as previously noted, topical FGF2 increased vascular density in ischemic wounds but did not improve wound healing (404). Fourth, placebo effects are surprisingly strong, which has made detection of a true therapeutic effect by angiogenic therapy more difficult. It is unclear why placebo treatment yields strong and sustained clinical improvement (456). Some researchers have suggested that patient selection bias and subsequent regression to the mean can partly account for the placebo effect (474). It is also possible that social and psychological improvements that result from participating in a clinical trial lead to true improvement. Regardless of the underlying causes, such results emphasize the requirement for double-blind randomized studies with long follow-up to ascertain the efficacy of therapeutic angiogenesis. Currently, no treatment based on therapeutic angiogenesis for ischemic conditions is clinically approved.

While angiogenic therapies appear to be well tolerated, the long-term side effects are unknown. Angiogenic factors could potentially trigger an angiogenic switch in latent tumors or induce vascular growth in atheroma (133). It is disconcerting that the list of angiogenic substances overlaps substantially with that of atherogenic ones (135). Because effects on tumor growth or atherosclerosis may require years before they are clinically apparent, well-powered trials to detect such effects are extremely costly.

Controlled release of growth factors

Even when injected directly into ischemic tissue, angiogenic growth factors have a limited half-life on the order of a few days at best (319,320). Most of the injected material is quickly lost from the injection site, with typically <1% remaining after a few days (485); the growth factors are eventually cleared by the liver, kidneys, and lungs (320). As a result, therapeutic efficacy has required the application of large doses, typically well in excess of 100 μg for small-animal studies. Unfortunately, such large doses are accompanied by exposure outside the injection site, which can lead to undesirable side effects. Although the use of gene therapy has helped to prolong the time during which growth factor concentrations are in the desired range, even here the duration of plasmid expression is typically limited to a few weeks, particularly for viral delivery (636). The difficulty in maintaining a therapeutic concentration of growth factor at the desired site has been blamed for the modest clinical findings to date for angiogenic therapies.

To address these issues, numerous studies have examined the use of porous biomaterials to serve as carriers for the local, slow release of growth factors. As shown with FGF delivery to the heart, the use of carriers can increase the retention of injected material at the desired location by over two orders of magnitude (Table 1) (485). Moreover, carrier-mediated delivery allows for maintenance of spatial growth factor gradients, which can aid in the angiogenic response; superfusion with growth factor solution, which results in small or nonexistent concentration gradients, did not yield any change in vascular architecture in a rat skin chamber (432).

Table 1 Fraction of FGF2 That Was Retained at Myocardial Infarct 3 Days After Administration

Route of administration	Fraction retained (%)
Intravenous	Less than 0.1
Intracoronary	Less than 0.1
Intramyocardial	1.8 ± 0.9
Intramyocardial with controlled release	32.0 ± 5.2

Note. Unless noted, FGF was applied by injection from solution. Data are, with permission, from (485).

Inert carriers In the simplest formulations, the carriers are passive; that is, they do not interact specifically with the growth factors. For instance, suspending crosslinked agarose microspheres in a solution of FGF2 results in diffusion of FGF into the pores of the microspheres (40). When FGF-loaded microspheres were injected into a pig coronary artery, they occluded the distal microvessels and reduced blood flow, but they also eventually resulted in larger microvascular densities in the myocardium, compared to injection of unloaded (FGF-free) microspheres. The vascular improvement is presumably the result of growth factor diffusing out of the microspheres to induce angiogenesis in surrounding tissue. (The increase in vascular density was not accompanied by improvements in ventricular function, another example of the disconnect between vascular growth and functional outcome.) Application of FGF2 to the rat cerebral cortex in an agarose gel carrier caused vascular growth (106).

Other types of porous carriers, such as sponges or films, can also serve as reservoirs of growth factor. Sponges of polytetrafluoroethylene (PTFE) were able to deliver FGF1 to the dog myocardium, and caused hyperplasia of vascular smooth muscle in infarcted tissue, which led to near-obliteration of lumens (35). Gelfoam, a gelatin sponge, served as a carrier of FGF1, which increased the angiographic signal and survival of ischemic rabbit skin flaps (213). Delivery of VEGF or the indirect angiogenic factor TNFα from a Gelfoam implant increased vascularization of ischemic rabbit skin flaps, but only VEGF increased flap survival; direct application of VEGF to the wound bed (i.e., without Gelfoam) had no effect (546).

Porous carriers have also been used for the controlled release of plasmids that encode for angiogenic proteins. Subcutaneous Matrigel or collagen released adenoviral VEGF-A, FGF1, or FGF2 and caused vascularization of the surrounding tissue (396, 397, 597). The ability of alginate gels to entrap plasmid DNA enabled the release of plasmid to be coupled to gel degradation (297). Mixtures of alginates allowed plasmid to be released over 2 to 3 weeks, which led to increased vascular density in the ischemic mouse hindlimb compared to injection of naked plasmid. Subcutaneous PLGA sponges released plasmid for PDGF-BB over several weeks in the rat, which resulted in increased vascularity in the surrounding granulation tissue 2 to 4 weeks after implantation (506). Although naked plasmid is readily taken up and expressed in skeletal muscle (640), extended exposure to low levels of plasmid may be helpful in tissues that take up minimal amounts of plasmid naturally, such as skin.

Reactive carriers While materials that passively store growth factor within pores can be effective reservoirs, they provide little versatility in tailoring the release kinetics, because the only mechanism of release is diffusion. Essentially all subsequent studies in controlled release have examined the development of carriers that not only provide pores for the uptake of growth factors, but that also interact with the proteins to slow their release. Given that many angiogenic factors (particularly, the FGFs and VEGF-A) bind strongly to heparin, it has been natural to use heparin-conjugated materials as a carrier for these factors. As seen during heparin affinity purification, heparin-Sepharose beads can be loaded simply by immersing the beads in growth factor solution. When loaded with FGF2 and injected around an occluded coronary artery, such beads (embedded within an alginate binder) induced collateral growth in the pig heart, increased flow, and improved myocardial function (Fig. 5) (350).

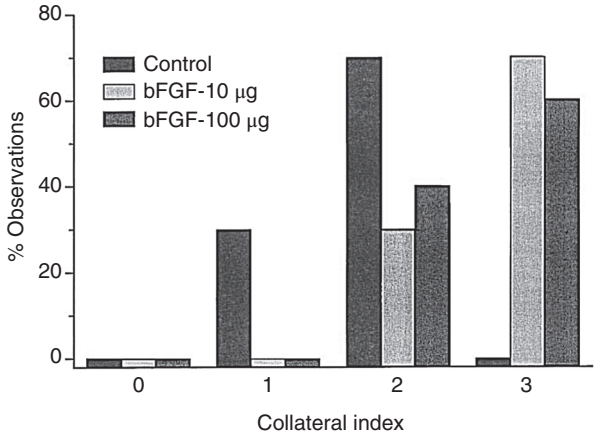

Figure 5 Dose-dependent collateral growth 2 months after implantation of FGF2-loaded heparin/alginate beads around occluded coronary arteries in the ischemic pig heart. bFGF, basic fibroblast growth factor (FGF2). Reproduced, with permission, from (350).

The highly anionic nature of heparin and the cationic properties of many angiogenic factors have motivated the use of other negatively charged biomaterials as carriers. In these materials, release of growth factors stems from diffusion, desorption from pores, and/or degradation of the carrier. An early example of anionic material for controlled release of angiogenic factors is sucralfate, a negatively charged derivative of sucrose; this material has been used extensively to test the angiogenic potential of compounds in the corneal pocket assay (111). Sucralfate is often delivered with the growth factor in a porous polymer; for instance, interposition of Gelfoam with FGF2 and sucralfate between an ischemic rat skin flap and the underlying wound bed resulted in increased numbers of collateral and SMA-positive vessels and increased flap survival (457).

Although Gelfoam itself appears to be near neutral at physiological pH and thus acts mostly as a passive carrier (188), charged gelatin scaffolds can be synthesized. Crosslinked acidic gelatin hydrogels released FGF2 in two stages: a quick release over a few hours, followed by gradual incomplete release over several days (558-560, 564). In contrast, basic gelatin gels, which do not bind FGF2 electrostatically, released the growth factor in a single quick bolus (559,560). The binding affinity of FGF2 to acidic gelatin was nearly a 1000-fold weaker than that to heparan sulfate (565). Nevertheless, release of the remaining FGF from acidic gels required degradation of the gels, a process that was faster in

gels with a smaller solid content. When implanted subcutaneously in mice, 15% gelatin gels degraded *in vivo* with a half-life of nearly 1 month and induced a sustained increase in vascular volume in surrounding tissue (560). Extremely dense gels (~35% gelatin) led to a smaller increase in vascular volume, which suggests that optimal FGF carriers should have intermediate degradation times and pore sizes (558). Consistent with an electrostatic mechanism for FGF sorption, incorporation of the anionic polymer carboxymethylcellulose caused gelatin microspheres to release FGF more slowly; the slower release was accompanied by a delayed, but more sustained, increase in vascular volume around subcutaneous implants (559). In the ischemic rat skin flap, administration of FGF2-loaded acidic gelatin microspheres to the underlying muscle at the distal end of the flap (where ischemia is most severe) led to dose-dependent generation of capillaries and SMA-positive vessels in the flap, which was accompanied by improved flap survival (153); application of soluble FGF without gelatin carriers had no benefit. Intra-arterial delivery of FGF2-loaded microspheres increased capillary densities in the ischemic hindlimb without compromising blood flow (216).

Alginate, a negatively charged polysaccharide, has also been heavily studied as a carrier for angiogenic factors. Growth factors are typically added to an alginate solution, which is subsequently gelled in the desired form by addition of calcium (435). Subcutaneous implants of alginate gel disks that contained VEGF-A or FGF2 demonstrated release over 2 weeks, and induced the formation of well-vascularized granulation tissue (327). The thickness of the vascularized region increased with VEGF dose, but vascular density did not.

Because alginates of different molecular weights and saccharide compositions are miscible and because they can be oxidized to hasten hydrolytic cleavage, they have been particularly suitable for generating customized carriers that release growth factor at defined rates. Mixtures of high- and low-molecular-weight oxidized alginates prolonged the release of VEGF-A to at least 1 month (524, 525). Intramuscular injections of VEGF-loaded alginate in the ischemic mouse hindlimb resulted in increased vascular densities that were independent of VEGF dose. Remarkably, the blood flow rate in the ischemic limb was restored to control (nonischemic limb) levels 1 month postimplant. Two injections at different locations were more effective than a single injection of the same total gel volume (524).

Collagen-based materials have also been used for controlled delivery of angiogenic factors, although growth factor uptake is likely not purely electrostatic. Subcutaneous implants of crosslinked type I collagen sponges released VEGF-A over a few weeks; the more heavily crosslinked the matrix, the slower the release (562). As observed with FGF-loaded gelatin gels, VEGF-loaded collagen increased the local vascular volume fraction, with the most crosslinked implants resulting in the highest and most sustained increases. FGF2-loaded collagen microspheres released slowly in the ischemic mouse hindlimb, which led to increased capillary densities and sustained rescue of the perfusion defect 1 month after implant (253); of note, injection of FGF solution without collagen had no vascular or physiological effect. When introduced with a collagen suspension in a rabbit ear ulcer model, only PDGF and TGFβ accelerated the formation of granulation tissue and the accompanying vascular ingrowth; treatment with FGF2 resulted only in surprisingly modest increases in vascularity (402), which contrasts with the strong effect observed when FGF2 was delivered in the same model without a collagen carrier (441). Instillation of FGF2-containing collagen underneath a rat skin flap led to much higher vascular density in the flap midsection, compared with instillation of collagen only; this treatment resulted in improved survival of ischemic flaps but no change in the ability of flaps to survive transfer (268). Tight binding of angiogenic factors to collagen and other matrix-based scaffolds can be achieved by engineering the growth factor to contain matrix-binding domains, such as that from PlGF2 (362). Retention of VEGF-A_{165} after injection in the ischemic rat heart was enhanced by using a VEGF that was coupled to a collagen-binding domain; the fusion protein caused greater capillary growth and cardiac function, compared to native VEGF (672).

Although the efficacy of matrix- and alginate-based delivery can be attributed to the ability of these materials to bind and slowly release growth factors, another possibility is that these materials protect the growth factors against degradation. For instance, VEGF-A that was released from alginate beads showed threefold to fivefold greater potency in an endothelial proliferation assay, compared to soluble VEGF that had not been exposed to alginate (435). Type I collagen protected FGF2 from tryptic digestion (253), and the ability of heparin to protect FGFs against denaturation is well-known (178).

Another large class of biomaterials that has proven effective as a vehicle for controlled release of angiogenic factors is synthetic biodegradable polymer, such as the copolymer of polylactic acid and polyglycolic acid (PLGA, copolymer of PLA and PGA). Because such polymers are typically not water soluble, special techniques are required to generate growth factor-loaded carriers. One approach is to emulsify aqueous growth factor solution with an organic polymer solution and then remove the organic solvent from the resulting water-oil-water double emulsions; the result are polymer microspheres that encapsulate growth factor (326). A second approach is to lyophilize growth factor solutions that contain a suspension of polymer particles. The latter approach usually adds a hydrophilic material, such as alginate or trehalose, as a cocarrier to stabilize the growth factor and promote its retention on the polymer surface (97, 436). Gas foaming of lyophilized materials with salt porogens and leaching of the salt is often used to generate pore space (192).

Regardless of the exact method used to load degradable polymers with angiogenic factors, numerous studies have shown the therapeutic efficacy of these materials. For instance, intracoronary infusion of poly(ethylene glycol-*co*-butylene terephthalate) microspheres that were loaded with

VEGF-A_{165} increased capillary density in ischemic pig heart, although they did not improve cardiac function (598). In the ischemic mouse hindlimb, PLGA scaffolds that were loaded with VEGF-A_{165} released the growth factor over several weeks (551). This slow, sustained delivery resulted in near-normalization of perfusion and a doubling of capillary densities in the underlying muscle. Subcutaneous injection of VEGF-loaded PLGA microspheres in an alginate gel carrier resulted in increased vascular densities, compared with alginate-free injection of VEGF-loaded microspheres or with injection of VEGF solution (326). Intramuscular injection of the same formulation in the ischemic mouse hindlimb yielded similar results (325).

A third major class of angiogenic biomaterials is based on the covalent coupling of angiogenic factors to a degradable matrix. This design is intended to mimic the ability of native extracellular matrix to serve as a reservoir of growth factors that are mobilized upon proteolysis of the matrix. For instance, transglutaminase can crosslink VEGF variants that contain a transglutaminase recognition sequence to a fibrin scaffold (128, 688). Because tethered VEGF can only be released by local proteolysis, the concentration of free VEGF around an implant is only elevated where cells are active. Subcutaneously implanted VEGF-coupled fibrin gels induced the growth of nonleaky vessels in the surrounding tissue (128). It has been suggested that a bolus of residual unconjugated VEGF could provide an initial chemotactic signal for vessels to sprout toward an implant (688). Implantation of fibrin gels that were covalently linked to VEGF-C with a protease-sensitive linker in a mouse skin wound resulted in increased retention of VEGF (compared to fibrin gels that contained free VEGF) and lymphatic growth, although fluid and solute drainage did not increase (184). Similar results have been obtained with growth factor-coupled, protease-sensitive synthetic hydrogels (439).

Transglutaminase-mediated coupling has been applied to load plasmids into fibrin gels (593). A short recombinant peptide served as a linker to fibrin and as a cationic agent for DNA condensation. Topical application of fibrin gels that were coupled to a plasmid for HIF1α increased vascularization of mouse skin wounds. Notably, the fraction of vessels that was SMA-positive was much higher in HIF-treated wounds than in VEGF-treated ones.

As with delivery of soluble angiogenic factors and their plasmids, controlled release has been applied to multiple growth factors in a single implant. Intramuscular injection of collagen microspheres that contained FGF2 and/or HGF in the ischemic mouse hindlimb showed that dual release led to synergistic increases in capillary density and mural cell coverage; bolus injection of the same factors had no effect, even at higher doses (363). Alginate hydrogels that were loaded with VEGF-A and PDGF-BB tended to release VEGF earlier and PDGF later (190). Intramyocardial injection of such gels to the ischemic rat heart resulted in elevated capillary densities that were similar to those obtained with injection of VEGF-loaded alginate, but in arteriolar densities that were higher than those obtained with injection of VEGF- or PDGF-only gel. In the ischemic mouse hindlimb, average vascular diameters were nearly twice as large when VEGF and PDGF were coreleased, compared to VEGF release only; corelease also led to a modest increase in hindlimb perfusion, compared to VEGF only (552). Codelivery of VEGF-A and Ang1 from a hybrid PLGA/alginate scaffold to the ischemic mouse hindlimb yielded equivalent capillary densities compared to delivery of VEGF or Ang1 alone, but skewed the distribution toward higher vascular diameters (514). Codelivery also led to a decrease in muscle fibrosis, equivalent to that achieved with VEGF alone and better than with Ang1 alone. Sustained delivery of two growth factors—VEGF-A_{165} to promote angiogenesis, insulin-like growth factor-1 (IGF1) to promote muscle regeneration—from intramuscular injection of loaded alginate gel resulted in fastest recovery from hindlimb ischemia in the mouse (56). Increases in vascular density depended only on the delivery of VEGF, and bolus coinjection of VEGF and IGF had no effect.

The most extreme example of multifactor controlled release may be the use of platelet-derived factors to promote angiogenesis. The α granules of platelets are a rich source of direct and indirect angiogenic factors, including VEGF, FGF2, IGF1, PDGF-BB, and TGFβ (193). Exposure of platelet-rich plasma to gelatin microspheres resulted in platelet degranulation and the release and entrapment of growth factors within the microspheres (52, 308). Intramuscular injection of such loaded microspheres in the ischemic rat hindlimb increased the angiographic scores and blood flow, compared to injection of platelet-rich or platelet-poor plasma without the carrier (308). Similar results were found in the ischemic hindlimb of diabetic mice, where controlled release of growth factors from platelet-rich plasma increased both total and SMA-positive vessel densities; in contrast, controlled release of factors from platelet-poor plasma had no effect (52).

Nanoparticles have also served as effective delivery vehicles. Because microvessels in ischemic tissues are hyperpermeable, intravenously injected VEGF-conjugated gold nanoparticles accumulated into the muscle of ischemic mouse hindlimb (272). Alternatively, nanoparticles could be injected directly into the muscle (176). The resulting delivery of VEGF resulted in increased vessel density and normalization of blood flow; intravenous injection of free VEGF had no effect. In a more elaborate approach, the receptor-binding portion of an angiogenic protein can be coupled to a long hydrophobic tail that directs the self-assembly of cylindrical nanofibers in aqueous solution (306, 627). The surface of these fibers displays a high density of receptor-binding epitopes, which should result in activation of growth factor receptors by oligomerization. Indeed, injection of VEGF-mimetic nanofibers in the ischemic mouse hindlimb led to increased capillary densities, perfusion ratios, motor function, and tissue viability, compared to injection of scrambled nanofibers or unassembled VEGF-derived peptide (627). VEGF-mimetic nanofibers persisted at the injection site 2 to 4 weeks after

delivery. Similar results were obtained with nanofibers that presented VEGF epitopes to promote angiogenic signaling and protease-sensitive sequences to allow degradation of the fiber (305).

Some extracellular matrix proteins promote angiogenesis independently of any added growth factors. The best-studied "angiomatrix" protein is Del-1, which promoted vascularization of the ischemic mouse hindlimb by activating α_V integrins on ECs (208, 679).

Cells as source of angiogenic factors

Leukocytes In the 1970s, Polverini, Clark, and their colleagues showed that stimulated macrophages and their conditioned media could induce angiogenesis in a corneal pocket assay (95, 445). At about the same time, Schaper showed that monocytes were often found in the process of extravasating across the endothelium during the initial stages of collateral development (491). These early studies led to the idea that macrophages may secrete growth factors that induce angiogenesis and arteriogenesis. Of the blood-derived cell populations, macrophages appear to be the most effective at promoting vascular growth, and of these, only activated macrophages can do so (553). The arteriogenic chemokine MCP-1 is induced by acute elevation of endothelial shear stress and recruits circulating monocytes, which presumably secrete endothelial and other growth factors upon extravasation (492, 522).

Activated macrophages secrete a large variety of substances that affect cell growth, such as the direct angiogenic factors VEGF-A and FGF2, other growth factors, and interleukins (553). Angiotropin, a unique macrophage-derived ribonucleopeptide, increased capillary density in the normal rabbit ear, stimulated fibrovascular growth into an artificial dermal graft, and rescued an ischemic rabbit skin flap from necrosis (209, 210). How much the beneficial effect stems from direct activation of ECs versus indirect activation of neighboring mesenchymal cells, such as fibroblasts and SMCs, is unclear.

Other subsets of inflammatory cells may be angiogenic. The neutrophil chemoattractant f-Met-Leu-Phe (fMLP) is angiogenic in the corneal pocket assay, but not on the chick chorioallantoic membrane, which suggests that it acts indirectly through leukocyte recruitment (367). Whether neutrophils are the primary mediator of the vascular growth by fMLP is unclear. $CD31^+$ T cells have been reported to be angiogenic (225).

Mesenchymal cells Noninflammatory mesenchymal cells, particularly bone marrow-derived mesenchymal stem cells, have also been examined for their ability to promote angiogenesis. These effects, and the ability of such cells to form vasculature, are discussed in the section on vasculogenic approaches. Other types of mesenchymal cells, such as SMCs, fibroblasts, and cardiomyocytes, induced angiogenesis when transplanted into ischemic heart, albeit modestly (337, 657). Mouse preadipocytes induced angiogenesis in a skinfold chamber (156).

Transduced cells Given the effectiveness of angiogenic gene therapy in animal models of tissue ischemia, introduction of transduced cells has been explored. Intramyocardial injection of rat cardiomyocytes that were transfected with VEGF-A_{165} in the ischemic rat heart increased capillary densities and blood flow (657). Coimplantation of VEGF-transduced adipose-derived stem cells with free fat grafts generated twice the capillary density of control (nontransduced) implants and increased graft survival (353). Implants of FGF2-transduced myoblasts, but not VEGF-transduced ones, increased vascularization of ischemic skin flaps (464). In principle, injection of cells rather than plasmids allows for the preselection of cell populations that express the angiogenic gene(s) at the desired levels. By comparison, *in vivo* release of plasmids leads to uncontrolled uptake; some cells may contain many copies of the plasmid, while others may have a few or none.

A preselection strategy has been used to analyze the role of local growth factor levels on vascular growth (426, 619). Polyclonal populations of myoblasts that were transduced with retroviral mouse VEGF-A_{164} secreted VEGF at a broad range of rates per cell (426). Intramuscular implants of polyclonal populations led to formation of hemangiomas. Implants of myoblast clones that secreted VEGF at the same rate as the average from a polyclonal mixture resulted in vascular growth without hemangiomas. This result suggested that the local VEGF distribution controls the architecture of the induced vascular growth. Monoclonal implants avoid "hotspots" of high VEGF expression that are found with polyclonal implants, and can thus reduce the development of hemangiomas. The capillaries that developed from monoclonal implants that secreted VEGF at a low rate were resistant to VEGF deprivation. Similar results were found in the ischemic mouse hindlimb (619).

Use of bicistronic vectors enabled implantation of myoblasts that expressed mouse VEGF-A_{164} and human PDGF-BB at defined ratios (37). Remarkably, when VEGF and PDGF were expressed from the same vector, even a polyclonal implant resulted in hemangioma-free vascularization, despite the delivery of some cells that expressed high levels of VEGF (and PDGF). In contrast, coinjection of two separate polyclonal populations of myoblasts—one that secreted VEGF only, the other that secreted PDGF only—led to formation of hemangiomas. These results implied the local balance of VEGF and PDGF secretion is what determines whether a normal capillary bed forms. Findings from cell implants were confirmed with adenoviral delivery of the same bicistronic vector to the ischemic mouse hindlimb, which led to increased vessel densities and blood flow and decreased muscle damage without the development of hemangiomas; as expected with PDGF expression, newly formed capillaries were well-covered by pericytes. These results contrast with the current view that sequential, rather than simultaneous, presentation

of VEGF and PDGF is needed for the formation of mature microvessels.

Myoblasts that overexpressed mouse VEGF-B_{167} were claimed to induce vessel growth in mouse myocardial infarct, but not in ischemic hindlimb (339).

Other inducers of angiogenesis

Heparin Compared with growth factors, heparin is modestly angiogenic at best. Intracoronary infusion of heparin in the ischemic dog heart led to increased collateral flow between an implanted artery and the myocardial circulation (603). Strangely, the total collateral flow did not change, which would imply that heparin reduced the development of collaterals that did not originate from the implanted artery. Pericardial injection of heparin did not improve collateral indices in the ischemic pig heart (310). Likewise, heparan sulfate did not improve vascular densities in the ischemic dog heart, although it did enhance the effect of FGF2 (596).

Notch inhibitors The Notch pathway modulates the emergence of "tip cells" at the forefront of nascent vascular sprouts (197); inhibitors of this pathway should promote angiogenesis. Indeed, the indirect Notch inhibitor DAPT increased the angiogenic response when coreleased with VEGF-A from an alginate gel to the ischemic mouse hindlimb (67,68). The highest levels of DAPT caused the highest capillary densities, but only weak improvements in blood flow. In the ischemic hindlimb of diabetic mice, sequential delivery of VEGF-A, DAPT, and PDGF-BB led to increased capillary and SMA-positive vascular densities, but did not show added benefit over delivery of VEGF and PDGF (67). Intradermal injection of viral plasmid for soluble Dll4, which serves as a competitive Notch inhibitor, resulted in lymphangiogenesis in the mouse ear; this effect synergized with that of VEGF-A gene therapy (676).

Lipids and related substances Early work by Goldsmith showed that omentum contained angiogenic activity in a chloroform-methanol lipid extract (175). This lipid fraction improved perfusion in ischemic cat hindlimb, regardless of whether intramuscular injection occurred locally or in a distant limb (174). Constant infusion of lipid fraction into demineralized bone allograft in a rat femoral defect led to vascular invasion throughout the entire graft by 6 weeks; blood flow also increased, but eventually returned to the level of controls after the lipid infusion was discontinued (417). Omental extract consists of a complex mixture of glycerides, glycolipids, cholesterol, and free fatty acids (368); the exact identity of the angiogenic lipid(s) in omentum remains unknown.

Other lipid-like molecules are angiogenic as well. Sphingosine 1-phosphate, a major lipid of platelets, induced vascular growth and recovery of blood flow when injected into ischemic mouse hindlimb (425). Retinoic acid induced lymphangiogenesis and restored drainage in a mouse tail lymphedema model (87). Slow release of prostaglandin E_1 from polymer microspheres into the ischemic hindlimb increased vascular density in normal and diabetic mice (138,220). Small doses of leukotriene B_4 promoted lymphangiogenesis in the edematous mouse tail, while large doses inhibited it (582).

Small organic molecules As described earlier for chemical mimics of hypoxia, low-molecular-weight organic inhibitors and promoters of signaling pathways can be used to induce angiogenesis (499). Compared to growth factors, these small molecules are easier to synthesize and purify, but may be more difficult to deliver in a sustained fashion. Combinatorial screening of chemical libraries has been used to isolate small angiogenic molecules, such as phthalimide neovascular factor 1 (molecular weight 229 Da) for stimulation of rat mesentery microvasculature (631). A potential advantage of such compounds is that they may be easier to incorporate into polymeric scaffolds, since they cannot denature and are generally nonpolar; it remains to be seen to what extent such compounds can successfully promote therapeutic angiogenesis in ischemic tissues.

Transition metal ions Divalent cations can induce angiogenesis, through mechanisms that are not entirely clear. The species that has been most explored for engineering microvessels is Co^{2+}, which can mimic the response to hypoxia (624). Controlled release of cobalt has been obtained with coated glass fibers, although the angiogenic efficacy of these materials *in vivo* is unclear (645). Other angiogenic cations, such as Cu^{2+} (and the copper-carrying protein ceruloplasmin), appear to act mainly through recruitment of inflammatory cells, since corticosteroids inhibit the angiogenic effect (367,683).

Oxygen and nitric oxide Given the importance of hypoxia in inducing the expression of VEGF, it is counterintuitive that oxygen can *promote* angiogenesis (575). The inspiration of 100% O_2 at hyperbaric pressures of 2.5 atm accelerated vascularization of elevated rat skin flaps, which was accompanied by higher tensile strength at the flap margins (373) and by less distal necrosis (359). Similarly, 100% O_2 at 2.4 atm caused much greater vascular growth in irradiated bone and its surrounding soft tissue in the rabbit, compared to normobaric air (20% O_2 at 1 atm) (365). In contrast, normobaric oxygen (100% O_2 at 1 atm) yielded no change in vascular density over normobaric air.

Hyperbaric oxygen therapy works synergistically with application of soluble angiogenic factors. In ischemic rabbit skin ulcers, the combination of 100% O_2 at 2 or 3 atm and topical PDGF-BB or TGFβ was able to increase vascularization and wound healing to levels seen in nonischemic wounds (675). No added benefit was found in using O_2 at 3 atm instead of 2 atm; 100% O_2 at 1 atm had no effect over control. In ischemic myocutaneous rat flaps, the combination of 100% O_2 at 2.5 atm and intra-arterial FGF2 led to greater distal vascular densities, perfusion, and flap survival than either O_2 or FGF delivery alone did (46).

In the ischemic mouse hindlimb, hyperbaric oxygen suppressed VEGF expression, but caused 2- and 20-fold increases in FGF2 and HGF expression, respectively (25). It appears that the net balance of the oxygen therapy is to increase the expression of angiogenic factors that overcompensate for the loss of VEGF. Oxygen therapy only showed these effects in the presence of existing tissue ischemia. Hyperbaric oxygen also increased the expression of the FGF2 receptor in ischemic hindlimb. It is important to recognize that the hyperbaric oxygen therapy is intermittent rather than continuous, and the duration of exposure is actually quite limited, on the order of 1 to 2 h per day (365, 373). Some have hypothesized that the intermittency of hyperbaric exposure results in switching between hyperoxic and hypoxic conditions in the tissue, which may be important for the observed angiogenic effects (25).

The vasodilator nitric oxide mediates the angiogenic effect of VEGF but not FGF2 (155,400,684). Exogenous delivery of nitric oxide is typically achieved through systemic injection of a precursor compound, such as L-arginine. It remains to be seen whether site-specific controlled release of nitric oxide is a feasible approach to induce vascularization.

Angiogenesis to engineer vascularized tissues

The ability of angiogenic growth factors to promote vascular growth near the site of release led to the idea that those same factors could be used to promote angiogenesis into a newly formed tissue. While it is possible to induce the formation of new vascularized tissue by injection of a vulnerary growth factor (470), by sequential layering of thin tissues (512), or even by deliberate localized injury (433), nearly all studies that use angiogenesis to engineer vascularized tissues have relied on porous scaffolds to serve as templates in which the new tissue forms and into which new vessels extend (60). Many of the scaffolds that have been used for controlled release of growth factors in therapeutic angiogenesis have been repurposed for slow, controlled vascularization of the scaffolds.

Scaffolds that promote angiogenesis

Growth factor-free scaffolds By itself, implantation of a porous scaffold is often sufficient to induce eventual vascular ingrowth. For instance, fibrovascular tissue grew within 1 to 4 weeks into the pores of subcutaneous polyvinyl alcohol sponges (PVA; pore sizes of 60-350 μm) and mesenteric polylactic acid membranes (PLA; pore sizes of 90-500 μm) (504, 563, 621). Transcutaneous implants of polyhydroxyethylmethacrylate (polyHEMA; pore size of ~40 μm) in the pig also led to vascular ingrowth (638). The consensus among studies with growth factor-free scaffolds is that an optimal pore size—not too large, not too small—exists for vascularization. In templated polyHEMA gels, which can be synthesized with nearly uniform pores, vascular density within the scaffold at 1-month postimplant was the greatest at pore sizes

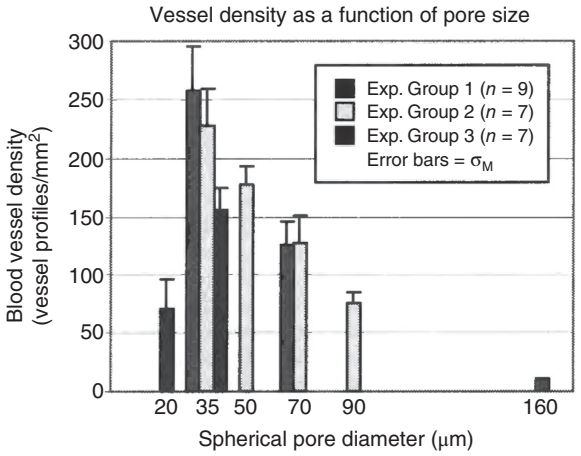

Figure 6 Density of vascular ingrowth into polyHEMA implants after 1 month. Reproduced, with permission, from (361).

of ~35 μm (Fig. 6) (361); surprisingly, vascularization did not depend on whether the scaffold was doped with collagen. For pore sizes of ~35 μm or less, vascular densities varied spatially, with largest densities at the surface of a scaffold and smallest densities at the core. Below a pore size of ~10 μm, essentially no vascular ingrowth occurred with PVA, cellulose, PTFE, and acrylic copolymer scaffolds (57,505). Similar results were found in sponges of type I collagen and chondroitin sulfate, for which pore sizes of 20 to 100 μm resulted in the fastest wound healing, and presumably in fastest vascularization as well (653). In hydroxyapatite and porous polyethylene implants, fibrovascular tissue grew fastest in scaffolds with pore sizes on the order of 400 μm (473). In micropatterned collagen implants, in which the pore size and geometry was controlled, vascular ingrowth was highest in scaffolds with 100-μm-wide pores (678). Surprisingly, prefilling the pores with a dilute collagen gel did not inhibit vascularization. In subcutaneous and intraperitoneal micropatterned poly(glycerol sebacate) implants, vascularization also occurred into 100-μm-wide pores (658).

The main stimulus for vascular ingrowth is most likely the influx of inflammatory cells that serves as the first stage of the foreign-body response. In scaffolds that permit vascularization, host inflammatory cells are often found within the scaffold pores, prior to vascular ingrowth (57). If the pores are too small to permit entry of leukocytes, then vascularization is invariably absent. With pore sizes of 1 to 10 μm, leukocytes penetrate into the scaffold even though vessels do not; it appears that leukocyte entry is necessary, but not sufficient, for vascularization. Not all inflammation is created equal, however: inflammation that accompanies infection of an implant can reduce vascularization (473).

A related approach is the use of lasers or needles to drill channels directly into an ischemic tissue; here, the tissue functions as the equivalent of a porous scaffold. Known as "transmyocardial revascularization" when applied to the myocardium, this technique is intended to provide channels for vascular ingrowth. Although some have found that lasing

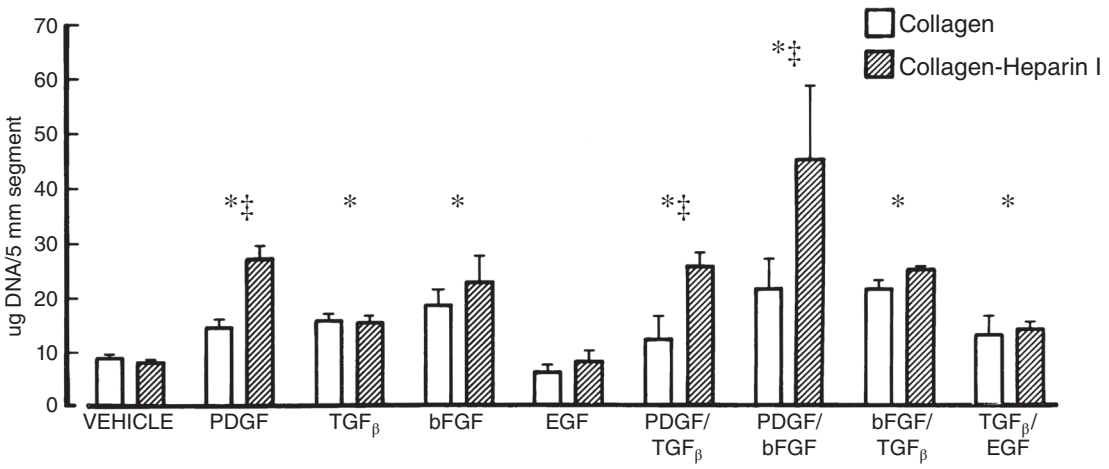

Figure 7 Cellularity of fibrovascular ingrowth into porous PTFE chambers in the presence of various growth factors after 10 days. Reproduced, with permission, from (541).

induced angiogenesis into the channels (120), the mechanisms and outcome remain controversial, as laser damage undoubtedly results in inflammation and a wound healing response [reviewed in (59)]. In particular, it is debatable whether laser-induced channels remain patent or are instead replaced by scar (91, 357).

Scaffolds for controlled release Although growth factor-free scaffolds can induce vascular ingrowth, the preclinical success of growth factor-based therapeutic angiogenesis naturally suggested that vascular ingrowth could be augmented if scaffolds released angiogenic factors. As seen with injection of vulnerary growth factors (PDGF, TGFβ, and FGF2), the controlled release of the same factors from subcutaneous porous chambers resulted in the formation of granulation tissue, but now within the pores of the chambers (Fig. 7) (541). Leukocytes and fibroblasts accumulated in the tissue ingrowth, and were accompanied by increases in capillary density, particularly when FGF was the released factor. FGF-releasing PVA sponges promoted more fibrovascular ingrowth into the scaffold pores than FGF-free implants did (563). Codelivery of FGF2 and FGF9 from subcutaneous Matrigel scaffolds synergistically increased both total and SMA-positive vascular ingrowth (150).

Similarly, controlled release of VEGF increases vascular ingrowth. VEGF-releasing polymer matrices promoted vascular formation within subcutaneous implants in the mouse (436). PLGA scaffolds that were loaded with VEGF-A_{165} were invaded by threefold to fivefold more capillaries than unloaded (VEGF-free) scaffolds were (131, 551). Remarkably, the density of SMA-positive vessels that developed within VEGF-releasing scaffolds not only exceeded that in unloaded scaffolds, but also that in the surrounding muscle (551). Nevertheless, the total vascular densities in the scaffolds were roughly an order of magnitude lower than those in the surrounding muscle (~100/mm^2 vs. ~800/mm^2). The host response to sustained VEGF release from PLGA scaffolds depended on the implantation site and host immune competence (81). Subcutaneous Matrigel plugs that released VEGF-A_{165} or VEGF-B_{167} promoted vascularization of the gel (528). The result with VEGF-B is surprising, given that VEGF-B gene delivery has shown weak therapeutic effect (468); the presence of Matrigel may enhance the vascularization potential of VEGF-B, since Matrigel contains many angiogenic growth factors, including FGF2, PDGF, and TGFβ (283).

Scaffolds that incorporate heparin or heparan sulfate have been used to release heparin-binding growth factors, such as FGF2 and VEGF. Immersion of collagen-heparan sulfate matrices in a solution of rat FGF2 led to binding of the growth factor, which was slowly released over several weeks (440). Subcutaneous implants of collagen-heparan sulfate-FGF resulted in greater vascular ingrowth than implants without either FGF or heparan sulfate did. Similarly, collagen-heparin scaffolds can be loaded with VEGF by immersion in VEGF solution, and these implants led to greater vascular ingrowth than growth factor-free implants did (544). The incorporation of heparin by itself also led to greater vascularization of collagen scaffolds, perhaps by potentiating the effect of endogenous angiogenic factors. Complexation of anionic dextran sulfate with VEGF-A enabled loading and slow release from subcutaneous Matrigel and intraperitoneal PLGA implants, which yielded greater vascularization than uncomplexed VEGF did (117).

Angiogenic factors can be covalently coupled to scaffolds for subsequent release. Fibrin scaffolds in which VEGF-A_{121} was covalently coupled were invaded by a higher density of vessels than scaffolds with passively adsorbed VEGF were (129). Of note, all vascular densities returned to control levels (i.e., those of VEGF-free fibrin implants) after 6 weeks. Fibrin that was coupled to mouse $VEGF_{164}$ and the protease inhibitor aprotinin to slow scaffold degradation led to stable vascularization after 3 months (480). Subcutaneous implants

of polyurethane scaffolds that contained a protease-sensitive polymer gel to which VEGF-A was covalently coupled were vascularized after 2 weeks (687).

Multi-step polymer processing provides the ability to deliver multiple growth factors sequentially. Preencapsulation of PDGF-BB in PLGA microspheres, followed by immobilization of the microspheres with VEGF-A_{165}, alginate, and more PLGA, generated scaffolds that released PDGF more slowly than VEGF (461). Sequential delivery of VEGF and PDGF resulted in the largest vascular density in the scaffold at 4 weeks postimplant, compared to sustained delivery of VEGF or PDGF only. In particular, sequential delivery yielded a density of SMA-positive microvessels that was nearly triple that achieved with single-factor delivery. In a note of caution, sequential delivery also resulted in the formation of multilayered and sinusoidal vessels. Scaffolds in which VEGF or PDGF were injected did not yield a sustained increase in vascular density.

The relative timing of signals that initiate angiogenesis versus those that mature newly formed capillaries is important in the final vascular outcome. PLGA scaffolds that released VEGF-A and Ang2 first, followed by PDGF-BB and Ang1, were invaded by greater densities of microvessels than scaffolds that released all four growth factors simultaneously were (62). Delayed release of PDGF and/or Ang1 yielded increased densities of SMA-positive vessels, compared to rapid release of VEGF and Ang2 only.

Sequential delivery of growth factors has also been achieved by using PLGA scaffolds that consist of spatially distinct VEGF- and PDGF-loaded layers (80). In the ischemic mouse hindlimb, scaffolds were invaded by higher densities of SMA-positive microvessels in the layers that contained PDGF. Surprisingly, PDGF-releasing scaffolds had larger SMA-positive microvessels, even in layers that did not contain PDGF. In principle, heterogeneous localization of VEGF and PDGF within a scaffold could be used to tailor the microvascular networks that develop in different regions of the scaffold. A similar strategy, using spatially distinct layers that released VEGF and antibody to VEGF, enabled the generation of pro- and antiangiogenic regions in an implanted PLGA scaffold and in the surrounding muscle, which yielded tissues of higher and lower vascular densities (666).

Despite the advantage of sustained delivery over bolus injection of angiogenic factors, the durability of the induced microvessels remains an issue. For instance, despite continuous pumping of VEGF-A, FGF2, and/or PDGF into subcutaneously implanted PVA sponges for over 1 month, the vascular density in these scaffolds peaked around 3 weeks postimplant and then decreased back to control levels at 5 weeks postimplant (177). Continuous pumping of VEGF-A_{165} into subcutaneous implants of porous polyurethane for 6 weeks showed that delivery of 150 ng/day led to an increase in vascular density over control (no VEGF) implants at 4 months (113).

Other types of substances have been released from scaffolds to promote vascular ingrowth. Slow release of adenoviral PDGF-B, FGF2, or VEGF plasmid from collagen gels in a subcutaneous PVA sponge yielded fibrovascular ingrowth, with less matrix in implants that released FGF or VEGF plasmid (121). When released from a Matrigel implant, sphingosine 1-phosphate synergized with VEGF or FGF2 to induce vascular ingrowth; by itself, sphingosine 1-phosphate did not cause vascularization (328).

Cobalt has been incorporated into glass scaffolds for bone tissue engineering, with the idea that the HIF-activating ion would be slowly leached from the scaffolds to induce angiogenesis (645). A low level (2%) of cobalt increased expression of HIF1α and secretion of VEGF by bone marrow-derived stromal cells that were cultured on the scaffolds. Although a higher level (5%) of cobalt also increased HIF1α expression, it decreased cell proliferation and did not increase VEGF secretion. Whether release of cobalt can be used to increase vascular ingrowth into bioactive glass, which serves as an osteoconductive scaffold for bone engineering, is unclear. Other activators of HIF1α induced vascular ingrowth into subcutaneous Matrigel or polyurethane sponge implants (335, 626).

Cells as source of angiogenic factors

Cells that secrete angiogenic factors, or that induce neighboring cells to do so, have been used to promote angiogenesis within scaffolds. For instance, scaffolds that contained bone marrow-derived stromal cells that were treated with cobalt (to activate the HIF pathway) resulted in greater vascular densities in subcutaneous and skull defect implants, although the amount of bone that formed did not increase (139). Subcutaneous implants of Matrigel that contained FGF2-transfected fibroblasts displayed vascular ingrowth after a week (597).

The binding properties of a scaffold can tailor the response to implantation of growth factor-expressing cells. Because type I collagen binds poorly to VEGF-A, collagen sponges served as a "buffer" to blur spatial variations in growth factor secretion from VEGF-transduced adipose stem cells within the sponge (162). In contrast, ovalbumin scaffolds (which bind strongly to VEGF) limited the diffusion of VEGF; although this property caused greater amounts of VEGF to be trapped in ovalbumin scaffolds, the VEGF distribution was expected to be more heterogeneous than in collagen scaffolds. Indeed, abnormally large vascular structures were found to grow into subcutaneous ovalbumin, but not collagen, scaffolds that contained VEGF-transduced cells. If the cells were preselected to cull the ones that expressed VEGF at high levels before incorporation into ovalbumin scaffolds, then the implants yielded vessels of normal size distribution.

Cells can also promote angiogenesis by remodeling the scaffold. In Matrigel implants, recruited macrophages appeared to digest "tunnels" that served as routes for ingrowth of fibrovascular bundles (13, 14), while pericytes formed networks that appeared to facilitate ingrowth of vessels (585).

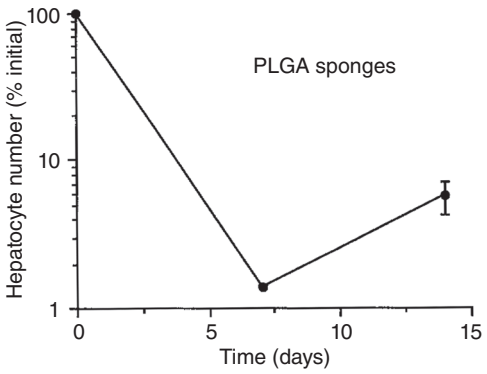

Figure 8 Number of viable hepatocytes in PLGA sponge implants as a function of time. Hepatocytes were added directly to scaffolds before implantation; scaffolds were not prevascularized. Reproduced, with permission, from (388).

Tissues in angiogenic scaffolds

Angiogenic ingrowth into scaffolds is intended to provide the vascular support needed to sustain the metabolism of living tissues. Although vessels do invade growth factor-free scaffolds, this ingrowth is generally insufficient to engineer tissues of physiological cellularity. For instance, hepatocytes that were transplanted in PLGA or PLA sponges to the rat mesentery displayed very poor long-term viability, with <10% of the initial grafted cells surviving at 2 weeks postimplant (Fig. 8) (388). The surviving cells were clustered near the surface of the implants, where vascular densities were highest. When hepatocytes were transplanted in PLGA scaffolds that contained VEGF-A, the 7-day surviving fraction was double that for VEGF-free implants (535). Transplantation of hepatocytes in PLA discs that released FGF2 led to twice the level of cell engraftment as seen in FGF-free implants (323).

Although these results show the potential of growth factor-loaded scaffolds to increase the viability of engineered tissues, it must be noted that the surviving cell fractions were still quite low. For highly metabolic cells such as hepatocytes, the degree and rate of vascular ingrowth—even when enhanced with angiogenic factors—may not be sufficient to rescue most of the implanted cells from ischemic damage.

These limitations suggested that scaffolds should be "prevascularized" by prior implantation before adding cells. Subcutaneous or mesenteric implantation of PVA sponges in the rat allowed vascular ingrowth to take place for 5 to 7 days before grafting of hepatocytes (605). Approximately 10% to 20% of grafted cells remained viable after 7 days. A subsequent study implanted PVA sponges, with and without FGF2, to the rat mesentery for 7-day prevascularization before grafting of hepatocytes (420). Long-term cell viability was 5% to 7% in FGF-loaded scaffolds, and <1% in control scaffolds. Prevascularization of myocardium with FGF2 enabled greater survival of injected cardiomyocytes (484). Nevertheless, it appears that grafting cells into scaffolds leads to modest cell viabilities, regardless of whether implants are prevascularized or not.

More promising results have been obtained by allowing the scaffolds to promote vascular and tissue ingrowth, such as for bone engineering. Coating of PLGA scaffolds with ceramic powder (Bioglass) made the scaffolds osteoconductive and osteoinductive (322). Implantation of Bioglass-coated scaffolds in a critical-sized rat skull defect led to generation of mineralized tissue after 3 months. Implants of scaffolds that were loaded with VEGF-A_{165} before Bioglass coating generated similar volumes of bony tissue, but with double the vascular density. In this case, vascularization did not augment tissue formation, probably because the scaffolds did not contain cells during implantation (in contrast to the hepatocyte-loaded scaffolds described above). Slow delivery of mouse VEGF from a PLA carrier into a femoral bone fracture induced vascularization of the surrounding soft tissue, but not existing bone; nevertheless, VEGF delivery enhanced repair of the fracture (550). Codelivery of VEGF, the osteoinductive factor BMP4, and mesenchymal stem cells (MSCs) in PLGA sponges yielded the greatest level of bone formation in subcutaneous implants (219). Delivery of VEGF only or VEGF and MSCs led to ingrowth of fibrovascular tissue instead. Similarly, sequential delivery of VEGF and BMP2 led to formation of vascularized bone in rat subcutaneous implant and femoral defect models, with VEGF and BMP2 having largely independent effects on vascularization and osteogenesis, respectively (263). On the other hand, in irradiated cranial bone defects, delivery of VEGF increased sustained vascularization of PLGA scaffolds and led to increased scaffold mineralization, compared to VEGF-free implants (248). Whether VEGF is sufficient for mineralization appears to depend on the bone model used.

Subcutaneous injection of Matrigel with FGF1 or FGF2 led to vascularization of the injection site in 1 week (429), with subsequent conversion of FGF2-releasing implants into adipose tissue over the next several weeks (257). PDGF exerted similar effects on vascularization and adipose formation, when injected with Matrigel. When FGF2 was first sorbed into gelatin microspheres before incorporation into Matrigel, subcutaneous injection of the Matrigel/gelatin/FGF mixture resulted in even more robust adipose generation than injection of Matrigel/FGF mixture did (561). It is reasonable that the combination of Matrigel and angiogenic factors should yield vascularized tissue, but why adipose is the favored result remains unclear. Some have suggested that Matrigel may bind and thus protect growth factors that can promote angiogenesis and adipogenesis (257). Alternatively, Matrigel may release additional substances that synergize with FGF or PDGF. By itself, subcutaneous injection of Matrigel can induce adipogenesis, but only in young mice. Given that pericytes are multipotent cells that can differentiate into adipocytes (101), it is possible that angiogenesis supplies the adipose progenitors that contribute to subsequent adipogenesis.

Although controlled release of FGF2 from collagen implants has been reported to induce vascular growth (253), vascularization by itself appears to be insufficient to transform the collagen into adipose (257, 561). To form adipose

within collagen scaffolds, it appears necessary to add both an angiogenic factor and adipose progenitors. Subcutaneous implantation of collagen sponges that contained human preadipocytes and FGF2-loaded gelatin microspheres generated adipose tissue within 6 weeks (279). Implants of collagen scaffolds that contained preadipocytes and free FGF2 also transformed into adipose, but to a lesser extent. The amount of adipose increased with number of preadipocytes, but not with FGF dose; in fact, high amounts of FGF (50 μg) caused local inflammation. Changing the FGF release rate by altering the degree of gelatin crosslinking did not affect the generation of adipose (278). When implanted in a rat fat pad, collagen scaffolds that contained FGF2-loaded gelatin microspheres were sufficient to generate adipose, presumably because the surrounding tissue served as an exogenous supply of preadipocytes (207). Intermediate FGF release rates yielded the greatest generation of adipose.

Controlled release of angiogenic factors has also been used to generate vascularized "bridges" to connect two tissues. For instance, implantation of an FGF1-loaded gelatin foam between two organs (e.g., liver and spleen) in the rat caused vascular ingrowth into the scaffold that connected the microcirculatory systems of the organs (580). Interposition of FGF1-loaded PTFE sponges (precoated with type I collagen) or fibrin glue between the thoracic aorta and ischemic myocardium in the rat induced vascular connections between the two tissues (141, 495). At extremely high doses (100-800 μg), however, FGF1 that was delivered from collagen or PTFE sponges did not induce vascular connections between the internal mammary artery and ischemic dog heart, and vascularization of the scaffolds was not observed (35). Instead, underlying vessels displayed smooth muscle hyperplasia that essentially obliterated the lumens of arterioles.

Intracranial delivery of VEGF- and heparin-releasing hyaluronan gels to a stroke lesion resulted in vascular and neuronal ingrowth that was accompanied by neurological improvement (414).

Arteriovenous bundles and loops

Flap prefabrication In the late 1970s, Erol discovered that overlaying a full-thickness skin graft onto an arteriovenous (AV) bundle of femoral vessels resulted in the generation of connections between the vascular system of the graft and the femoral vessels (136). After 2 weeks, the vascular connections were sufficiently numerous that the graft could be sustained purely by blood flow to the AV bundle. Subsequent work by Erol and Spira showed that a surgically constructed AV loop, such as a direct shunt or interpositional graft between femoral vessels, could also be used to generate new vascular growth that sustained skin grafts (Fig. 9) (137). These findings led to the development of reconstructive tissue flap "prefabrication", in which a major vessel is mobilized to vascularize tissue that will eventually be transferred to another location in the body (186); a delay between implantation of the vessel and flap transfer is not absolutely required

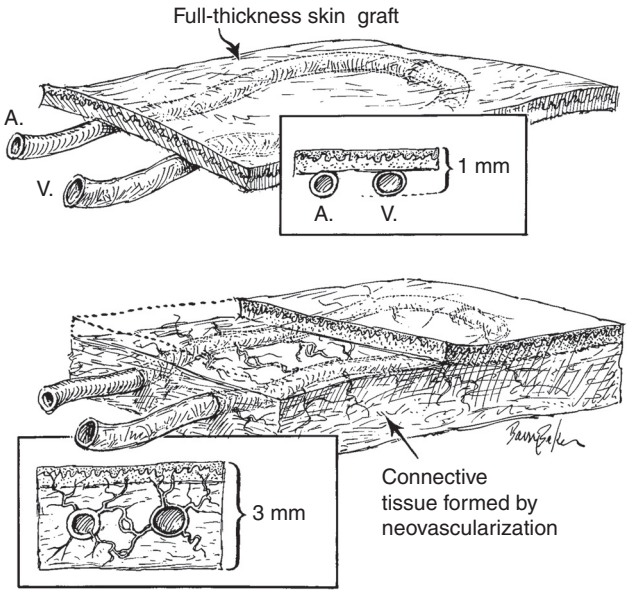

Figure 9 Strategy for vascularization of grafts from a surgically constructed arteriovenous (AV) loop. Angiogenesis from the loop invades into overlying tissue. A, artery; V, vein. Reproduced, with permission, from (137).

(618). Although flap prefabrication can be successful without the addition of angiogenic factors, these exogenous factors result in faster establishment of vascular connections between the flap tissue and the underlying pedicle (203). Addition of FGF2-containing heparin-Sepharose beads (in an alginate or ethylene-vinyl acetate binder) along a ligated vascular bundle enabled roughly half of the treated skin flaps to be perfused solely by the pedicle after 1 week; without FGF, the raised flaps were almost completely necrotic. Moreover, FGF treatment caused flaps to remain viable at 4 weeks even if their pedicles had thrombosed. Vascularization proceeded by angiogenesis from both the vascular bundle and existing vessels in the overlying skin. Only a small number of vascular connections was needed to maintain the viability of a raised flap. Similarly, instillation of TGFβ-containing collagen between skin and a vascular bundle accelerated the prefabrication of a skin flap that could be perfused solely by the pedicle (238); extensive vascular growth connected the pedicle and existing microvessels within the flap 3 days after delivery of growth factor. Introduction of PLGA microspheres that contained VEGF-A and/or FGF2 along an intramedullary vascular bundle in rat bone allotransplants caused increased capillary densities and faster bone formation; surprisingly, the combination of VEGF and FGF resulted in lower bone blood flow (314).

Vascularized chambers In the above examples of flap prefabrication, angiogenesis occurs from both the implanted large vessels and surrounding tissue, and only the short distance between the two vascular systems needs to be bridged. When engineering vascularized tissue de novo, however, the

vascular loop or bundle is the sole source of angiogenic growth into an initially avascular scaffold (656). Once vascularized, the scaffold can be left in place or transferred elsewhere by using the loop or bundle as an intact pedicle (574). Since angiogenesis must proceed throughout the scaffold to generate fully vascularized volume, this form of tissue generation is inherently slower than traditional flap prefabrication.

Comparison of different vessel configurations showed that vascular loops generated greater volumes of vascularized tissue than vascular bundles did (573). Although the underlying reasons are still unclear, it is likely that the extravasation of fibrinogen and blood cells at the suture holes that exist after construction of AV loops play a role in inducing angiogenesis. This possibility supports the earlier work of Vineberg, who found that wounding stimulated vascular growth from implanted arteries (614, 615). Ligated vascular bundles, however, generated smaller tissue volumes that were similar to those obtained with intact ("flow-through") vessels. This latter finding suggests that the higher flows that result from shunting blood in AV loops may play a more important role than vascular injury in promoting angiogenesis. Indeed, thrombosis of ligated bundles eliminated their ability to generate new tissue (103). In vascular loops, new blood vessels formed from the adventitia of the femoral vessels and directly from the femoral vein, but not from the interpositional artery or vein graft (573). In ligated vascular bundles, capillaries sprouted mainly near the distal ends of the femoral vessels, again in accordance with Vineberg's observations.

Regardless of the precise mechanism of angiogenesis, it is clear that the use of AV bundles and loops is a robust way to grow large volumes of vascularized tissue (Fig. 10). This strategy has been validated in rodents, large animals, and recently humans (393, 656); larger scaffolds resulted in greater final volumes of vascularized tissue (212). The type of tissue that results from an AV implant depends on the nature of the scaffold around the vessels. When an AV loop was implanted into a polycarbonate chamber without a scaffold, the chamber filled first with a provisional fibrin matrix that was then gradually infiltrated by fibrovascular tissue (349, 376). Similar fibrovascular growth was obtained when an AV loop was implanted in a PLGA scaffold (103). In contrast, implantation of an AV loop in Matrigel resulted in generation of adipose tissue. Unlike subcutaneous Matrigel implants [which require coimplantation of angiogenic factors to generate adipose (257)], inlaid AV loops caused Matrigel to be replaced by adipose in 4 weeks without any exogenous growth factors (103). AV bundles were much less effective in generating adipose, perhaps because vascular bundles are less angiogenic than loops; here, addition of FGF2 helped to increase the adipose volume fraction (622). FGF2, mouse VEGF-A_{120}, and rat PDGF-BB synergistically increased adipogenesis by 6 weeks (471). Matrigel is not unique in its ability to be transformed into adipose by an AV bundle and slow-release FGF2, as type I collagen showed similar results (610). An exhaustive study of scaffolds and angiogenic factors showed that vascular and adipocyte volume fractions were poorly correlated (586). For some types of scaffolds (e.g., a matrix gel that was derived from skeletal muscle), the delivery of FGF, VEGF, and PDGF to the AV bundle resulted in decreased vascularization, compared to growth factor-free controls.

Because these experiments typically encased the scaffold within an impermeable silicone or polymer chamber that only had holes for placement of the vascular pedicle, it was unclear where the adipose progenitors originated from. At first, local activation of preadipocytes was the favored mechanism; the existence of blood-borne adipose progenitors seemed unlikely. The loose perivascular adipose that surrounds the artery and vein in an AV bundle or loop may provide one source of progenitors; pericytes, such as those within the vasa vasorum, may be another source (140). It is also possible to provide adipose progenitors simply by implanting a pedicled fat flap into a rigid porous polymer chamber (119, 145, 393), or by implanting a free fat autograft with the AV bundle and FGF2-loaded Matrigel (262). The degree of vascularization was largely indifferent to the different chamber configurations (e.g., sealed or open, with or without fat graft), but adipogenesis was never observed when the pedicle was thrombosed (262). Angiogenesis was necessary, but not sufficient, for adipogenesis.

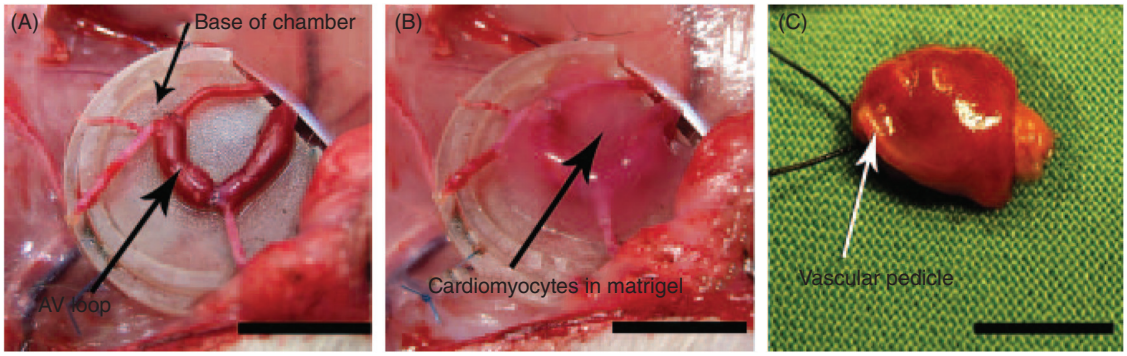

Figure 10 Generation of centimeter-scale tissue with an arteriovenous (AV) loop in a polycarbonate chamber. (A) AV loop overlaid on a polycarbonate base. (B) Addition of rat cardiomyocytes and Matrigel around the AV loop. (C) Explant of pedicled myocardium that formed by 4 weeks. Scale bar refers to 1 cm. Reproduced, with permission, from (394).

Addition of adipose-derived stem cells (ASCs) to the scaffold can help ensure that progenitors are well distributed and ready to respond to vascular ingrowth. In a collagen-chitosan scaffold with a femoral AV bundle, addition of VEGF-loaded microspheres increased vascular density by 2 weeks, and ASCs increased the generation of adipose after 4 weeks (673). Just because ASCs led to adipogenesis, however, does not guarantee that the adipose was derived from those cells; in fact, it appears that most of the vascular and adipose growth was host- rather than graft-derived (549).

To date, a pilot study in humans has shown the feasibility of generating >200 cm^3 of vascularized adipose tissue using AV bundles, but the success rate was low (393). The patient population (women who had undergone mastectomy several months to years prior) may have selected for comorbidities or local tissue scarring that inhibited adipose growth.

Surprisingly, coimplantation of skeletal muscle with an AV loop also resulted in generation of adipose tissue (375). Implantation of nonviable, freeze-thawed muscle or muscle extract led to the same result. Only the implantation of cultured myoblasts avoided adipogenesis in the AV loop-containing chamber; in this case, skeletal muscle fibers were obtained. The abundance of basement membrane in skeletal muscle (living and nonviable), Matrigel, and muscle extract, and its absence from cultured cells, are consistent with the idea that all that is needed to form vascularized adipose is an AV loop and basement membrane. It is intriguing that adipose tissue appears to be the default outcome in both subcutaneous (257) and chamber-based implants of Matrigel; influx of macrophages is critical for this result (116). Implantation of rat cardiomyocytes and Matrigel with an AV loop generated spontaneously contracting heart tissue after several weeks (394).

Engineering bone with an AV loop or bundle has required the addition of separate signals for angiogenesis and osteogenesis (5). Subcutaneous insertion of an osteoconductive porous hydroxyapatite scaffold in rats led only to the ingrowth of fibrovascular tissue. Insertion of a hydroxyapatite or bone-derived scaffold that contained a channel inlaid with a ligated epigastric bundle or a femoral AV loop resulted in vascularization throughout the scaffold, but no bone formation (5, 285). Incorporation of BMP2 into the scaffold promoted deposition of bone matrix; the effect of BMP2 addition was greatly enhanced in scaffolds that were vascularized by an implanted bundle (5). Surprisingly, the addition of FGF2 and BMP2 did not yield greater bone formation than BMP2 alone; while FGF release did increase vascular growth from the implanted epigastric vessels and osteoid deposition in the scaffold pores, it caused lower bone formation at 4 weeks (407, 408). When osteoblasts were injected into bone-derived scaffolds 6 weeks after the scaffolds had been implanted with or without an AV loop, cell viability was much higher in the samples that were prevascularized with the vascular loop; nevertheless, bone formation was rare in both types of implants (18). These studies suggested that vascular growth can compete with osteogenic cells for space within the scaffold pores. Thus, while angiogenesis is required for engineering bone, it is not always a positive factor; healing critical-sized bone defects with AV loop-containing scaffolds remains a challenge (19).

AV bundles have also been used to revascularize freeze-thawed (nonviable) bone allotransplants (634, 635), although the results are not as striking as those seen with viable allotransplants (314). Outgrowth from rat saphenous vessels that were placed in an intramedullary position in frozen bone allografts generated capillaries into the bone. The addition of PLGA microspheres that contained VEGF-A and FGF2 seemed to result in higher capillary densities, compared to PLGA alone. Sustained release of VEGF, with or without FGF, increased bone formation and blood flow.

Angiogenesis in microphysiological systems

Recent advances in materials engineering have enabled the development of microfluidic devices that contain microscale vascularized gels (641). These devices are commonly based on "soft lithography," a collection of techniques that use crosslinked silicone elastomers to pattern biological materials (649). Selective delivery of angiogenic factors into different compartments of a device can expose ECs on the surface of a gel to growth factor gradients that promote angiogenesis (Fig. 11) (92). In many implementations, fibroblasts are placed in a nearby compartment and serve as the source of growth factors (277). Once ECs have invaded the gel (most commonly, fibrin) and reached its opposite end, flow can be established through the newly formed vessels. Delivery of solutes through the lumens has shown that the permeability of such vessels is similar to that of venules *ex vivo*.

Because microfluidic devices afford tight control over perfusion pressures and flows, such devices have been used to elucidate how mechanical stress affects angiogenesis. Negative transendothelial pressures, in which the basal pressure exceeds apical pressure, promote EC sprouting (276, 612). Shear stresses above ~10 dyn/cm^2 also appear to promote angiogenesis (160), although contrary results have been reported (539). Increases in shear stress are often accompanied by a positive transendothelial pressure, which would attenuate the angiogenic response; indeed, when transendothelial pressures were held constant and close to zero, increased shear stress promoted sprouting (449). Elevated EC junctional shear stress may mediate the angiogenic effects of a negative transendothelial pressure (160).

Aside from investigation of the cell biology of angiogenesis, these devices can be used to study angiogenic vascularization of cell spheroids and other types of tissues (409).

Physical signals in angiogenic vascularization

Although the delivery of growth factors and their plasmids is by far the most popular strategy for angiogenic vascularization, it is also possible to use physical signals to induce angiogenesis *in vivo*. These signals include solid and fluid stresses, such as those imparted by tissue deformation and

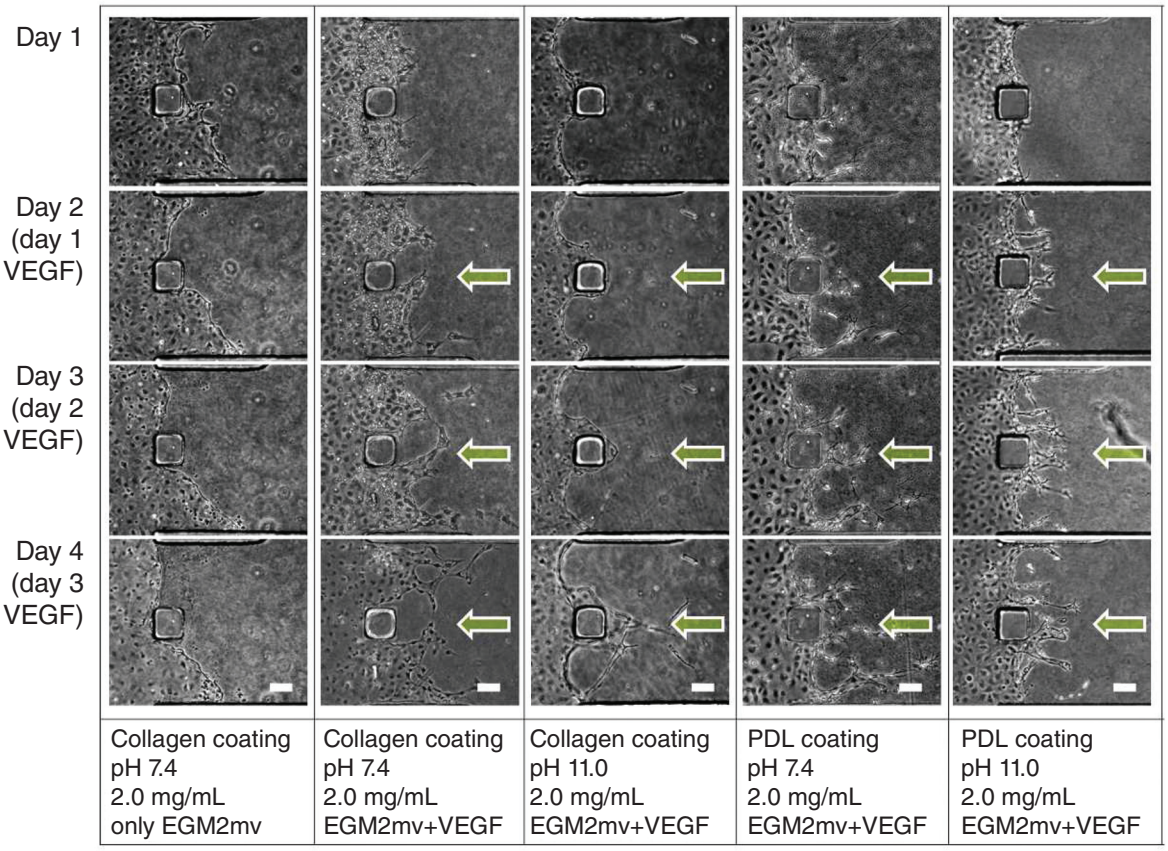

Figure 11 VEGF-driven angiogenesis into collagen gels in microfluidic devices. Treatment of devices with poly-D-lysine (PDL) enabled ECs to sprout from a monolayer toward a VEGF source. Arrows denote the direction of VEGF transport. Scale bars refer to 100 μm. EGM2mv, endothelial cell growth media. Reproduced, with permission, from (92).

blood flow, and the stiffness of an implanted scaffold. Using such signals to control vascular growth is not as well developed as methods based on biochemical factors, and how to best manipulate the physical environment to obtain vascular growth remains to be determined.

Over a century ago, Thoma recognized that increased blood flow led to generation of vascular networks (579). Indeed, chronic vasodilation increases vascular densities in heart and skeletal muscle (682). Elevated shear stress is partly responsible for transducing the effects of increased flow into angiogenesis *in vivo* (222-224). Endothelial production of nitric oxide mediates this effect (41). In fact, the arteriolar growth that is promoted by VEGF gene therapy appears to result mainly from flow-mediated stimulation of upstream vessels, rather than from a direct effect of VEGF (466). Flow-mediated increase in VEGF expression may also partly account for the vascular growth (380).

In subcutaneous implants of collagen scaffolds, the angiogenic effect of scaffold crosslinking was found to be at least as strong as that of VEGF delivery (654). Caution is warranted in interpreting this result, however, as crosslinking also increased the scaffold pore size. A different mode of vascularization has been described when a mechanically compliant scaffold contracts, such as during wound healing; here, vessels are "pulled" by the retracting scaffold to fill the residual volume (269).

Vasculogenic Vascularization

Vasculogenic vascularization refers to the development of microvessels through the self-organization of individual ECs or progenitors. In contrast to angiogenesis, in which vessels invade a graft, the vessels that form in vasculogenesis develop within the graft; this type of vascularization is considered "intrinsic" to the graft. In reality, the distinction between the two processes is not so clear-cut, and vessels that form by vasculogenesis can subsequently undergo angiogenic sprouting as well. Although strictly speaking not vasculogenesis *per se*, the development of vascular networks from suspensions of microvascular fragments or preaggregated ECs (e.g., spheroids) is also a form of intrinsic vascularization and is thus discussed in this section.

Summary of developmental vasculogenesis

Formation of the embryonic blood vascular system begins when hemangioblasts, the common precursor to blood cells

and ECs, organize into "blood islands" in the yolk sac by embryonic day 7 (389). Hemangioblasts are contained in the *Brachyury*-expressing VEGFR-2$^+$ cell population in the mouse and human, and differentiate into hematopoietic stem cells and angioblasts, the true precursor to ECs (88, 142, 221, 264). Angioblasts, which share markers with hemangioblasts and are VEGFR-2$^+$VE-cadherin$^+$ cells (415, 651), organize into interconnected solid cords that subsequently undergo tubulogenesis to form the first vascular network by embryonic day 9 in the mouse (124, 389). Further growth of the vascular system proceeds by angiogenesis.

In contrast to the blood vascular system, the lymphatic system does not form from angioblast-like cells. Instead, lymphatic sacs form by budding of *Prox1*-expressing cells from the venous system on embryonic days 10 and 11 (633). These sacs then extend and grow by lymphangiogenesis (477, 478).

Therapeutic vasculogenesis

The challenge in harnessing developmental vasculogenesis for treatment of tissue ischemia is that true angioblasts are known to exist only in the early embryo and in small quantities, compared to the numbers anticipated for therapeutic benefit in the adult. As a result, essentially all studies of therapeutic vasculogenesis have examined to what extent readily available adult-derived ECs or progenitors can substitute for embryonic angioblasts. Early work focused on differentiated ECs, but it was quickly realized that blood- and bone marrow-derived endothelial progenitors held much greater promise. Because vasculogenic cells can both form new vasculature and secrete angiogenic factors, one of the main issues when interpreting studies of therapeutic vasculogenesis is the relative contributions of vasculogenesis versus angiogenesis to the observed vascular growth.

Differentiated endothelial cells

Injection of allogeneic or syngeneic aortic ECs into the cryo-injured rat myocardium resulted in modest levels of vascularization within the scar (270). Only cells that were grafted 2 weeks after injury were effective; when grafted earlier, ECs did not persist at the scar and were likely cleared by ongoing inflammation. When ECs were labeled with BrdU before injection, some vessels in the scar were found to contain labeled cells. VEGF levels within the scar were the same whether ECs or culture media was injected. These findings implied that the increase in vascular density resulted partly from vasculogenesis, but not from VEGF-induced angiogenesis. It is important to recognize, however, that the absolute vascular densities obtained with EC injection (~15 vessels/mm^2) remained orders of magnitude lower than those in native myocardium. Other studies have found no increase in vascular densities from EC injection beyond those of controls (226, 252). When injected into ischemic hindlimb, ECs that were differentiated induced pluripotent stem cells (iPSCs) or reprogrammed from fibroblasts with defined factors increased blood flows and vascular densities, but with only <10% of injected ECs persisting at the injection site (189, 475); rescue occurred in roughly half of injected limbs (189, 446). Given the modest levels of vascularization and the sensitivity of EC suspensions to lack of matrix adhesion and blood flow, the injection of EC suspensions to treat ischemic tissues is currently out of favor; instead, these cells have been much more successfully applied when introduced within a scaffold for engineering vascularized tissues.

Endothelial progenitor cells

In 1997, Isner and co-workers reported that semipurified CD34$^+$ or VEGFR-2$^+$ cells from human peripheral blood could differentiate on fibronectin-coated dishes into cells that resembled ECs in their molecular footprint [e.g., positive for VEGFR-2, CD31, and endothelial nitric oxide synthase (eNOS)] (23). In contrast with true ECs, however, these "adult angioblasts" or endothelial progenitor cells (EPCs) were round or spindly, did not form cobblestone monolayers in culture, and expressed the hematopoietic stem cell marker AC133 (Fig. 12A) (23, 431). EPCs grew as colonies that

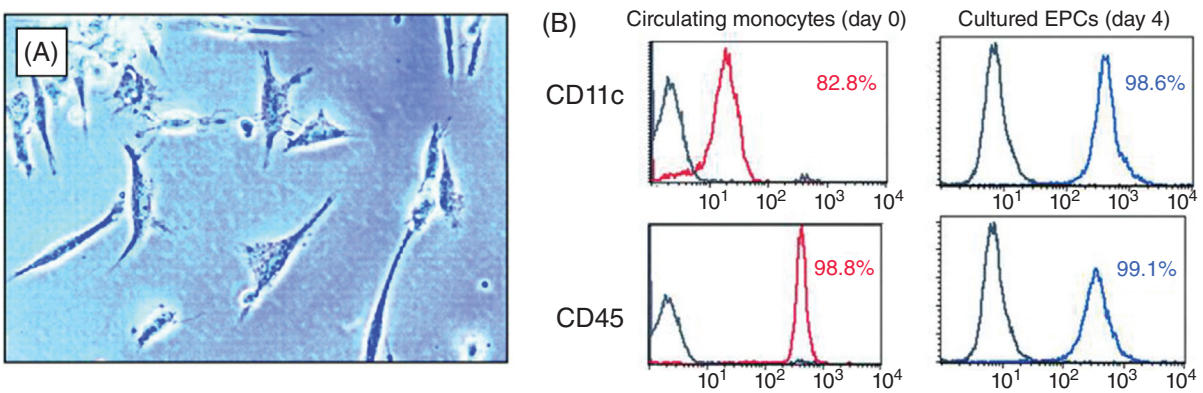

Figure 12 Derivation and characterization of EPCs. (A) Spindle-shaped EPCs that were derived from 1-week-old cultures of CD34-enriched peripheral blood-derived mononuclear cells. Reproduced with permission from (23). (B) Flow cytometry of EPCs and monocytes. EPCs express blood cell markers, including CD45 and the monocyte activation marker CD11c. Adapted, with permission, from (459).

surrounded residual $CD34^{-}CD31^{+}$ T cells (225). When injected intravenously, *lacZ*-expressing or DiI-labeled EPCs homed to ischemic hindlimbs in mice and rabbits (23). Remarkably, these EPCs incorporated into capillaries, expressed CD31, and became histologically indistinguishable from ECs. Transfection with VEGF-A rendered EPCs even more effective in increasing vascular density and in restoring blood flow to ischemic tissue (235). Nevertheless, homing of EPCs is extremely inefficient: only ~3% of intravenously or intraventricularly injected cells deposited in the heart, even in the presence of ischemic injury; the vast majority of EPCs accumulated in the spleen (1).

Although EPCs are mobilized into the blood after myocardial infarction and other ischemic episodes (515), the quantities are insufficient to restore the vascular function of the affected tissues, and methods to increase the level of mobilization are needed. Given the common embryonic origin of the hematopoietic and vascular systems, treatments that mobilize hematopoietic stem cells and/or progenitors into the circulation should have similar effects on EPCs. Indeed, administration of granulocyte-macrophage colony-stimulating factor (GM-CSF) for 1 week resulted in increased counts of circulating EPCs in the rabbit (566). Induction of hindlimb ischemia led to a further increase in EPC mobilization and subsequent homing and differentiation into ECs in the ischemic tissue. Similar results were obtained with mobilization of $CD34^{+}$ human EPCs by granulocyte colony-stimulating factor (G-CSF) and their homing and differentiation in the ischemic rat myocardium (290). The ability of EPCs to be mobilized by such factors implies these cells normally reside in bone marrow. Cord blood, which is a rich source of hematopoietic stem cells, is enriched in EPCs (401). Liver and intestine also appear to be a source of EPCs (3).

VEGF-A can mobilize EPCs into the blood and cause them to home to a distant organ (24, 250). This mobilization requires activity of eNOS and the resulting induction of the protease MMP-9 in bone marrow (2); similar mechanisms apply to mobilization of EPCs by estradiol (236). In skin wounds in the diabetic mouse, topical $VEGF\text{-}A_{165}$ resulted in a threefold to sevenfold increase in EPCs in circulation (159). The EPCs integrated into newly formed vessels both in the treated wound and in adjacent ones that were not treated with VEGF. Thus, VEGF-mobilized EPCs provided a systemic benefit to wound healing that enhanced the angiogenic effects of local VEGF application. This result also implied that previous work in VEGF-induced therapeutic *angiogenesis*—and by extension, any angiogenic strategy based on mimicking hypoxia—should be re-evaluated for possible contributions from circulating EPCs.

Once mobilized, EPCs home to ischemic tissues using the same molecular signals that facilitate homing of leukocytes and other blood-borne cells to activated vessels during inflammation. EPCs bind to vascular surface VCAM-1, ICAM-1, and selectins with the ligands VLA-4 ($\alpha_4\beta_1$ integrin), CD18 (β_2 integrin), and PSGL-1, respectively (77, 245, 607, 661). Endogenous plasma and tissue levels of the chemokine SDF-1 were maximal 6 h and 3 days after induction of ischemia, respectively, while levels in bone marrow reached a minimum after 1 day (115); these changes are consistent with SDF-mediated mobilization of EPCs from bone marrow to blood to ischemic tissue. Indeed, homing of EPCs to VEGF-releasing or ischemic tissues required expression of SDF-1 in the local tissue (74, 183). Intramuscular injection of SDF-1 into the ischemic mouse hindlimb increased the numbers of EPCs that incorporated into the muscle vasculature; of note, SDF-1 also increased VEGF-A expression in the ischemic tissue, which implies a positive feedback loop between the two signals (650). Controlled release of SDF-1 and VEGF from intramuscular injection of alginate gel into ischemic hindlimb promoted homing of EPCs to the same site, with subsequent increase in vascularization and recruitment of myeloid cells (12). Release of SDF-1 from a polymer chamber that contained an arteriovenous loop also resulted in homing of EPCs, but no increase in vascularization, perhaps because the loop-containing chamber was itself already highly angiogenic (529). Platelets that adhere to endothelium at sites of injury provide another source of SDF-1 to recruit EPCs (545). The proliferation rate of recruited EPCs appears to correlate with the degree of ischemia (576). Angiogenic therapies, such as transfection with COMP-Ang1, can indirectly upregulate SDF-1 and thus concentrate EPCs locally (664).

For clinical applications, mobilization of EPCs by cytokines may be insufficient, and methods to increase the concentration of EPCs even further may help. Human EPCs can be expanded in culture media that was originally designed for human microvascular ECs; this media contains a mixture of angiogenic and other growth factors, including VEGF, FGF2, IGF1, and epidermal growth factor (EGF) (251). Under optimized culture conditions, each milliliter of peripheral blood yields ~5000 EPCs that can be grown to yield ~35000 EPCs for injection. Expanded human EPCs homed to ischemic rat myocardium and incorporated into vessels there (258). Alternatively, EPCs can be directly injected at the ischemic site in large numbers. For instance, injection of $CD34^{+}$ EPCs into a raised skin flap resulted in greater vascularization and flap survival, compared to injection of saline or ECs; EPCs, but not ECs, incorporated into capillaries (303). Intramyocardial injection of $CD31^{+}$ autologous pig EPCs or $CD34^{+}$ human EPCs into ischemic pig or rat myocardium, respectively, preserved heart function and generated EPC-incorporated capillaries (261). Surprisingly, intracardiac injection of cultured EPCs led to greater vascularization in the ischemic mouse hindlimb than injection of freshly isolated EPCs did, which suggests that other cells that copurified in the initial isolation may hinder the vascularization process (251). Perhaps most importantly, injection of cultured EPCs restored the capillary density of the ischemic limb to that of the normal one (~500 vessel/mm^2). EPCs can also be introduced by encapsulating them into an alginate implant that gradually releases VEGF and thereby promotes outward migration of cells into the surrounding ischemic tissue (523).

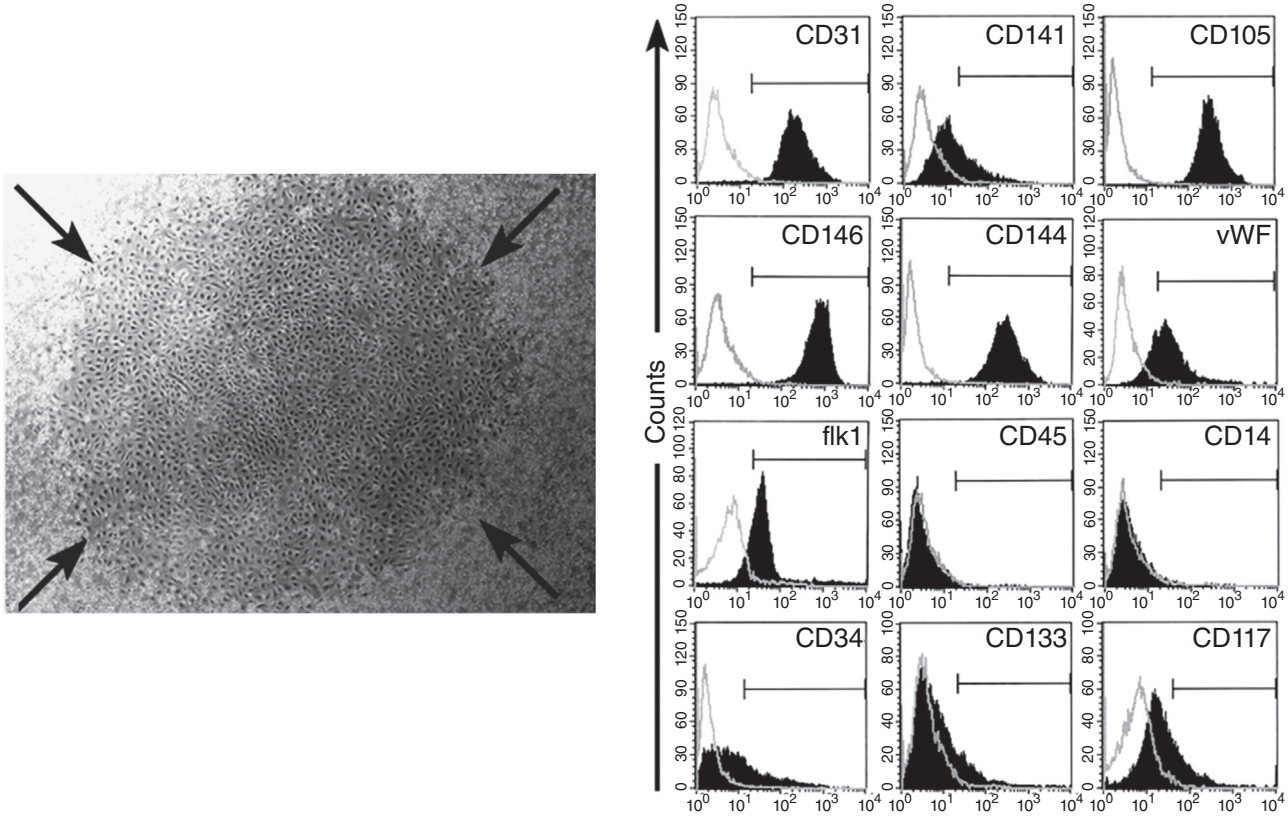

Figure 13 Derivation and characterization of EOCs. (*Left*) Colony of cord blood-derived EOCs that emerged after mononuclear cells were cultured for 9 days. (*Right*) Flow cytometry of EOCs. EOCs express EC markers, such as CD31 and CD144 (VE-cadherin), but do not express markers of blood cells, such as CD45 and the macrophage/monocyte marker CD14. Adapted, with permission, from (232).

Endothelial outgrowth cells

$CD34^+$ cells from peripheral blood contain at least two distinct populations that can differentiate into ECs (226). Culture on fibronectin-coated dishes yields spindle-shaped EPCs, as discussed above. Culture of cells that did not adhere by the first day and that were subsequently plated onto fibronectin/gelatin-coated dishes eventually yielded cobblestone monolayers that expressed typical EC markers (Fig. 13, *left*) (510). These endothelial "outgrowth" cells (EOCs) were morphologically indistinguishable from ECs, particularly those derived from large vessels. Like ECs and unlike EPCs, EOCs did not express the stem cell marker AC133 (398). Other methods for obtaining EOCs from peripheral blood have been developed, the simplest being to culture blood-derived mononuclear cells in endothelial growth media for several weeks (226). Intraventricular injection of EOCs and EPCs led to their incorporation into microvessels and equivalent levels of vascularization in the ischemic mouse hindlimb (~500 vessels/mm^2); in contrast, injection of ECs or culture media led to much lower capillary densities. Moreover, EOCs and EPCs improved perfusion ratios and limb salvage by equivalent amounts, and their combination was synergistic (226,662). Given the morphological and biochemical similarity between EOCs and ECs, it is surprising that EOCs induce such robust vascularization.

The relationship, if any, between EOCs and EPCs remains unclear. Mononuclear blood cell cultures invariably yield EPCs in the first days, followed by EOCs over several weeks; this temporal sequence suggests that EOCs may derive from rare EPCs that lose their spindle shape and switch to EC-like spreading. In support of this view, DiI-labeled EPC cultures eventually yielded faintly labeled EOCs (226). On the other hand, the ability of EOCs to be derived from initially nonadherent blood cells and the expression of monocyte/macrophage markers and secretion of angiogenic factors by many EPCs and not by EOCs suggest that EPCs and EOCs do not share a common lineage (Fig. 12B and 13, *right*) (185, 459, 510, 604, 662). Although EPCs and macrophages share common surface markers, EPCs are not true macrophages, which display a much weaker vasculogenic potential (604). Further confusing the issue are that EPCs can also be derived from initially nonadherent cultures (660), and that "EPCs" is sometimes used to refer to cultures that display the cobblestone growth of EOCs (149, 372, 591). Moreover, a third source of vasculogenic cells—circulating ECs—exist in the blood and most likely originate by sloughing off from vascular walls (344). Even today, EPCs and EOCs are distinguished mainly by morphology and culture time. Until precise molecular-level definitions [akin to the clonogenic identification of hematopoietic stem cells (517)] become accepted, the relation between EPCs and EOCs will remain unclear.

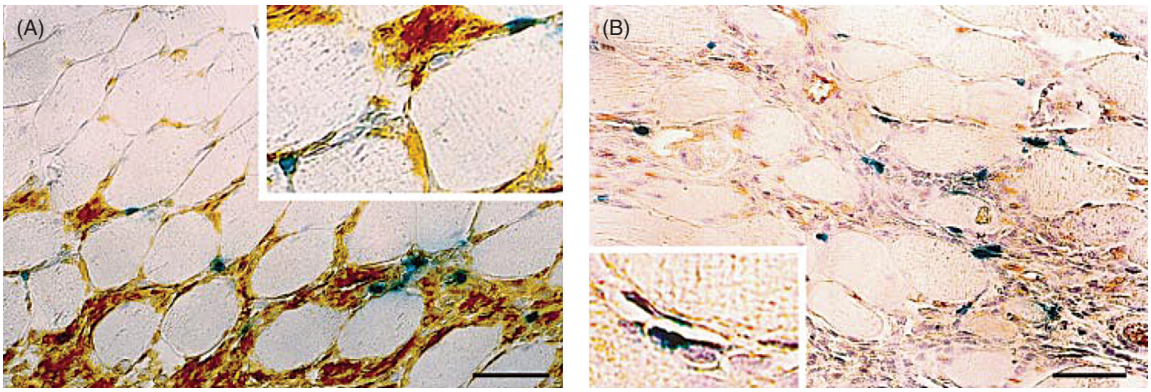

Figure 14 Incorporation of bone marrow-derived, *lacZ*-expressing EPCs at sites of ischemia. Sections were costained by X-gal (blue) and for lectin (A) and CD31 (B). Scale bar refers to 25 μm. Reproduced, with permission, from (22).

Regardless of the true origin of EOCs (including the possibility that they are an artifact of *in vitro* cell culture), these cells have been isolated from many sources besides peripheral blood. As with hematopoietic stem cells, cord blood is a particularly rich source of EOCs (232).

Bone marrow

Arguably, the most unsettled issue in therapeutic vasculogenesis is whether unpurified bone marrow (BM) can serve as an equivalent substitute for EPCs. Experiments with genetically labeled BM transplants showed that BM-derived endothelial progenitors incorporated into capillaries in mouse skin wounds and ischemic myocardium and expressed VEGFR-2 and Tie2 (Fig. 14) (22). These findings were consistent with the increased vascularization found after intramyocardial, intramuscular, or subcutaneous injection of autologous BM in animal models (122, 123, 230, 587). In rat myocardial scar and in mouse skin flaps, injected BM cells contributed modestly to new endothelium (228,587). Intramyocardial injection of BM mononuclear cells or stromal cells into the ischemic pig heart resulted in their incorporation into 10% to 30% of vessels (252, 588). Intramuscular injection of BM-derived mononuclear cells into the ischemic rabbit hindlimb led to incorporation into vascular networks and increased angiographic scores, blood pressure ratios, and blood flow (516). In contrast, injection of bone marrow-derived fibroblasts had no effects beyond those of saline controls. When injected intramuscularly, BM-derived stromal cells contributed to both endothelial and smooth muscle layers in the ischemic rat hindlimb (6). Angiogenic gene therapy synergized with BM mononuclear cells to improve vascularization (260, 288).

If the effects of BM injection result mainly from the EPC subpopulation, then injection of EPCs alone or a greater number of BM cells that contain an equivalent number of EPCs should yield similar results. Experiments in the ischemic rat heart, however, showed that intramyocardial injection of human $CD34^+$ EPCs increased capillary densities and decreased cardiac fibrosis, while BM mononuclear cells that contained the same number of EPCs did not (259). In fact, BM injection resulted in hemorrhagic infarction. Human ECs were present in the myocardium after injection of EPCs, but much less so after injection of unpurified BM. Given that <2% of BM consisted of $CD34^+$ cells, the BM injection introduced a large excess of leukocytes that may have precipitated or exacerbated the hemorrhage. Indeed, injection of autologous BM in the ischemic rat myocardium greatly increased the tissue concentration of inflammatory cytokines IL-1β and CINC (289). Still, these results are difficult to reconcile with studies in which no adverse effects of local BM injection were noted.

Whether BM cells contribute to endothelium has also come into question. The initial studies were mirrored by several spectacular claims in the stem cell literature of BM transdifferentiation into cardiomyocytes, hepatocytes, neurons, skeletal myotubes, and many other nonhematopoietic cell types (9, 58, 144, 301, 422). Such claims were often based on detection of lacZ or GFP expression that could only have originated from graft-derived cells. The scrutiny that such claims engendered led to several counterclaims, in which the contribution of BM to nonhematopoietic tissues was declared to be minimal (73, 620). Several studies specifically examined the contribution of BM to endothelium and found it to be essentially nil (104, 179, 452, 685). BM-derived cells were found instead in a perivascular location and contributed to pericytes and tissue leukocytes (244, 452, 685). Vigorous debate about potential strengths and flaws of these studies even led some authors to question the correctness of the original series of EPC studies by Isner and co-workers (265). Vascular injury by the irradiation used in BM transplants appears to prime tissues for vasculogenesis from BM-derived cells (532).

Confounding the issue is the ability of BM cells—particularly, mesenchymal stem cells (MSCs)—to secrete angiogenic and vasculogenic factors. Compared to ECs, MSCs produce orders-of-magnitude higher amounts of VEGF-A, FGF2, PlGF, and the arteriogenic factor MCP-1 (281, 282); MSCs also secrete large amounts of SDF-1 (239). Injection of *GFP*- or *lacZ*-expressing MSCs into the ischemic mouse hindlimb improved blood flow and limb function, but did not result in colocalization of GFP or lacZ with vessels (282). Injection of MSC-conditioned media had similar

effects, which implies that any direct contribution of MSCs to vessels is redundant (281). Indeed, intramyocardial injection of MSCs with or without EPCs did not lead to incorporation of MSCs into vascular endothelium (555), although contrary results have been reported (112, 526). BM-derived cells rescued a defect in PDGF-BB expression and vascularization in mouse heart grafts (126). Mononuclear cells secreted several angiogenic factors and inflammatory cytokines, including FGF2, VEGF, Ang1, IL-1β, and TNF-α, and comprised ~10% of macrophages after injection into ischemic pig heart (252). Subcutaneous injection of BM cells or MSCs restored lymphatic continuity in a mouse lymphedema model, but again, whether these cells contributed directly to endothelium is unclear (98, 513). Some studies have suggested that macrophages may transdifferentiate into lymphatic ECs *in vivo* at sites of injury (266, 364, 513).

Another issue is that some cell types seem able to form structures that resemble vessels and conduct blood but are not comprised of ECs, a process known as "vascular mimicry" (Fig. 15) (202, 358). Originally described in melanoma, vascular mimicry would enable a functional vasculogenesis that could increase blood supply without actually increasing the density of EC-lined vessels. As one might expect, the existence of such nonendothelial vasculogenesis is highly controversial (53, 201, 369).

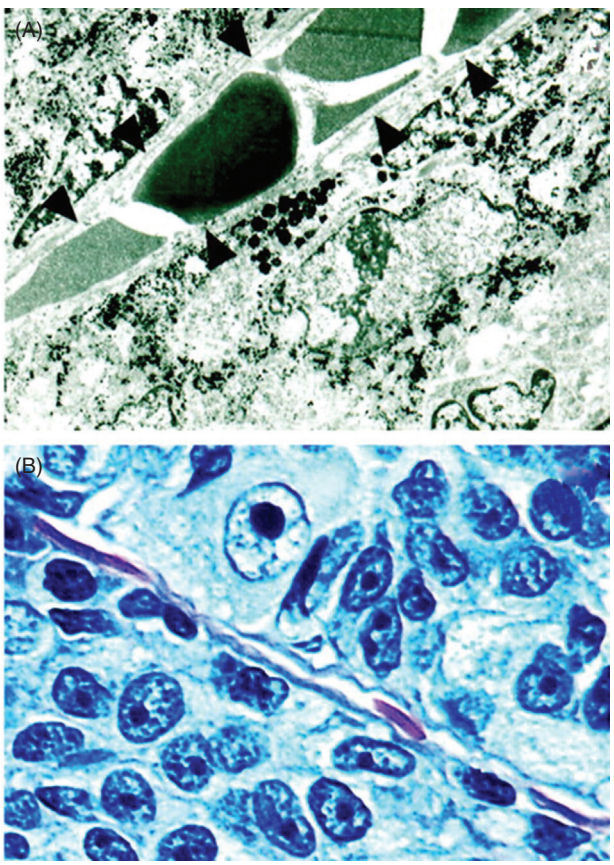

Figure 15 Vascular mimicry in uveal melanoma. Red blood cells (*top*, arrowheads) fill vessel-like structures that do not appear to be lined by ECs. Reproduced, with permission, from (358).

Other sources

Adipose-derived stem cells (ADSCs) have also been examined for their vasculogenic potential. In principle, these cells are easier to obtain than the BM-derived MSCs and display near-identical differentiability as MSCs do (689). Cells from cultured mouse adipose stromal-vascular fraction (SVF), a population that is enriched in ADSCs (but also contains ECs), enhanced vascularization to the same degree as BM-derived mononuclear cells did (444). Only SVF cells from white adipose tissue were effective; SVF cells from brown adipose were equivalent to saline controls. Cultured human SVF cells and dedifferentiated human adipocytes also increased vascular density and flow in the ischemic mouse hindlimb and incorporated into vessels (70, 444). As with BM, whether the vascularization potential of ADSCs or SVF cells is mainly the result of vasculogenesis or angiogenesis remains unclear. ADSCs secrete several angiogenic factors, including VEGF, HGF, TGFβ, and the lymphangiogenic factor VEGF-C (460, 513). Compared to MSCs and mature adipocytes, ADSCs secreted more SDF-1, which helped recruit EPCs to the ischemic rat heart and mouse hindlimb (229, 296). Half of injected ADSCs were already apoptotic by day 3, which would argue against a persistent vasculogenic benefit (229). In skin wounds, ADSCs synergized with platelet-rich plasma to induce vascularization, but resided only in a perivascular location (54). Subcutaneous injection of ADSCs reduced mouse-tail lymphedema, but resulted in rare incorporation of ADSCs into regenerated lymphatics (513). On the other hand, injection of freshly harvested $CD34^+CD31^-$ human ADSCs into the ischemic mouse hindlimb increased capillary densities, with ~8% of vessels showing endothelial incorporation of human cells (384). At bone fractures, ADSCs incorporated into vessels at early but not late stages of healing (518).

It has been claimed that blood and BM also contain circulating progenitors for SMCs (321, 482, 511, 531), although dissenting views certainly exist (489). Intravenous, intraventricular, or intramyocardial injection of human peripheral blood-derived $CD34^+$ cells resulted in contribution of the cells to vascular smooth muscle in the ischemic rodent myocardium (237, 659). Cord blood-derived mononuclear cells differentiated into SMCs *in vitro*, and intravenous coinjection of SMCs and EOCs resulted in synergistic vascularization of ischemic hindlimb, in part via Ang1/Tie2 signaling; strangely, the injected SMCs were not observed to have incorporated into the vasculature (149). Although vasculogenic therapies do not require injected cells to contribute to the smooth muscle lineage, the ability of some cell populations to serve as smooth muscle progenitors (or as bipotent progenitors of both ECs and SMCs) may aid in the generation of mature vascular networks.

Clinical trials

If the 1990s can be considered the heyday of clinical trials for angiogenic therapies, then the 2000s holds the same

status for trials of vasculogenesis (458). The association of higher EPC counts with spontaneous myocardial salvage in patients supported the idea that exogenous increases in EPCs could help repair ischemic tissue (418). As with angiogenesis, Phase I/II trials of vasculogenic therapies with EPCs, BM-derived mononuclear cells, AC133$^+$ BM cells, and other cell populations showed safety of the approach and hints of clinical efficacy (543, 594). Initial enthusiasm was tempered by the inconsistent findings of angiogenic clinical trials; as Laham and Oettgen aptly stated, "[w]herever this field takes us, it is likely to follow the well-known pathway of incredible results in the setting of unrealistic expectations followed by disappointments and cautious optimism" (309). This statement proved to be prophetic, as randomized controlled trials of vasculogenic therapies have shown modest benefit over placebo [reviewed in (463) for critical limb ischemia].

The same sources of discrepancy between preclinical and clinical results for angiogenic therapies also hold for vasculogenic therapies. For instance, in contrast to animal studies, human trials use recipients with one or more comorbidities. The quantity and VEGF-induced migration of circulating EPCs in humans is inversely correlated with the number of risk factors for coronary arterial disease (206, 609) and the likelihood of future cardiovascular events (497, 628). Although the effects of these deficits may be minimized by collection, concentration, and direct injection of EPCs into the ischemic tissue, EPCs from patients in clinical trials are more senescent and are functionally impaired in their response to local growth factors (194, 206, 609). Old age, a major risk factor, impaired both the vasculogenic potency of donor BM-derived stromal cells and the responsiveness of the recipient, in a rat model of hindlimb ischemia (681). Notably, an old recipient of old cells (akin to the clinical situation) responded equivalently as a young recipient that was injected with culture medium only. In heart grafts into old rats, only BM from young rats was able to rescue aging-impaired angiogenesis and to incorporate into vessels (126). A second source of discrepancy is that clinical endpoints do not rely on vascularization *per se*, but rather on improvements in blood flow and limb or heart function. In fact, it has been claimed any functional benefit of injected cells in the ischemic heart can largely be attributed to softening of the myocardium, rather than by any specific vasculogenic effect (50). As with angiogenic therapies, placebo effects are surprisingly strong, which make detection of a true clinical effect difficult.

Long-term side effects of vasculogenic therapies are unknown. As seen with angiogenic factors, BM-derived cells promote vascularization and atherogenesis, in part through the induction of inflammatory mediators such as MCP-1 (135). Given the ability of BM-derived cells (particularly EPCs) to promote vasculogenesis within tumors, vasculogenic therapy may induce tumor progression (355). Currently, use of autologous cells that are processed onsite and then reinjected does not require clinical approval. No therapies with cultured autologous cells for therapeutic vascularization have been approved.

Vasculogenesis to engineer vascularized tissues

In the early 1980s, Montesano and coworkers showed that a monolayer of cultured bovine capillary ECs on a type I collagen gel could spontaneously reorganize to form tubes when overlaid by a second collagen gel (387). Later work found that initially dispersed ECs reassembled into "networks" on Matrigel and on laminin-supplemented collagen gels (304). Thin sections showed the presence of open lumens within the tubes, but evidence of fully interconnected networks was scant. Nevertheless, these studies led to the hope that ECs could be coaxed to undergo vasculogenesis if provided with an appropriate scaffold to reorganize on top of or within. In principle, incorporation of parenchymal cells and ECs within a scaffold could lead to generation of microvessels that enable perfusion of the developing construct. In contrast to angiogenesis, which starts at the surface of a scaffold and then invades deeper, vasculogenesis could result in synchronous vascularization throughout the scaffold and thereby decrease the ischemic time at the center of the tissue.

As with therapeutic vasculogenesis, many different types of ECs and progenitor cells have been studied for their ability to undergo vasculogenesis in scaffolds. In contrast to the use of spindle-shaped EPCs in therapeutic vasculogenesis, however, definitive ECs—isolated from existing vessels or differentiated from rare progenitors and grown as cobblestone monolayers—have been the main cell source when promoting vasculogenesis in engineered tissues.

Prevascularization of scaffolds by vasculogenesis

ECs and EOCs Embedding differentiated ECs into a collagen or Matrigel scaffold rapidly leads to apoptosis, and even the tubes that do form regress within a few days (494). ECs require survival signals from cell spreading, which is restricted within typical scaffolds of submicrometer pore size (78). Nevertheless, subcutaneous implantation of human umbilical vein EC (HUVEC)-containing collagen-fibronectin gels in the mouse yielded perfused, thin-walled microvessels after 1 month (Fig. 16A) (494). Virtually all the vessels were derived from HUVECs and not from the recipient mouse. Moreover, only gels that contained aggregated "cords" of ECs (the precursors to tubes) at the time of implant yielded perfused vessels. Transfection of ECs with a caspase-resistant Bcl-2 mutant before embedding in a collagen-fibronectin gel protected the ECs from apoptosis and increased vascular density nearly fivefold after implantation, compared to control transfection. These vessels became invested by a mouse-derived mural coat after 1 month, and matured to form hierarchical microvascular networks by 2 months (Fig. 16B, C) (130).

These findings served as a basic template for subsequent studies that varied the type of ECs, the method used to inhibit EC apoptosis, the scaffold composition, and the degree of *in vitro* culture (a form of "prevascularization") before implantation. Since HUVECs are unlikely to be clinically obtainable for most patients, autologous ECs have been

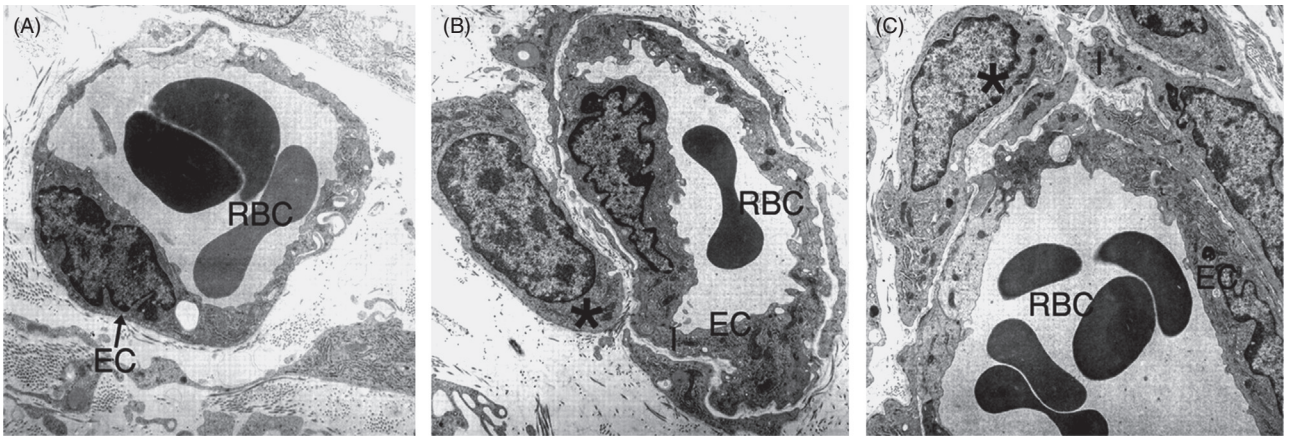

Figure 16 Fine structure of vessels that formed from normal and *Bcl2*-expressing HUVECs in collagen-fibronectin gels 1 month after implantation. (A) Implants of normal HUVECs. (B, C) Implants of *Bcl2*-expressing HUVECs. The lumens are perfused with red blood cells (RBC); asterisks denote mural cells. Reproduced, with permission, from (494).

examined for vasculogenic potential in scaffolds. Adult ECs exhibit limited proliferation and resistance to apoptosis, and most attention has focused instead on ECs that are differentiated from progenitor cells *in vitro*. In particular, endothelial outgrowth cells (EOCs, also known as "endothelial colony-forming cells" or ECFCs) from bone marrow, peripheral blood, or cord blood have shown considerable promise in generating vascularized tissues (28, 660). Subcutaneous implants of collagen-fibronectin gels that contained human EOCs yielded human EC-lined vessels by 4 weeks; notably, similar implants with human EPCs showed virtually no vascularization (660). When coimplanted with 10T1/2 fibroblasts, cord blood-derived EOCs or iPSC-derived ECs organized into much longer-lived vessels than peripheral blood-derived EOCs did (28, 293, 486). When coimplanted with human SMCs in Matrigel, early-passage cord blood-derived EOCs yielded larger vascular densities (~100/mm^2) than late-passage cord blood-derived EOCs, early-passage peripheral blood-derived EOCs, and microvascular ECs did (10-20/mm^2); as expected, higher EOC concentrations resulted in higher vessel densities (372). Generation of perfused vessels in Matrigel occurred mainly between days 3 and 5 (255). Vascularization of Matrigel or gelatin implants with cord blood-derived EOCs and SMCs or MSCs required early infiltration of the scaffold by myeloid cells, particularly neutrophils (343, 371). Neutrophil recruitment and vascularization were more effective when EOCs and MSCs had yet to assemble into vascular networks before implantation (343). Ranked, the vasculogenic potentials of different ECs are: early-passage cord blood-derived EOCs > HUVECs > late-passage cord blood-derived EOCs ~ peripheral blood-derived EOCs ~ microvascular ECs (83, 372).

Lymphatic ECs have also shown vasculogenic capability in scaffolds (26, 360). Remarkably, coimplantation of blood- and lymphatic vessel-derived ECs in fibrin gels led to generation of distinct vascular networks, without comingling of the different ECs in the same vessel (Fig. 17) (360). The underlying mechanisms of this self-segregation are unclear, although platelet-derived signals appear to be important in the natural development of the two distinct vascular systems (72).

Methods to promote EC survival within scaffolds have been developed. Incorporation of VEGF-releasing alginate microparticles in collagen-fibronectin gels that contained *Bcl2*-transduced HUVECs led to increased vascular densities (~100/mm^2) and diameters in implants (241). Surprisingly, nearly all of the improved vascularization resulted from an increase in HUVEC-lined vessels, rather than from any VEGF-induced angiogenesis. Even more surprisingly, the addition of unloaded (i.e., VEGF-free) microparticles resulted in similar vascular densities, which suggests that the alginate may concentrate and slowly release local survival factors.

A different strategy to promote EC survival in implants is to provide mesenchymal cell support, as these cells produce numerous stabilizing factors, such as Ang1 (114). Numerous mesenchymal cell types, including fibroblasts, SMCs, BM-derived stromal cells, MSCs, and ADSCs, have been coimplanted with ECs (11, 28, 29, 181, 372, 591). Coimplantation of human saphenous vein SMCs with EOCs yielded perfused vessels in Matrigel, but EOCs by themselves did not (372). Human MSCs displayed the same potency as mouse 10T1/2 fibroblasts in stabilizing newly formed vessels from HUVECs in collagen-fibronectin implants (29). They resulted in a several-fold increase in vascular density in several types of implanted scaffolds (11). Likewise, ADSCs increased the total and SMA-positive vascular densities in cord blood-derived EOC-containing collagen-fibronectin implants (591). A feedback loop, in which ADSCs secreted VEGF to support EOCs or ECs that secreted PDGF-BB to support ADSCs, may underlie the enhanced vascularization (590, 591). Nitric oxide, likely EC-derived, mediates the association of mural cells with newly formed vessels (256). In HUVEC-containing fibrin implants, lung fibroblasts induced greater vessel densities than MSCs or ADSCs did; on the other hand, only MSCs and ADSCs generated vessels that expressed the smooth

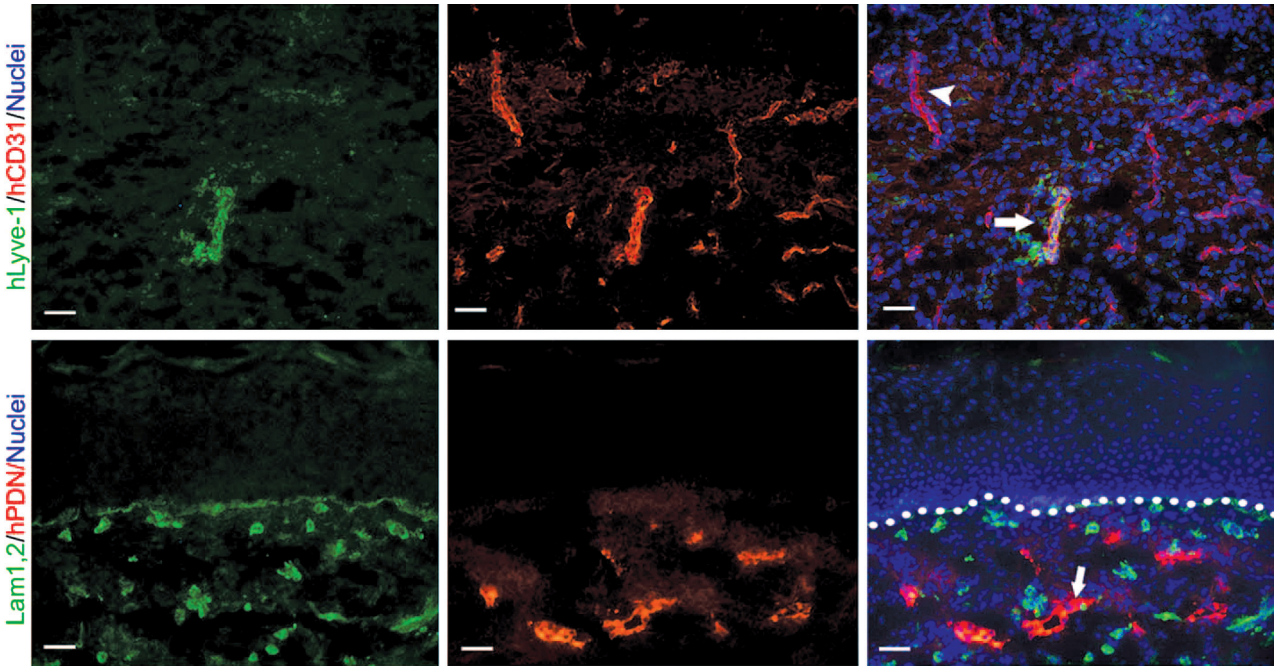

Figure 17 Organization of human microvascular ECs into blood and lymphatic vessels in a dermal graft after 2 weeks. Immunostains are shown for CD31, blood vessel marker laminin (Lam1,2), and lymphatic markers LYVE-1 and podoplanin (hPDN). Scale bars refer to 100 μm. Adapted, with permission, from (360).

muscle marker calponin (181). The higher the density of fibroblasts, the greater the vascular density and perfusion (83). Invariably, the mesenchymal cells migrated to a perivascular location, with little evidence of transdifferentiation into ECs (29,590). Implantation of mesenchymal cells in scaffolds without ECs resulted in minimal vascularization, which was recipient-derived (372,591). Lymphatic ECs appear to be able to undergo vasculogenesis only in the presence of fibroblasts (26, 360).

Many types of scaffolds, including fibrin, Matrigel, biodegradable polymers, and a self-assembling peptide-based matrix (Puramatrix), have shown the ability to be vascularized by implanted ECs or EOCs (11, 416, 436). Regardless of the scaffold used, the resulting vascular densities are generally an order of magnitude lower than that of the surrounding native tissue.

EPCs and mesenchymal cells To what extent EPCs, hematopoietic cells, and mesenchymal stem cells from bone marrow (MSCs) or adipose tissue (ADSCs) contribute to vascular structures in scaffolds remains unclear. Some studies have claimed that these cells can differentiate into ECs and form perfused vessels within implants. In FGF2-loaded Matrigel implants, 6% to 26% of ECs in the vascular ingrowth were BM-derived (399). In subcutaneously implanted PVA sponges, ~10% of the ECs in the vascular ingrowth were BM-derived (104).

Other studies observed minimal incorporation of EPCs, MSCs, or ADSCs into newly formed vessels (162). Subcutaneous implants of EPC-containing collagen-fibronectin gels yielded minimal vessels ($<1/mm^2$) by 4 weeks (660). Subcutaneous injections of MSC-containing Matrigel developed denser, more persistent, and better-organized vascular networks than VEGF- or FGF2-loaded Matrigel did, with vascular densities of $\sim 100/mm^2$ by 4 weeks (7). Although MSCs contributed both to endothelial and smooth muscle layers, the vast majority (~99%) of vessels in the Matrigel were derived from the recipient, which suggests that angiogenesis rather than vasculogenesis is the dominant effect of MSC therapy; consistent with this interpretation, VEGF antibodies reduced the vascular density in MSC-containing implants to control levels. Similar results were obtained for implants of collagen or fibrin gels that contained MSCs (11). In SVF-containing Matrigel implants, depletion of ECs from the SVF eliminated vasculogenesis, which implies that SVF-derived ADSCs are unable to form vessels (292).

Multicellular endothelial structures One way to hasten the generation of perfused microvessels in implanted scaffolds is to replace individual, dispersed ECs with multicellular structures that more closely resemble normal vascular networks. Allowing ECs to assemble into a network *in vitro* before implantation shortened the time to perfusion with blood *in vivo* by roughly a week; the total time between preparation of the EC-laden scaffold and perfusion remained ~2 weeks (82). Prevascularization of jejunum-based scaffolds for different culture times and subsequent abdominal implantation showed that longer times *in vitro*, up to 3 weeks, resulted in reduced vascular densities *in vitro* but greater densities *in vivo* (291). Thus, vessels that formed by

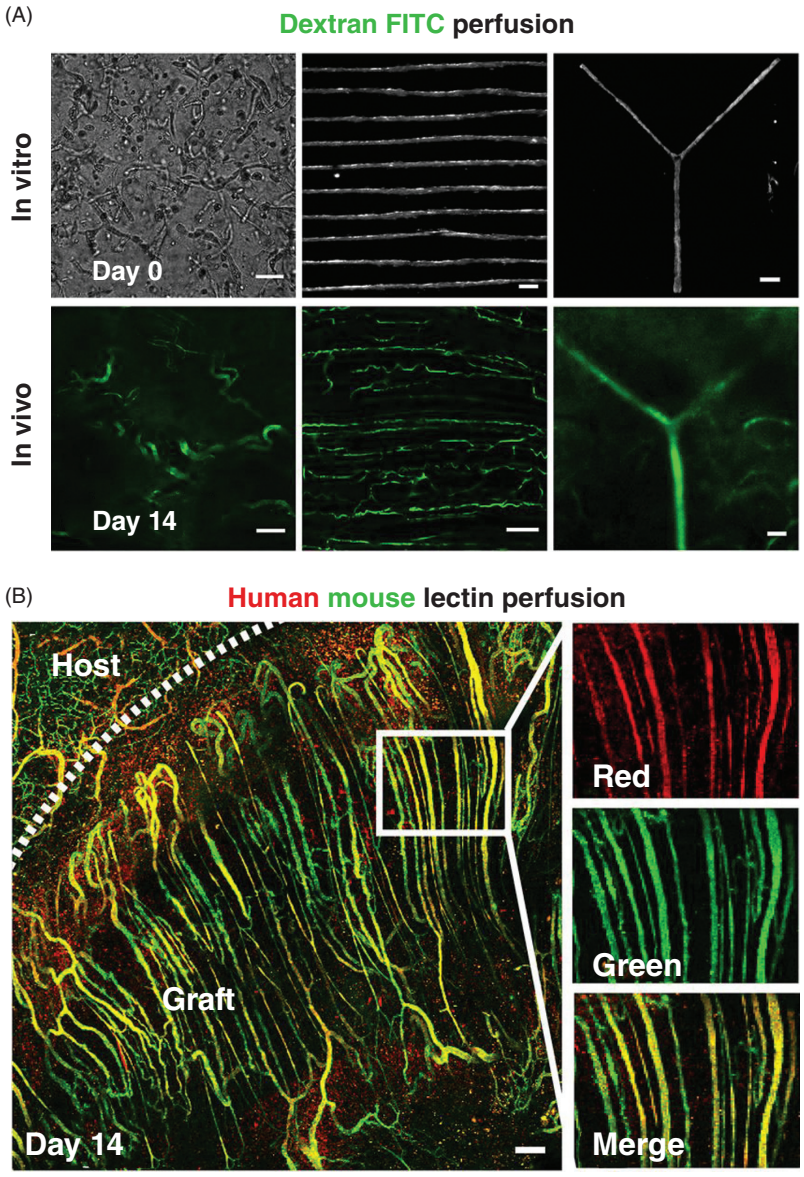

Figure 18 Transformation of endothelial aggregates into perfused vessels. (A) Random self-organized HUVEC networks and patterned HUVEC cords, before and after implantation. (B) Two-week-old implants after perfusion with species-specific lectins. Vessels in implants consist of ECs that are derived from graft (human, red) and host (mouse, green). Scale bars refer to 250 μm (A, *upper left*), 500 μm (A, *upper middle* and *upper right*), 100 μm (A, *lower*), and 150 μm (B). Reproduced, with permission, from (38).

vasculogenesis were not necessarily maintained *in vivo*. Other types of aggregated structures, such as EC spheroids, EC-coated microstructures, and patterned EC cords, have been used to generate vascular networks *in vivo* (Fig. 18) (4, 8, 38, 617).

A related strategy is to use freshly isolated microvascular fragments as the vascular source (Fig. 19). Here, the source tissue (primarily adipose tissue, as it is plentiful and accessible) is minced and digested to release microvessels that are collected and embedded within a scaffold. Subcutaneous, epicardial, or perimuscular implants of microvessel-containing collagen or polymer scaffolds showed perfusion near the scaffold surface after 1 to 3 days and deep within the scaffold after 10 to 14 days (316, 443, 507, 509). *In vitro* culture of microvessels before implantation appears to destabilize their mural coat and thereby render them more able to inosculate with host vessels upon implantation (317). Perfused vessels deep within a scaffold were almost completely derived from the implant, even after several weeks (507). Embedded microvessels secreted FGF2 and (to a lesser extent) VEGF, and the enhanced surface perfusion can be attributed partly to angiogenic ingrowth from the host (315, 316). Despite these promising results, the clinical potential of microvascular fragments is tempered by their reduced potency from older

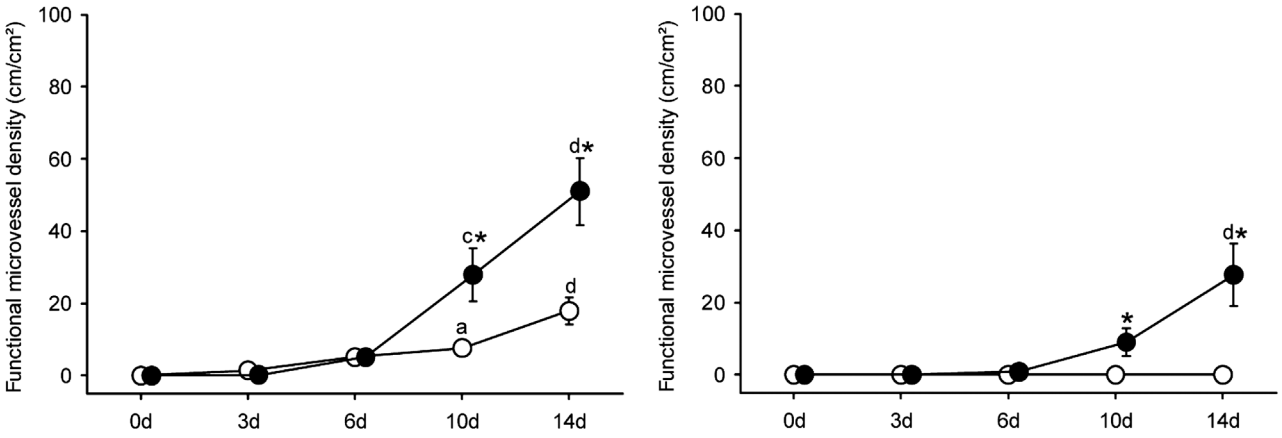

Figure 19 Increase in perfused vascular density over time in subcutaneous polymer implants that contained adipose-derived microvascular fragments (black circles) or that did not (clear circles). (*Left*) Vascular density near the surface of implants. (*Right*) Vascular density at the center of implants. Adapted, with permission, from (316).

donors and a tendency to generate intra-scaffold hemorrhage (315).

Engineering tissues by vasculogenesis

Engineering vascularized tissues by vasculogenesis has consisted largely of supplementing a scaffold with ECs or EOCs, mesenchymal cells, and the parenchymal cells of interest. Culture of *Bcl2*-transduced HUVECs or EOCs and keratinocytes on an acellular dermal scaffold resulted in a vascularized skin equivalent (493, 508); similar results were obtained with HUVECs, fibroblasts, and keratinocytes in a collagen-chitosan scaffold (592). When grafted on a wound, the scaffolds integrated well, with primarily human microvessels at the epidermal-dermal interface at day 4; in contrast, EC-free scaffolds were vascularized by angiogenesis, with host-derived vessels reaching the epidermis only after 2 weeks. A similar approach, but with both lymphatic ECs and blood vessel-derived microvascular ECs, resulted in artificial skin that contained functional blood and lymphatic networks (360). Subcutaneous or intramuscular implants of PLA/PLGA scaffolds that were prevascularized with HUVECs, fibroblasts, and myoblasts yielded vascularized muscle constructs after 2 weeks (334); when implanted around the femoral vessels, such constructs incorporated the vessels and could subsequently be transposed as a pedicled muscle flap (503). HUVECs, fibroblasts, and cardiomyocytes generated vascularized myocardial patches after 2 weeks (Fig. 20) (332); this strategy was successful even when the scaffold was solely cell-generated (547). Implants of EOCs, ADSCs (as mesenchymal support), and pancreatic islets or adipocytes in collagen-fibronectin scaffolds yielded vascularized islets or adipose, respectively (591).

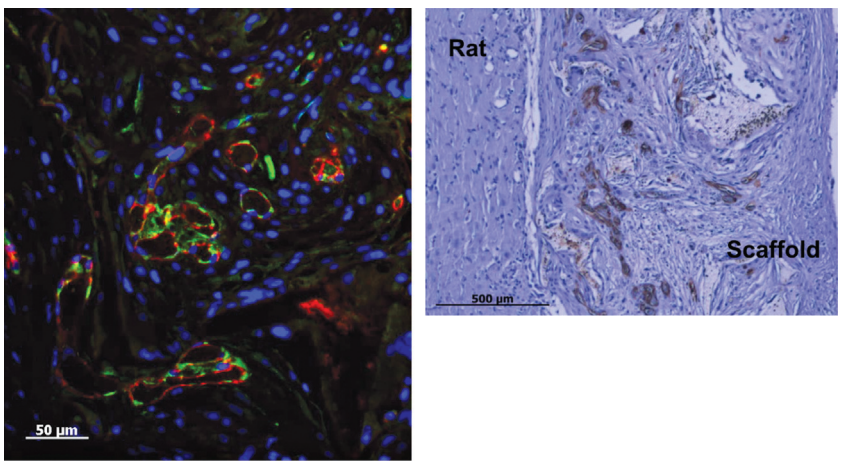

Figure 20 Vascularized myocardial constructs that contained human cardiomyocytes, HUVECs, and mouse fibroblasts in a PLA/PLGA sponge after 2 weeks. (*Left*) Immunostain for human CD31 (red) and von Willebrand factor (green). (*Right*) Immunostain for human CD31 (brown). Reproduced, with permission, from (332).

Multicellular EC aggregates can also serve as the vasculogenic cells when engineering tissues. For instance, incorporation of microvascular fragments into an artificial skin (Integra) increased perfusion of the implant by day 6, generation of blood vessels (nearly all implant-derived) and lymphatic vessels (~60% from the implant), and epithelialization of the wound by day 10; focal hemorrhage occurred at day 6 but did not appear to impede generation of vascularized skin (151). Similarly, implantation of fibrin gels that contained hepatocyte/fibroblast spheroids and preaggregated EC cords yielded human hepatic tissue that grew in response to host (mouse) liver injury (548).

Regardless of the specific vasculogenic approach used, one must keep in mind that a vascularized tissue is viable, but not necessarily functional. For instance, coimplantation of myoblasts and microvascular fragments in a collagen scaffold into a full-thickness muscle defect resulted in a well-vascularized construct that still underwent fibrotic transformation (336). Moreover, although the initial vascular networks (particularly deep within a construct) are generated by vasculogenesis, these vessels are often eventually replaced by angiogenic ingrowth. In a subcutaneous implant of HUVEC-coated, islet-containing collagen gels, the vessels within the implant transitioned from initially human-derived to largely host (mouse)-derived over 2 weeks, although this change did not seem to hinder the restoration of normoglycemia (617).

Vasculogenesis in microphysiological systems

The development of microfluidic technologies has enabled the perfusion of self-organized EC networks *in vitro* (218, 277, 395). In these studies, suspensions of ECs or EOCs (with or without mesenchymal cells) are allowed to organize into open networks within microscale extracellular matrix gels in a microfluidic device (Fig. 21A). By applying pressure across the ends of each gel, perfusion can be initiated within the nascent lumens once they make contact with the microfluidic channels (Fig. 21B). Careful attention must be paid to interfaces within the device to minimize leakage, and a combination of vasculogenesis within the scaffold and angiogenesis from the microfluidic channels generated leak-free junctions (625).

These perfused *in vitro* microvascular networks are primarily intended for microphysiological studies. For studies of cancer progression, tumor cells and candidate therapeutics can be delivered intravascularly in EC networks (79, 205, 243, 536). For studies of engineered tissues, parenchymal cells such as cardiomyocytes can be added to the self-organizing ECs and the resulting networks perfused to maintain tissue viability (483). The addition of C2C12 myoblasts or MSC-derived osteoblasts increased vascular permeability several-fold (243).

Physical signals in vasculogenic vascularization

The physical properties of a scaffold play an important role in determining the outcome of vasculogenesis *in vivo* and *in vitro*. Vasculogenesis in EC/EOC- and MSC-containing collagen and fibrin gels of different concentrations showed that intermediate scaffold concentrations of 5 to 10 mg/mL were optimal (11, 105, 286). Higher concentrations showed minimal vasculogenesis, and it appeared that these scaffolds resisted the remodeling needed to support EC migration and rearrangement. Lower concentrations resulted in vessels that could not resist hemorrhage, most likely because these scaffolds were mechanically weak. Correlation between vascular density and scaffold stiffness suggested that lower stiffness resulted in greater vascularization; it is not known whether a causal relation exists between the two, since it is difficult to vary stiffness independently of all other physical parameters.

Vasculogenesis in anisotropic environments invariably leads to generation of vessels that are preferentially oriented along the main axis of anisotropy. Such conditions can be induced by nonuniform contraction or strain of compliant scaffolds (298, 391, 472), by assembly of fibrous matrices in a magnetic field (392), by interstitial flow (198, 410), or by allowing EC self-organization to take place in a confined channel (455). The resulting aligned microvessels may prove

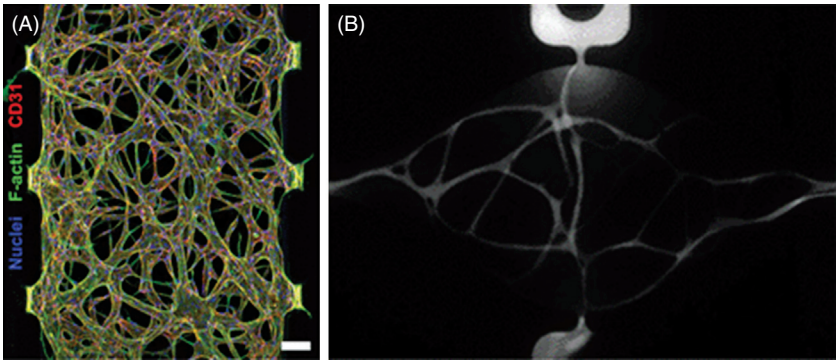

Figure 21 Perfused vascular networks in microscale fibrin gels within microfluidic devices. Fibroblasts, in the same or separate gel, were included to promote vasculogenesis. In (B), the resulting networks were perfused with a solution of fluorescent dextran. Reproduced, with permission, from (277, 395).

to be advantageous when engineering tissues that contain highly aligned vascular networks, such as in the heart, skeletal muscle, and kidney. Intramuscular implants that matched vascular and recipient tissue alignment yielded stronger grafts than those in which the vessels were perpendicular to the tissue fibers (472). On the other hand, isotropic vascular networks proved to be as effective as aligned ones in perfusing an epicardial patch (462).

As seen with angiogenic sprouting from flat EC monolayers in microfluidic devices, EC networks that are formed by vasculogenesis preferentially sprout against the direction of interstitial flow (275).

Microfluidic Vascularization

Microfluidic vascularization refers to the direct, physical construction of perfusable vessels in a graft or scaffold (583). Like vasculogenesis, this form of vascularization is intrinsic, since the vasculature is built into the graft or scaffold. Unlike vasculogenesis, however, no biological step of tubulogenesis is required for perfusable structures to form. Similarly, angiogenic sprouting is not required, although it can help enhance the ability of microfluidic structures to vascularize a region. Given that no natural biological analog of microfluidic vascularization exists, essentially all of the work in this area has originated from the surgical and engineering communities.

Flap-based revascularization

It has long been appreciated that the surgical vascular anastomosis of a single biological or prosthetic graft can bypass an arterial stenosis to revascularize an ischemic tissue. What is less appreciated is that tissue flaps, which contain intact microvascular networks, can perform the same function. Flaps based on the omentum through the gastroepiploic artery have been used to augment the circulation to the dog hindlimb (170) and brain (173); the flaps are partially transected to allow them to reach the extremities. In the ischemic human limb, omental flaps have been successfully used to salvage tissue that would otherwise be susceptible to amputation (172,217). This procedure relies on growth of vessels to connect the flap vasculature to that of the ischemic tissue, a process that is similar to the vascular growth that underlies the Vineberg procedure for revascularization of ischemic myocardium.

Similar flap-based approaches have been applied for the treatment of lymphedema. The same pedicled omental flaps that can salvage ischemic tissues also reduced the severity of edema in 30% to 50% of patients (171). In mice and rats, myocutaneous flaps prevented the development of lymphedema after ligation of tail lymphatics (534). Lymphatics display a strong ability to regenerate; even in free flaps, which have no lymphatic continuity with host lymphatics after implantation, bridging between the lymphatics in the flap and those in the distal tissue occurs quickly to mitigate the development of lymphedema (533). Fibrosis at the flap border appears to be the main impediment to lymphatic regeneration (31).

Engineering microvessels with microfluidic scaffolds

To apply a microfluidic approach beyond the tissue flaps that are surgically accessible, the ability to generate microfluidic scaffolds (those that contain perfusable channels or networks) is critical. These perfusable structures can then serve as templates for the growth of open microvessels.

Generation of microfluidic scaffolds

Lithographic methods In 2000, Vacanti and colleagues proposed that the micropatterning techniques that were (and are still) widely used in the microelectronics industry could be adapted to the patterning of biomaterials (249). Initial work focused on silicon micromachining, in which planar, tree-like patterns on an etched silicon wafer provided a template for the growth of ECs (Fig. 22); once the cells formed a monolayer, they could be detached from the silicon surface for further processing.

Subsequent studies focused on developing similar micropatterning technologies to build perfusable, and not just patterned, structures in more biologically relevant materials (65,90,168). These studies required the adaptation of silicone-based micropatterning techniques, such as those used to create microfluidic devices. This so-called "soft lithography" proved to be particularly well suited for patterning fragile biological materials, such as protein gels, because it takes place under ambient conditions without exposure to harsh chemical treatments (649).

One strategy for forming biomaterials that contain microfluidic networks (so-called "microfluidic biomaterials") relies on additive processing, in which two or more distinct parts are combined to form the microfluidic material. The earliest example of this strategy used micropatterned molds to form an alginate hydrogel on which a negative pattern was

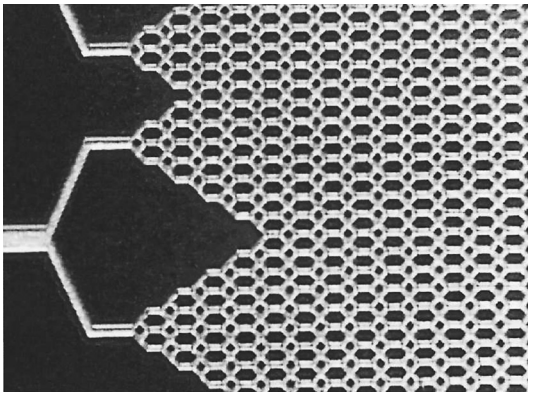

Figure 22 Branching vascular-like pattern that was etched into a silicon wafer. Such patterns could be seeded with ECs to generate patterned cultures. Feature widths were on the order of ~10 μm. Adapted, with permission, from (249).

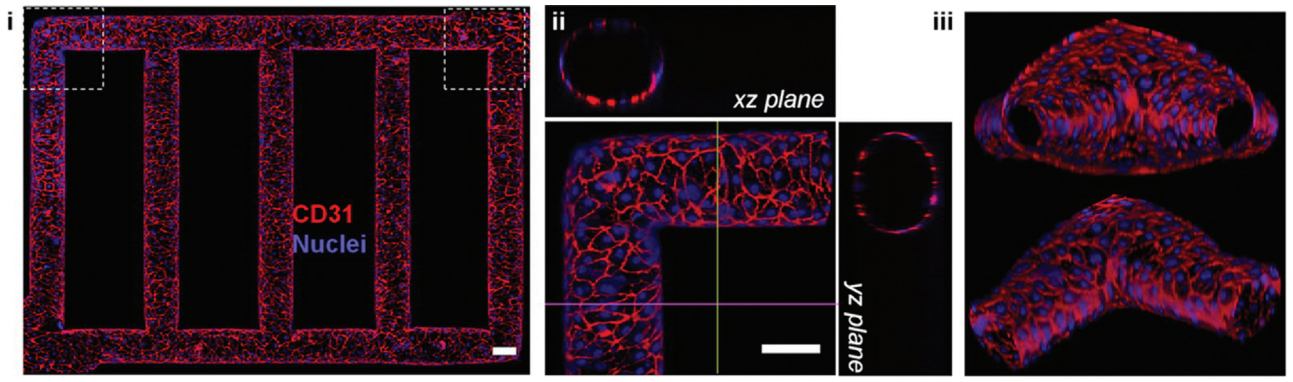

Figure 23 Vascularization of a microfluidic type I collagen scaffold that was formed by combining a micropatterned and planar gel. Both *en face* and reconstructed 3D views of HUVEC-seeded structures are shown. Scale bar refers to 100 μm. Reproduced, with permission, from (677).

replicated (65, 89). Coupling the patterned gel to a second, flat gel generated a composite that contained internal channels through which fluid could be pumped. Since alginate is not adhesive to ECs, these channels were left unvascularized, but they were able to sustain the metabolism of bovine chondrocytes that were embedded within the alginate (89). Similar avascular microfluidic hydrogels provided exchange of solutes with an underlying porous substrate (66). Adaptation of the same design to type I collagen gels and agarose-gelatin gels required the development of methods to secure two separate gels so that they would not detach under perfusion (448,453,677). When seeded into the collagen channels, individual ECs rapidly attached and proliferated to line the channels with a monolayer of cells (Fig. 23).

A second strategy is based on negative processing, in which material is removed to leave behind channels. Many removable materials have been investigated, including waxes (578, 611), gelatin (168), thermoreversible polymers (648), and metals (90). The negative material is first patterned, either by micromolding or direct printing, before being encapsulated in a scaffold that is compatible with EC adhesion. Removal of the patterned material (e.g., by raising the temperature to melt the material and allow it to be flushed out) results in a scaffold that contains channels. In scaffolds that are made of extracellular matrix proteins, seeded ECs grow to line the channels (Fig. 24). Alternatively, vascular cells can be grown on matrix fibers that are later digested to yield open lumens (39).

The widespread availability of laser and inkjet printing, and recent advances in three-dimensional (3D) printing technologies, have led to the exploration of methods that can directly draw 3D scaffolds that contain channels for vascularization (385). Early work retrofitted commercial inkjet cartridges so that they contained hydrogel precursor solution with or without ECs (55,637). Printing an "ink" that consisted of thrombin droplets onto a solution of fibrinogen caused local polymerization of fibrin; rasterizing the ink ejection point in 3D yielded fibrin structures that contained channels (107). The resolution of these structures was low, and channels had corners and were bowed inward, in part because the inks tended to diffuse quickly in the medium that they were printed into. Printing inks of higher viscosity allowed the printed structures to hold their shape while the surrounding material set in place (51, 648). Switching between different ink cartridges, each of which contains a unique solution, allowed 3D printing of different cell types and materials (254). As long as one of the inks is compatible with EC adhesion, it is possible to generate perfused microvessels in 3D-printed scaffolds (with the caveat that "microvessel" often refers to structures

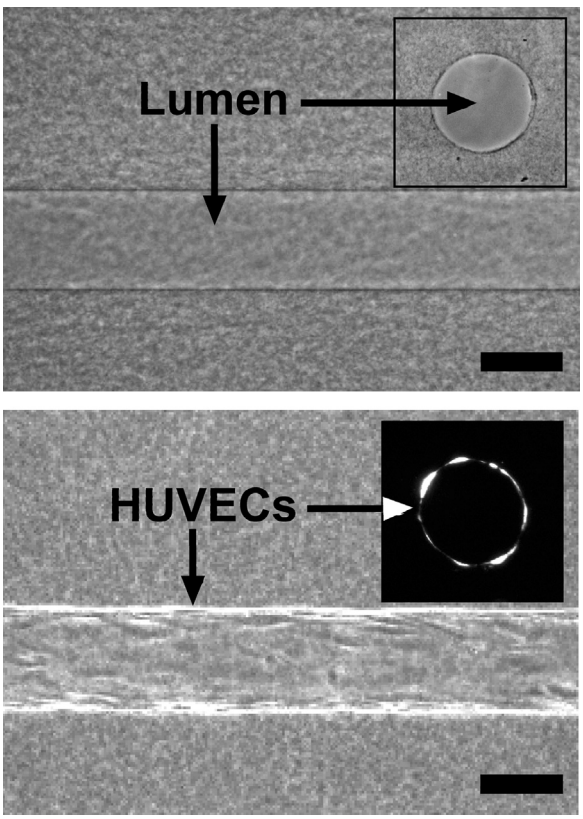

Figure 24 Vascularization of a microfluidic type I collagen scaffold that was formed around a removable needle. (*Top*) Unseeded scaffold. (*Bottom*) Scaffold that was seeded with HUVECs. Insets show cross-sectional views. Scale bar refers to 100 μm. Reproduced, with permission, from (90).

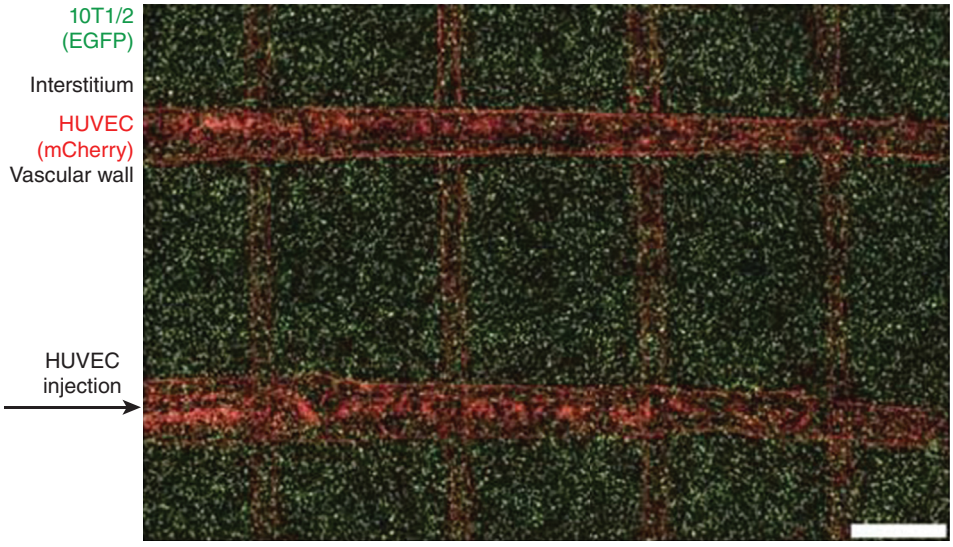

Figure 25 Vascularization of a microfluidic fibrin scaffold that was formed around a sacrificial 3D-printed network of sugar-based fibers. Channels were seeded with HUVECs (red), and the scaffold bulk contained 10T1/2 mouse fibroblasts (green). Scale bar refers to 1 mm. Reproduced, with permission, from (382).

much wider than the 100 µm limit typically encountered in the microvascular physiology community); sacrificial inks to define the vascular architecture include gelatin, Pluronic block copolymers, and glassy mixtures of saccharides (Fig. 25) (294, 295, 330, 331, 382). In general, the versatility of 3D printing is unmatched, and it excels at drawing complex customized shapes at the scale of whole organs. Nevertheless, the resolution and speed of printing techniques remain below those of micromolding approaches; in particular, routine construction of interconnected channels with appropriate cross-sectional shape for microvascular engineering remains a challenge.

Optical methods have also been explored for generating microfluidic scaffolds in photosensitive materials. The material can either be photopolymerized to create solid regions that together encompass the desired channels, or photodegraded to create channels within a preformed solid. For instance, polyethylene glycol gels that contained photodegradable acrylates in the polymer backbone enabled complete degradation of the gel with UV light (284). Focusing and scanning a UV source into a preformed gel with a two-photon microscope allowed channels and other complex structures to be drawn into the gel. At a sufficiently high intensity, such as that available with pulsed lasers, channels can be ablated into collagen (17). As with 3D printing, the pixel-by-pixel drawing of patterns into or onto photosensitive materials can be slow. Seeding of optically patterned scaffolds with ECs yielded microvascular networks (Fig. 26) (196).

Decellularization of organs An entirely different method of creating microfluidic scaffolds treats tissues with specialized solutions that decellularize the tissue, leaving the original extracellular matrix intact (32). These solutions typically contain detergents to solubilize cell membranes, chaotropic agents to disrupt intermolecular bonds, and enzymes to digest DNA and RNA. Early work used

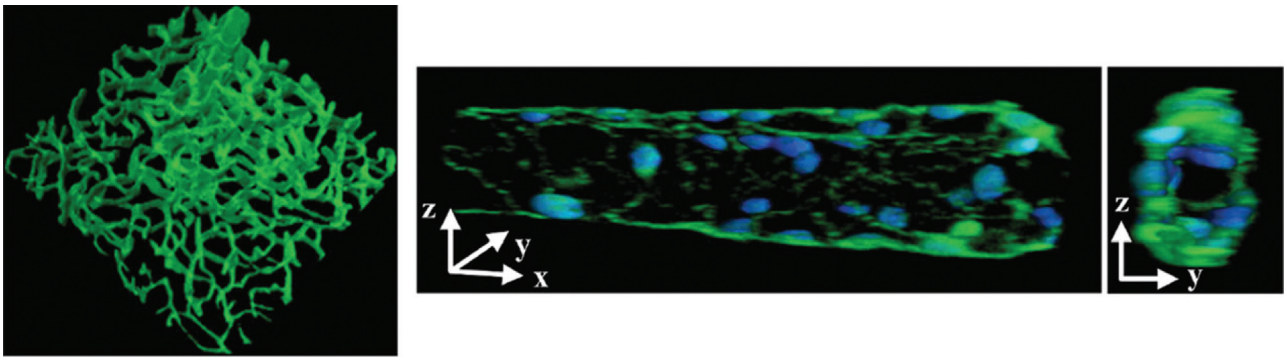

Figure 26 Vascularization of a microfluidic polyethylene glycol gel that was patterned by photodegradation. (*Left*) Visualization of interconnected channels by perfusion with fluorescent dextran. (*Right*) 3D confocal reconstruction of a vascularized channel that was stained for ZO-1 (green). Reproduced, with permission, from (196).

decellularization mainly to reduce the antigenicity of xenografts, such as pig heart valves (163). While such decellularized tissues retained the geometry of any vascular networks that they contained, it was not obvious how these channels could be accessed, given the low mechanical stiffness of decellularized tissue. Moreover, because these tissues were decellularized by immersion in the appropriate solution, diffusion-limited transport meant that decellularization was practical for small tissues only, on the order of one centimeter in size and smaller.

The potential of decellularization to generate microfluidic scaffolds for vascularization was only realized in 2008, when Ott and Taylor showed that an entire heart could be perfused with decellularization solutions (Fig. 27) (424). As with small tissues, decellularization of the heart yielded the extracellular matrix, but now as a perfusable structure and with organ-scale size and shape. It is important to appreciate that the architectural complexity of this type of 3D microfluidic scaffold is far beyond anything that can be engineered by lithography. Subsequent work demonstrated that other whole organs and large tissues—lung (423,437), liver (606), kidney (538), pancreas (166), small intestine (374), and skin flap (200)—could be perfusion-decellularized.

Vascularization of microfluidic scaffolds

Whether created by lithography or decellularization, microfluidic scaffolds are vascularized by perfusing a suspension of ECs into the channels *in vitro*. ECs are able to distribute through channels that are wider than ~30 μm and grow to form perfusable vessels (90). In narrower channels, ECs can form plugs, which require additional signals to promote cell migration along the channels (346). Seeding lymphatic ECs into blind-ended channels generated lymphatics (581). An alternate strategy used electrical desorption to transfer a monolayer of ECs from a thin metal rod onto a channel in collagen gel (502).

When formed in microscale extracellular matrix gels, these microvessels have been used in microphysiological systems (641). Since the vessel wall can be viewed in side

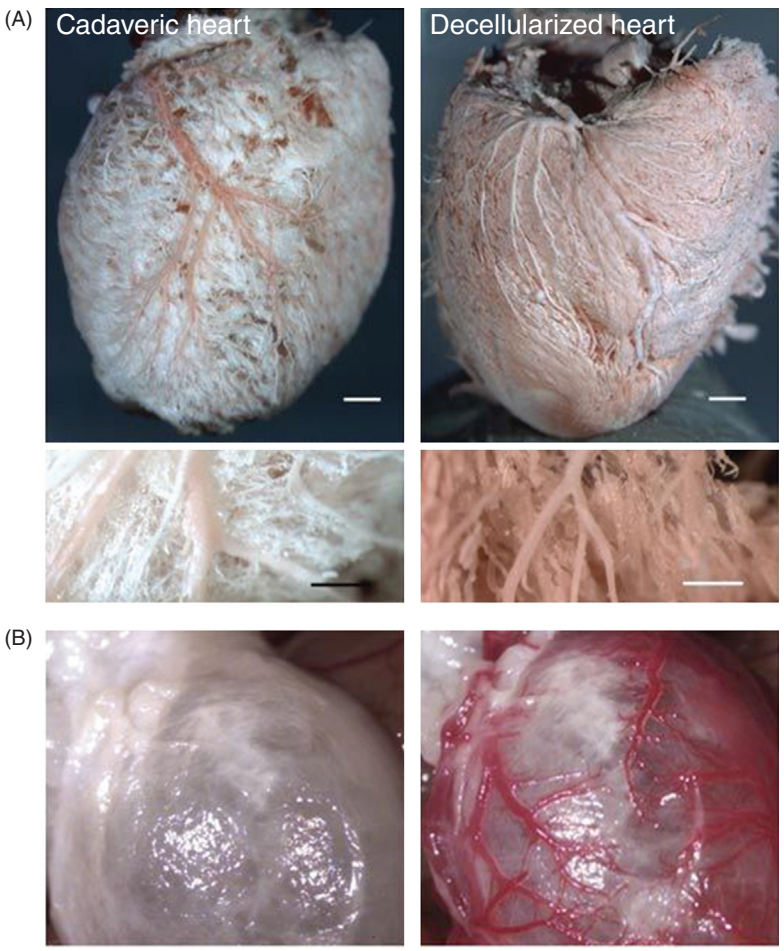

Figure 27 Perfusion-decellularization of whole hearts preserves the vascular architecture. (A) Corrosion casts of native and decellularized hearts. Scale bars refer to 1 mm (*top*) and 250 μm (*bottom*). (B) Transplanted decellularized heart before and after reestablishment of blood flow. The recipient animal was heparinized to minimize thrombosis. Reproduced, with permission, from (424).

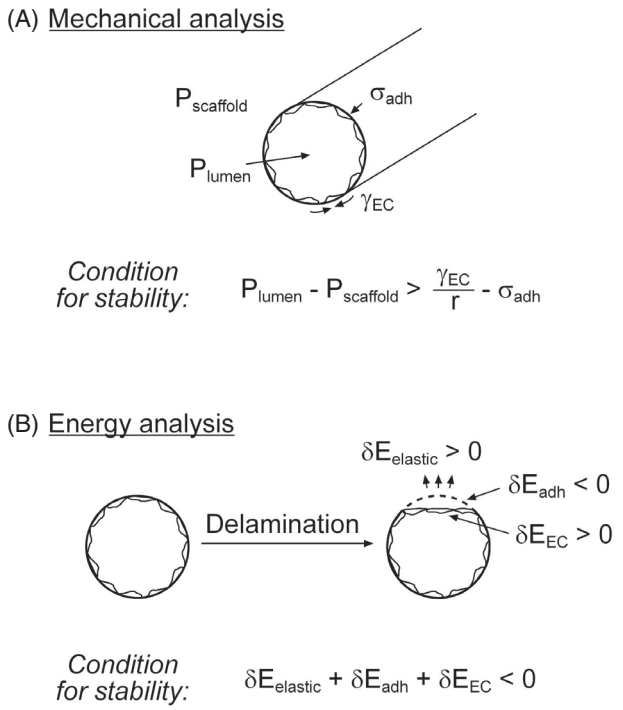

Figure 28 Physical mechanisms for stable vascularization of microfluidic scaffolds. Maintenance of vascular adhesion can be viewed as (A) a balance of outward (stabilizing) and inward (destabilizing) stresses, including pressures, contractile stress, and adhesion stress, or as (B) a balance of adhesion (stabilizing) and stored elastic (destabilizing) energies. Adapted, with permission, from (643).

profile and at high resolution, such vessels are particularly well-suited for studies of vascular permeability, inflammation, and angiogenesis (90,273,411,447,677). Solute permeability tends to be comparable to those of explanted microvessels and up to an order-of-magnitude higher than those observed *in vivo*. Vessels in these gels can also be used to perfuse microscale biomaterials and tissues (64,246,340). Skin equivalents that incorporate vessels can be used to study vascular absorption of topically applied substances (390). The addition of adipocytes to the gel increased vascular permeability (340).

Application of such vessels *in vivo* has been challenging. Subcutaneous implants of vascularized microfluidic gels were able to provide perfusion to the ischemic hindlimb; surprisingly, only channels of diameter 400 μm were effective (383). Because these implants were not surgically anastomosed to the recipient circulation, perfusion relied on angiogenic sprouting and inosculation with host vessels, and the advantage over implants of EC cords or microvessel fragments is unclear. The near-impossibility of suture anastomosis with vessels of ~100 μm diameter has led to the exploration of ways to couple these vessels to larger ones that can be more easily stitched (341,540,668).

Perfusion of decellularized organs with ECs has yielded vascular networks in all organs tested *in vitro*. Methods to enhance EC coverage include precoating the decellularized channels with antibodies to CD31 (287), or changing the position of the organ during seeding (542). In general, EC coverage does not appear to be complete, but is sufficient to sustain blood flow *in vivo* for several hours. Orthotropic implants of revascularized lungs provided functional gas exchange in rats for 2 h, albeit with intra-alveolar hemorrhage (437).

Physical signals in microfluidic vascularization

Because the vascular geometry can be readily controlled in microfluidic scaffolds, these structures enable routine study of how physical signals affect vascularization. Long-term maintenance of the EC lining appears to depend on the mechanical stress at the EC-scaffold interface. Signals that reduce this stress—increased vascular pressure, decreased scaffold pore pressure, reduced EC contractility, decreased vascular permeability—promote vascular stability (Fig. 28A) (333,449,642,644). Scaffold crosslinking also increases vascular stability, in part by reducing the amount of elastic energy available to detach the EC layer (Fig. 28B) (76). High flow promoted vascularization by decreasing vascular permeability and increasing transendothelial pressure (449).

Conclusions

Methods for microvascular engineering are now quite sophisticated and have led to numerous positive preclinical findings, although translating those studies to clinical practice remains a challenge. Each decade has introduced a new approach: angiogenesis in the 1980s, vasculogenesis in the 1990s, and microfluidic vascularization in the 2000s. History has shown that roughly a decade of preclinical development takes place before clinical trials begin; thus, one expects the first trials of microfluidic scaffolds to be proposed soon.

Several themes have emerged from these decades of research. First, combinatorial approaches work better than single strategies. Synergies between direct and indirect angiogenic factors, between vasculogenic and stromal cells, and between angiogenesis and vasculogenesis have shown that using multiple ways to form microvessels usually leads to more robust results. These synergies suggest that it may be just as fruitful to design therapies based on the removal of factors that inhibit vascularization, rather than on the addition of ones that promote it. The recent development of microfluidic vascularization will suggest new combination therapies.

Second, vascular persistence is not guaranteed. Whether formed by angiogenesis or vasculogenesis, microvessels tend to regress over time *in vivo* and become replaced by host-derived vessels. In microfluidic scaffolds, which provide a template for vascular growth, the vessels must receive additional perfusion- or scaffold-derived signals to be stable over the long term *in vitro*. Since vascular densities are tightly matched to metabolic needs *in vivo*, engineering persistent vascularization may be easier in the presence of metabolically active parenchymal cells. The challenge is that parenchymal

cells need vessels to survive, so which component to make first poses a tough dilemma. Perhaps an iterative process of growing vessels and parenchyma in alternating stages would yield more durable vascularization, compared to forming vessels exclusively first.

Third, comorbidities affect the vascular outcome. Old age, and the chronic conditions that often accompany it, greatly reduces the effectiveness of vascularization therapies. To increase chances of clinical success, trials in younger and/or healthier patients may be advantageous.

Fourth, vascularization is necessary, but can be insufficient, for tissue functionality. Having a sufficient vascular density only enables survival of a tissue. For simple tissues, such as skin and adipose, creating vascularized "flesh" is enough. For more complex tissues, such as lung, liver, and kidney, the vessels provide functions beyond perfusion. In many tissues, the vessels display organ-specific fenestra, discontinuities, or tight junctions, and organ-specific 3D architectures (267); the endothelium can secrete soluble factors that regulate parenchymal function (454).

How to obtain tissue-specific vascular architecture and function remains a mystery. It seems likely that the use of pluripotent stem cell-derived ECs will increase, especially since such stem cells may provide a route to obtain tissue-specific ECs, which tend with dedifferentiate upon extended culture *in vitro*. A very recent study demonstrated that iPSCs can be differentiated into ECs that form perfusable vessels with a blood-brain barrier (347). Robust methods to generate other types of tissue-specific ECs and to preserve their phenotype upon assembly into vessels are sorely needed. Greater emphasis on the entire suite of microvascular functions, including autoregulation of blood flow, antigen presentation, and enzymatic processing of hormones, may help shed light on this issue.

Acknowledgements

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