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Building Vascular Networks

Hojae Bae[1,*], **Amey S. Puranik**[2,*], **Robert Gauvin**[3], **Faramarz Edalat**[4,5], **Brenda Carrillo-Conde**[2], **Nicholas A. Peppas**[2,6,7], and **Ali Khademhosseini**[4,5,8,†]

[1]Department of Maxillofacial Biomedical Engineering and Institute of Oral Biology, School of Dentistry, Kyung Hee University, Seoul 130-701, Republic of Korea

[2]Department of Chemical Engineering, University of Texas at Austin, Austin, TX 78712, USA

[3]Quebec Center for Functional Materials (CQMF), Université Laval, Québec, QC GV1 0A6, Canada

[4]Center for Biomedical Engineering, Department of Medicine, Brigham and Women's Hospital, Harvard Medical School, Cambridge, MA 02139, USA

[5]Harvard–Massachussetts Institute of Technology (MIT) Division of Health Sciences and Technology, MIT, Cambridge, MA 02139, USA

[6]Department of Biomedical Engineering, University of Texas at Austin, Austin, TX 78712, USA

[7]College of Pharmacy, University of Texas at Austin, Austin, TX 78712, USA

[8]Wyss Institute for Biologically Inspired Engineering, Harvard University, Boston, MA 02115, USA

Abstract

Only a few engineered tissues—skin, cartilage, bladder—have achieved clinical success, and biomaterials designed to replace more complex organs are still far from commercial availability. This gap exists in part because biomaterials lack a vascular network to transfer the oxygen and nutrients necessary for survival and integration after transplantation. Thus, generation of a functional vasculature is essential to the clinical success of engineered tissue constructs and remains a key challenge for regenerative medicine. In this Perspective, we discuss recent advances in vascularization of biomaterials through the use of biochemical modification, exogenous cells, or microengineering technology.

Tissue engineering research that combines cells and materials (biomaterials) is one of the most promising avenues to addressing the limited supply of organs for transplantation (1). Bioengineers are currently on the lookout for biomaterials that induce the formation of a vascular network. Vascularization strategies are crucial for the in vitro synthesis of complex tissues and organs for transplantation, because—unlike engineered skin, cartilage, or bladder tissue—cell viability and optimal function of the construct cannot be sustained through diffusion alone.

The formation and long-term survival of blood vessels within a material requires the integration of biochemical and biophysical cues. Moreover, proper maturation of an in vitro–generated vasculature is crucial for the in vivo success of engineered tissues (2). Vascular growth and remodeling are coupled with developmental and wound-healing processes and have been implicated in the progression of various pathological states, such as

[†]Corresponding author. alik@rics.bwh.harvard.edu.
[*]These authors contributed equally to this work.

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inflammation, cardiovascular diseases, and cancer. Most of these processes involve endothelial cells, which line the interior of blood vessels and form a thin layer of cells called the endothelium. This vasculature maintains tissue homeostasis by delivering the required oxygen and nutrients and removing waste products.

Incorporation of a microcirculation into engineered tissues presents multiple challenges, including the formation of microscale vascular conduits for blood flow, a functional endothelium that regulates vascular activity, and specialized cell types that perform the physiological function of the tissue of interest. Several approaches have been developed to address these challenges, including (i) the incorporation of biomolecular cues (3, 4) within the material, (ii) the seeding of vascular or vascular-inducing cells in the scaffold (5), or (iii) the use of microfabrication technologies to engineer branched microfluidic channels within biocompatible materials (6). Successful application of these approaches, either individually or in combination, is expected to enhance therapeutic opportunities by building functional tissue and organ systems for regenerative medicine.

VASCULOGENESIS AND ANGIOGENESIS

To engineer vascular constructs de novo or induce vascularization from preexisting blood vessels and capillaries, one must understand the molecular mechanisms of blood vessel formation in vivo. During embryogenesis, angioblasts migrate to various regions of the developing embryo and differentiate into endothelial cells in response to local cues [for example, growth factors and extracellular matrix (ECM) components]; the endothelial cells then form a vascular plexus—a network built by connections (anastomoses) between blood vessels (7) (Fig. 1). But this process—called vasculogenesis—is not limited to the embryonic period and can occur in adults through the recruitment and participation of bone marrow–derived endothelial progenitor cells (8, 9).

Another mechanism of blood vessel formation is through the sprouting of existing blood vessels, a process known as angiogenesis (10) (Fig. 1). Angiogenesis is a sequential, multistep process that begins with activation of a quiescent endothelium by angiogenic factors—vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), and angiopoietin-2 (ANG-2)—released from hypoxic or tumorigenic tissues. This step spurs degradation of the basement membrane—a thin layer of ECM between the epithelial cell layer and the endothelial cell lining of blood vessels—through up-regulation of matrix metalloproteinases (MMPs) followed by migration of an endothelial “tip cell” from the leading edge of a vascular sprout; this leading edge defines the direction of the newly growing sprout. Migration is mediated by signaling through proteins on the endothelial cell surface such as integrins, NOTCH [delta-like ligand 4 (DLL4) and JAGGED1], VEGF receptors, and neuropilins. Then, stimulated by VEGF, vascular endothelial (VE)–cadherin, and Hedgehog proteins, the endothelial cells adjacent to the tip cells begin to proliferate and elongate to form capillary sprouts, which then assemble to form a vessel lumen.

After the activation and proliferation stages, a nascent blood vessel must mature to become functional. Maturation occurs through signals such as transforming growth factor–β (TGF-β), platelet-derived growth factor B (PDGF-B), ephrin-B2, and NOTCH, which together cause vascular smooth muscle cells (pericytes) to cover and stabilize the endothelial cell channels in a process known as arteriogenesis (7). Tissue-engineering strategies may benefit from generating materials that can guide these biological events in the formation of vascular networks.

BIOMATERIALS FOR VASCULARIZATION

Biochemical modification of scaffolds

The considerable amount of research on angiogenesis has been pivotal in developing solutions for vascularization challenges associated with most tissue constructs and has made growth factor incorporation one of the primary material vascularization strategies investigated for three-dimensional (3D) engineered tissues (11–13). A large body of work attempting to elicit angiogenesis with the use of growth factors has shown that synthetic and natural scaffolds functionalized with growth factors such as VEGF, basic FGF (bFGF), and PDGF could trigger the formation of vascular structures when implanted in vivo (14, 15). Although these proangiogenic molecules lead to sprouting of capillary beds within the constructs, they lack the ability to direct growth of blood vessels or enable interconnectivity between the capillary networks prior to implantation.

The need for materials that could be chemically and mechanically tailored to transport and release bioactive molecules—while meeting regulatory biocompatibility and biodegradability standards—has spawned further research into the use of hydrogels (hydrated materials made from a cross-linked network of hydrophilic polymers) as scaffolds for engineered tissue constructs (16). The clinical success of these hydrogels depends on the integration of factors that induce rapid endothelial cell ingrowth and that stabilize the vascular network as it forms.

In addition to the classic angiogenic growth factors and peptides, recent research has evinced the multifaceted roles in angiogenesis played by other molecules, which may serve as tools for biomaterials innovation. For instance, an in vivo study by Phelps *et al.* made use of a poly(ethylene glycol) diacrylate (PEGDA)–based degradable scaffold that housed a panel of responsive elements, such as cross-links that were cleavable by proteases, RGD cell adhesive domains, and conjugated VEGF (17). In vitro studies in which NIH3T3 fibroblasts were seeded in these hydrogel scaffolds demonstrated that both adhesive ligands and MMP-degradable sites were necessary for cells to spread. Furthermore, upon implantation into a mouse model of hind-limb ischemia, VEGF-conjugated scaffolds resulted in rapid vascularization of the biomaterial that remained stable for at least 4 weeks.

The formation of stable vascular networks can be attributed to the widespread and prolonged availability of VEGF, likely a result of the controlled release feature of MMP-degradable implants. Diffusing VEGF was able to meet the proliferative demands of the host endothelial cells that infiltrated the scaffold aided by scaffold-associated RGD-based adhesive molecules. Consequently, endothelial-cell invasion and proliferation within the scaffold was shown to enhance the perfusion of newly formed vessels.

New data that demonstrate the importance of ephrin receptors in the mediation of cell adhesion, repulsion, and migration have inspired its use in scaffolds designed to promote vascularization (Fig. 2A). For example, Saik *et al.* developed MMP-sensitive PEGDA hydrogels immobilized with ephrin A1 ligands, which stimulate a wide range of receptors that induce vascularization (18). The efficacy of ephrin A1 ligand conjugation was demonstrated 14 days after implantation, by comparing vascular network parameters—vessel density, branch points, and lacunarity—of MMP-sensitive, PDGF-BB–containing PEGDA hydrogels with or without immobilized ligand. The biodegradable, bioactive hydrogels immobilized with ephrin A1 ligand produced a denser vasculature in the mouse cornea pocket relative to the non–ligand-containing scaffold.

Another approach to creating a conducive microenvironment for vascularization is the use of natural-derived materials. For example, a fibrin matrix that has been enzymatically

conjugated with two multifunctional recombinant fibronectin (FN) fragments capable of binding growth factors such as VEGF, PDGF and bone morphogenetic protein-2 (BMP-2) and integrins such as $\alpha_5\beta_1$ and $\alpha_v\beta_3$ has been shown to be a potent proangiogenic material (19). FN III9-10/12-14–functionalized fibrin matrices enhance wound healing responses in diabetic rats via localization of growth factors and integrins, which in turn boost angiogenesis through endothelial cell recruitment and maintenance of neovessel integrity by smooth muscle cells and mesenchymal stem cells (MSCs) in the tissue.

The improved interaction between growth factors and integrins resulting from the customized FN are mainly responsible for neovascularization in cutaneous wounds and superior granulation tissue morphogenesis in bone tissue repair. Combining scaffolds with recombinant proteins that can locally implicate native biomolecules paves the way for in vivo neovascularization of host tissue. For both safety and efficacy purposes, this controlled and localized delivery of growth factors may be preferable to loading scaffolds with randomly encapsulated or chemically immobilized growth factors.

Because blood vessel formation is a highly organized process in vivo, the spatial and temporal presence of specific signaling molecules is an important aspect of the biochemical modulation of biomaterials. Thus, it is crucial to engineer materials with properties that mimic the in vivo complexities of the blood vessel formation process. For instance, the sequential expression of specific angiogenic molecules, as well as the patterned localization of these molecules within 3D spaces can be important for enhancing biochemical modulation approaches. Fortunately, these goals are within reach as a result of advances in controlled-release technology (20, 21), novel chemistries, and micro- and nanoengineering tools such as micromachining, photolithography, and molding (6, 22).

Cell-based approaches

Because of the emergence of new cell sources, such as stem and progenitor cells, there is an increased focus on strategies that involve encapsulation of endothelial cells and other supporting cell types within biomaterials. Such an approach can take advantage of cell signaling, differentiation, and migration as well as the dynamic interactions between cells that provide the biochemical environment required for the ensuing tissue remodeling.

Initial work on the prospective merits of such a cell-based approach for vascularizing skin tissue made use of a coculture of human umbilical–vein endothelial cells (HU-VECs) and human-derived fibroblasts and keratinocytes (skin cells) housed within a collagen-based scaffold (5). Biochemical coordination between the ECM generated by the fibroblasts and growth factors such as VEGF and TGF-β secreted by neighboring cells in the presence of HUVECs promoted the development of a vascular network both in vitro in the experimental model and in vivo after implantation in a mouse model (23, 24). Furthermore, recent studies highlight the benefits gained from the addition of epithelial cells—those that line the body cavities such the gut and lungs—to stabilize and regulate the size and formation of capillaries within the vascularized model. Thus, various cell types can play a complementary role in tailoring vascularization in vitro (25).

Successes with the use of cocultures of HUVECs and other cell types for vascularizing skin tissue has paved the way for application to other tissue types that require denser vascular networks. For instance, Alajati *et al.* validated the concept of implanting a coculture of endothelial cells with ECM-forming fibroblasts or bone-forming osteoblasts in vivo (26). HUVECs and osteoblasts were encapsulated within a scaffolding material composed of VEGF and FGF-2 within Matrigel (a murine tumor–derived material that comprises ECM proteins and glycosaminoglycans), fibrin, and thrombin, and the biomaterial was implanted subcutaneously in a severe combined immunodeficiency (SCID) mouse model for up to 20

days. Subsequent analysis showed this approach was capable of forming a durable perfused vascular networks in vivo. Moreover, incorporation of FGF-2 in the matrix before implantation provided molecular assistance to cell-secreted VEGF in conferring stability upon the developing vasculature after biomaterial implantation. Stability of the vascular network in the implant was shown to be a consequence of the interaction of host mural cells with the perfused vasculature, which remained functional for up to 60 days.

The development of stable vascular networks was also observed in mice when HUVECs and fibroblasts were combined with mouse myoblasts for vascularization of skeletal muscle tissue (27) and with cardiomyocytes for vascularization of cardiac tissue (28). In still another study, HUVECs were used to promote the differentiation of human MSCs into an endothelial lineage, promoting the formation of 3D vascular structures for up to 2 weeks in a Matrigel-based ECM (29). Similarly, the combination of HUVECs and human MSCs seeded in a polymeric scaffold were shown to form mature vascular networks in vivo 4 to 7 days after implantation, thus accelerating the functional remodeling of the implant when used as a bone graft (30).

For cell-based therapies, the scaffold is a crucial component that regulates the dynamic vascularization process. Research in this area has made use of either natural biodegradable materials, such as collagen and Matrigel, or synthetic biodegradable scaffolds, such as poly-L-lactic acid (PLLA) or poly-D,L-lactic-co-glycolic acid (PLGA). However, long-term evaluations of these materials have reflected shortcomings in their mechanical strength, durability, and even immunogenicity, thereby creating an opportunity for the creation of more suitable scaffolds for endothelial cell–based vascularization. It is possible that the inherent limitations of previously investigated natural scaffolds can be overcome by incorporating biochemical functionality, within natural hydrogels, that controls mechanical strength and rate of biodegradation, thereby combining the advantages of natural scaffolds with robust synthetic materials.

For instance, mechanically robust gelatin-based photocrosslinkable hydrogels can encapsulate and allow synergistic interaction between endothelial colony–forming cells (ECFCs) and α-smooth muscle actin (α-SMA)–expressing MSCs to form a microvasculature in vitro and in vivo that is composed of endothelial cell–lined functional blood vessels (Fig. 2C) (31). Furthermore, this microvasculature abets perfusion of red blood cells in the tissue construct in vivo after biodegradation of the hydrogel scaffold, the rate of which was controllable by the degree of methacrylation of the polymer and the extent of cross-linking in the material (Fig. 2D).

Microengineering strategies

With the emergence of microengineering technologies, it is becoming increasingly possible to recreate vascularized materials through microfabrication-based approaches. The advantages include the ability to control the initial architecture of the microvascular network and enable immediate perfusion through the scaffolds. Microfabricated devices with integrated microvasculature can be optimized to provide a uniform distribution of fluid flow and mass transfer across the scaffolding material; thus, cells are provided with an adequate supply of nutrients in capillary channels ranging from a few millimeters down to micrometers. Such fabrication techniques benefit greatly from the advances in noninvasive imaging technology that can be used to aid the design and function of microengineered vascular networks (Fig. 2B) (32).

To engineer a functional vasculature network, endothelial cells could be seeded in microengineered scaffolds containing conduits to form a confluent endothelium on the walls of the vascular channels (33). In one study, a 3D tissue construct composed of

endothelialized hollow vascular structures was produced using a self-assembled monolayer (SAM)–based cell deposition technique and a hydrogel photocrosslinking method to provide a robust hydrogel-based scaffold for endothelial cell attachment (Fig. 2E) (34). Moreover, Chrobak *et al.* validated the hypothesis that the existent flow and shear conditions within such microscale channels are favorable for endothelium sustainability (35). These studies have demonstrated that microfabricated tissues composed of a fluidic network designed to mimic the human vasculature may one day be inosculated in vivo.

Microengineering techniques such as photolithography (a process that uses light illumination through a mask to generate structures from light-sensitive materials) and molding (a process that uses a hollowed-out pattern to which a deposited material conforms) are inherently planar; therefore, 3D structures mostly result from stacked 2D structures that comprise channels with rectangular cross sections instead of channels with the circular cross sections ubiquitous in nature (36). Microengineering techniques such as direct ink writing and omnidirectional printing within a gel reservoir have recently been developed to create 3D vascular structures in vitro (37, 38). Despite their enormous potential, these approaches still await further improvements in the integration of other specialized cell types required to enable functionality of the tissue structures that surround the vascular network. In a recent study, a printing approach was used to generate a micropatterned sugar-based sacrificial layer around which cell-laden hydrogels could be built; the sugar-based layer is then dissolved, creating a 3D microarchitecture consisting of microvascular networks (39). However, despite these advances, new technologies are required to more accurately recreate the complexity of native tissues.

Another way to recreate the human tissue vasculature is with modular approaches in which smaller building blocks are assembled in a controlled manner (40). This approach can be used to modulate the cell microenvironment and macroscale properties of relatively large and complex engineered tissues (41), especially when the tissue requires perfusion and needs to perform a specialized physiological function. Recent work has shown the potential of modular assembly to produce vascularized tissues in vitro (42). Arrays of microgels with precisely defined structures and channels are produced by microfabrication and subsequently assembled to yield 3D structures with interconnected lumens, resembling native vasculature (43).

This biofabrication process was validated by placing endothelial and smooth muscle cells inside microgels in a precise and concentric fashion that mimicked the organization of blood vessels, strengthening the assembly with a secondary cross-linking step, and showing that these assemblies could be perfused with fluids. The precise design of microscale components combined with the capability of linking them together into higher-order structures represents a promising way to build vascularized 3D biomaterials in vitro.

CHALLENGES: FORM AND FUNCTION

Most recently developed vascularizing approaches for biomaterials attempt to mimic cellular communication that occurs in tissues and organs through the use of multiple cell types and biochemically modified scaffolds. These attempts to match natural biological and mechanical cues through the use of ECM components have inspired the creation of scaffolds that incorporate growth factors, integrin-binding peptides, vasculature-forming cells, and physical features that promote diffusion and transport. Studies have revealed that scaffold degradability and a combination of MMP-degradable moieties and adhesive components collectively promote ingress of endothelial cells into the biomaterial. Furthermore, in vitro release studies, such as those that compare proteolytically released VEGF with fibrin-

localized VEGF or that correlate VEGF release with vessel density, should expand our ability to precisely tune preformed tissue constructs.

However, the ability to quickly achieve a stable vascular network that can offer functionality to an ischemic organ or tissue implant remains a major challenge. Thus, for successful clinical translation of biomaterials, it is essential that researchers identify parameters that can be controlled to promote and regulate angiogenesis. The long-term in vivo function of various engineered vascular networks must also be assessed. Thus, further research into mechanisms for regulating the spatial aspects of vascularization is essential for further success in tissue engineering. Although microfabrication technologies need more in vivo studies to validate their claims of structural and functional integrity, scaffolds that recruit endogenous growth factors and stimulate the host to form a vascular network appear safe, efficient, and cost-effective. A combination of these approaches may be most suitable for creating constructs that can yield functional vasculature within the host.

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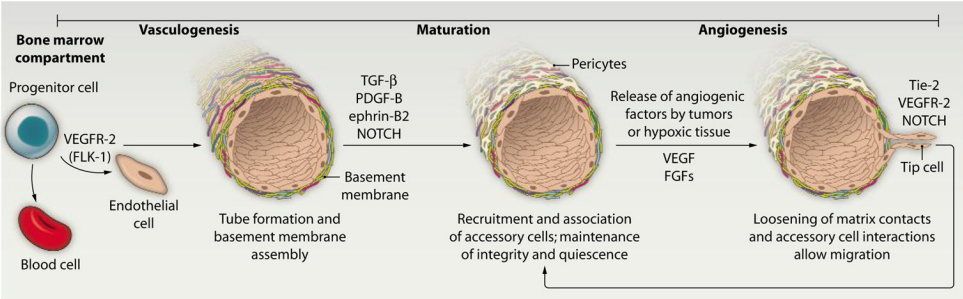

Fig. 1. Vasculogenesis and angiogenesis

Two distinct mechanisms of blood vessel formation. Vasculogenesis gives rise to the primitive vascular plexus during embryogenesis. Stimulated by tumors and hypoxic conditions, angiogenesis remodels and expands the vascular network.

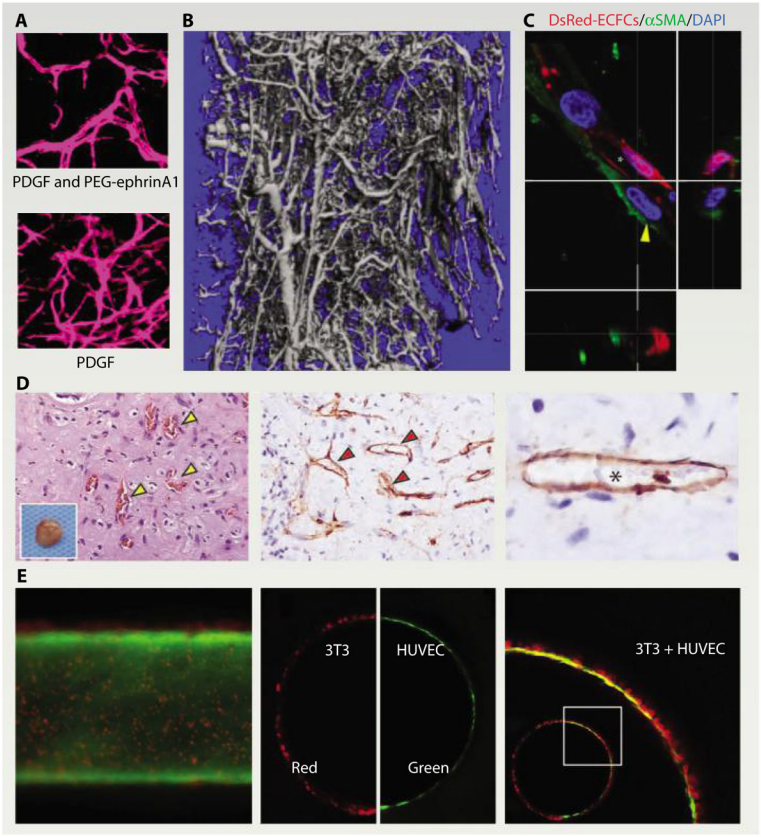

Fig. 2. Cue gardens

Biochemical cues can be integrated in hydrogels to induce vascularization. (**A**) Biomimetic hydrogels with platelet-derived growth factor (PDGF), immobilized RGDs, and poly(ethylene glycol) (PEG)-ephrinA1 (top panel) showed more robust response than hydrogels with PDGF only (lower panel) when implanted into the mouse cornea micropocket. [Reprinted from (18) with permission from the American Chemical Society] (**B**) Microcomputed tomography (micro-CT) image of a porous poly(L/DL-Lactide) (PLDL) copolymer filled with growth factor–treated, RGD-alginate hydrogel several weeks after implantation in a rat 8-mm segmental defect model; formation of vascular network is shown (32). [Reprinted from (32) with permission from Elsevier] (**C**) Formation of endothelial colony–forming cell (ECFC)—lined lumens within a gelatin methacrylate (GelMA) hydrogel that contains both ECFCs (DsRed) and mesenchymal stem cells (MSCs) (7 days after seeding the hydrogel with cells). ECFC-lined lumens were surrounded by MSC-derived pericyte cells that expressed α-smooth muscle actin (α-SMA) (yellow arrow). [Reprinted from (31) with permission from Wiley-VCH Verlag] (**D**) ECFC- and MSC-containing GelMA hydrogel implants were retrieved from the subcutaneous tissue of the dorsum of 6-week old nude mice 7 days after implantation; functional vascular network formation in vivo is shown. Top panel, image of hematoxylin and eosin (H&E)–stained GelMA explants. Numerous blood vessels containing murine red blood cells are shown (yellow arrowheads). The dark purple spots indicate nuclei of cells, and shades of light purple in the background indicate GelMA hydrogel. The inset shows the macroscopic view of the GelMA explant; middle panel, immunohistochemistry shows human cluster of differentiation 31 (CD31)–positive engineered microvessels; bottom panel, single human CD31–positive microvessel at higher magnification carrying murine erythrocytes (star). [Reproduced from (31) with permission from Wiley-VCH Verlag] (**E**) A microfluidic

hydrogel containing microvascular-like structures fabricated using self-assembled monolayer (SAM)–based cell transfer. Left panel, double-layer construct generated using 3T3 cells (red) encircling a human umbilical–vein endothelial cell (HUVEC) (green) monolayer; middle panel, confocal cross-sectional image of the vascular construct; right panel, merged confocal image of the construct. Inset shows the entire channel section. [Reproduced from (34) with permission from Elsevier]