CellPress

Special Issue: Biofabrication

Review

Vascularization and Angiogenesis in Tissue Engineering: Beyond Creating Static Networks

Jeroen Rouwkema[1,2,3,*] and Ali Khademhosseini[1,2,4,5,6,*]

Engineered tissues need a vascular network to supply cells with nutrients and oxygen after implantation. A network that can connect to the vasculature of the patient after implantation can be included during *in vitro* culture. For optimal integration, this network needs to be highly organized, including venules, capillaries, and arterioles, to supply all of the cells with sufficient nutrients. Owing to the importance of vascularization for the clinical applicability of tissue engineering, many approaches have been investigated to include an organized vascular network in tissue constructs. This review will give an overview of recent efforts, and will propose future perspectives to engineer the optimal, functional vascular network.

Importance of Vascularization in Tissue Engineering

One of the goals of tissue engineering is to generate tissues that can be used as alternatives for donor material to repair or replace damaged tissues or organs [1]. Tissues generated for this purpose will generally be of a size larger than the diffusional limit for nutrients and oxygen [2]. Therefore, a need for a system to distribute nutrients within the tissue is apparent. During culture in the lab this distribution can be facilitated by using, for instance, perfusion bioreactors, but after implantation the tissue will have to rely on a vascular network to supply nutrients to all cells within the tissue. As part of the foreign body response, a vascular network will generally invade an implant. However, this is a process that takes days or weeks, meaning that cells in the middle of the tissue will be starved of nutrients for a considerable amount of time [3], resulting in suboptimal tissue integration or cell death.

To decrease the time that is needed to vascularize an engineered tissue, researchers have been exploring the possibility of adding a vascular network before implantation. This network has the potential to connect to the vasculature of the patient, resulting in a much faster perfusion of the implant [4]. Theoretically, the perfusion will be instantaneous if the pre-engineered network in the implant is sufficiently organized, and can be microsurgically connected to the patient during the implantation procedure.

An optimal vascular network in an engineered tissue needs to possess several characteristics. One of the key tasks of a vascular network is to supply all cells in a tissue with sufficient nutrients. This means that all of the cells need to be within a distance of 200 μm from a vessel, which is generally regarded as the diffusion limit of oxygen and nutrients within a tissue [5]. To reach this

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Engineered tissues of a clinically relevant size need a vascular network to supply the cells with nutrients and oxygen. Including a vascular network before implantation can aid in this need, by connecting to the vasculature of the patient.

To supply all cells with sufficient nutrients, and to successfully connect to the patient vasculature, the engineered vascular network needs to be highly organized. Using microfabrication technology such as photo patterning and bioprinting, the initial organization of vascular networks can be designed and controlled.

The geometry of vascular networks can also be controlled by adapting local microenvironments. The patterning of mechanical signals, fluid flows, or the availability of growth factors leads to directed vascular organization.

[1]Department of Medicine, Biomaterials Innovation Research Center, Brigham and Women's Hospital, Harvard Medical School, Cambridge, MA, 02115, USA
[2]Harvard-MIT Division of Health Sciences and Technology, Massachusetts Institute of Technology, Cambridge, MA, 02319, USA
[3]Department of Biomechanical Engineering, MIRA Institute for Biomedical Technology and Technical Medicine, University of Twente, Enschede, The Netherlands
[4]Wyss Institute for Biologically Inspired Engineering, Harvard Medical School, Boston, MA, 02155, USA

fine distribution while minimizing the pressure that is needed for the blood flow, the vascular network should be organized as a vascular tree, where larger vessels branch into smaller vessels, which ultimately branch into capillaries that are distributed throughout the tissue volume. Apart from that, the vascular network should act as a barrier that selectively controls the passage of materials from the vessels to the surrounding tissue to prevent excessive outflow of fluid leading to tissue edema. Finally, to supply the tissue with nutrients shortly after implantation, it should be possible for the network to easily connect to the vasculature of the patient, or to be microsurgically connected to it. For the microsurgical approach, vascular structures with a diameter of several hundred micrometers are needed.

Vascularization is currently regarded as one of the main hurdles that need to be taken to translate tissue engineering to clinical applications at a large scale [6]. As such, it is one of the main research topics in the tissue engineering community. This review will give an overview of current strategies that have been explored in the past to prevascularize engineered tissues, and will propose future perspectives to engineer the optimal vascular network within an engineered tissue.

Angiogenesis and Vascular Remodeling

To include an organized vascular network in an engineered tissue, it is important to understand the process of vascular formation and remodeling. A starting point for this process is to look at the formation of the vascular network during embryonic development and growth. Two processes can be distinguished during the formation of a natural vascular network: vasculogenesis and angiogenesis. Vasculogenesis is the process that takes place during early embryonic development where **angioblasts** (see Glossary) differentiate into endothelial cells, and proliferate within a previously avascular tissue to form a primitive capillary network [7]. Vasculogenesis can also occur in adults to revascularize a tissue following extensive damage or during tumor growth [8,9]. During these so-called postnatal vasculogenesis processes, bone marrow-derived endothelial progenitor cells are mobilized into the circulation, home to the tissue repair site, and differentiate into mature endothelial cells to form a primitive vascular network [8].

Most of the processes involved in vascular organization and remodeling, both in embryonic development and afterwards, are angiogenic processes (Box 1). Angiogenesis is defined as the formation of new vessels from an existing vascular network. These vessels can be formed by sprouting angiogenesis, where endothelial cells form sprouts starting from pre-existing vessels, or intussusceptive angiogenesis, where tissue pillars are inserted within existing capillaries to split the vessels [10]. Angiogenesis is mainly driven by the need to supply tissues with sufficient nutrients and oxygen. As such, angiogenesis is regulated to a large extent by oxygen levels within tissues. Hypoxic tissues secrete growth factors and chemokines that activate vascular growth and remodeling [11]. Endothelial cells are stimulated to break out of their stable position in the vessel wall and jointly coordinate sprouting, branching, and new lumenized network formation [12]. When perfusion of the tissue has increased and the supply of oxygen meets the demand, quiescence can be re-established resulting in a stable vascular network.

The process of angiogenesis initially leads to the development of capillaries. This can be followed by a process called arteriogenesis, where the vascular structures further mature and increase in diameter and the vascular wall thickness [13]. **Mural cells** proliferate, and acquire specialized characteristics, including contractile components [14]. The mechanisms governing arteriogenesis are not yet completely understood, but the remodeling process is mainly mediated by fluid flow shear stresses [15].

Natural Organization of Endothelial Cells in Engineered Tissues

Many research efforts in prevascularized tissue engineering have relied on the spontaneous organization of endothelial cells to form vascular networks on scaffolds [16–18], in extracellular

[5]College of Animal Bioscience and Technology, Department of Bioindustrial Technologies, Konkuk University, Hwayang-dong, Gwangjin-gu, Seoul 143-701, Republic of Korea
[6]Department of Physics, King Abdulaziz University, Jeddah 21569, Saudi Arabia

*Correspondence:
j.rouwkema@utwente.nl (J. Rouwkema) and alik@rics.bwh.harvard.edu (A. Khademhosseini).

Box 1. Growth Factor Regulation of Angiogenesis

Angiogenesis, both during growth and in disease states such as cancer, is a complex process regulated by multiple factors. Understanding these processes is important to tune vascular organization in engineered tissues. VEGF is one of the key players, being involved in most morphogenic events [82]. Stimulation with VEGF results in various distinct responses in endothelial cells, including proliferation, migration, differentiation, and survival [83]. In vascular patterning during sprouting angiogenesis, endothelial tip cells guide and lead the vessel sprout, while proliferation of neighboring stalk cells results in the lengthening of the sprout. Gradients of VEGF regulate the shape of the newly formed sprout, by directing tip cell migration and regulating stalk cell proliferation [84]. The tip cells determine the amount of sprouts and the direction in which the sprouts migrate, while the stalk cells lengthen the sprout and form the vascular lumen [85].

Basic fibroblast growth factor (bFGF) is another factor that plays multiple roles in the initiation of angiogenesis by promoting the migration, proliferation, and differentiation of endothelial cells [86]. In addition to stimulating capillary growth, the mitogenic effect of bFGF for smooth muscle cells induces the growth of larger vessels [87].

PDGF is mainly involved in vascular maturation and remodeling [88]. PDGF targets mural cells and recruits them to vascular structures [89]. Apart from that, PDGF has been shown to directly affect endothelial cells by, for instance, inhibiting the angiogenic response to bFGF [90].

Angiopoietins play an important role in the stabilization and destabilization processes of vascular networks. There are two different angiopoietins, with opposing effects in angiogenesis. Angiopoietin 1 (Ang1) is expressed by vascular mural cells and non-vascular normal and tumor cells [91], and helps to maintain a stable state in vessels [92]. Upon activation of its receptor, Ang1 mediates a range of effects such as tightening of endothelial cell junctions, promoting endothelial cell survival, and increasing endothelial–mural cell interaction [93]. Angiopoietin 2 (Ang2) is primarily produced by endothelial cells during vascular remodeling [91] and competes with Ang1 for binding to the same receptor. The effect of Ang2 stimulation depends on the presence or absence of VEGF. Binding of Ang2 in the presence of VEGF sensitizes endothelial cells to proliferation signals mediated by other proangiogenic factors [93], increases the remodeling of basal lamina, and stimulates the migration of endothelial cells [92,94], thus resulting in vascular remodeling. In the absence of VEGF, Ang2 leads to endothelial cell death and vessel regression [94].

matrix analogs [4,19], or in cellular aggregates with other cells [20,21]. These studies show that endothelial cells are capable of forming vascular networks, often without the addition of specific cues or growth factors. First, the endothelial cells form a primitive network within a previously avascular tissue, which is similar to vasculogenesis. Subsequently, this network can organize further in a process that is similar to angiogenesis and arteriogenesis. Depending on the culture conditions and the cell type with which the endothelial cells are combined, the morphology of the vascular networks ranges from immature cord-like networks with a limited amount of lumen [21,22], to more mature networks containing well-developed lumen [4,19] (Figure 1A). The addition of mural precursor cells such as embryonic fibroblasts or mesenchymal stem cells (MSCs) results in maturation and stabilization of the vascular structures, indicated by an increase in the amount of lumen [4]. This effect is beneficial for the amount of blood that can be distributed in the tissue because luminal structures are needed for transporting fluids. Apart from that, mural cells play a role in the regulation of vascular permeability, which means that stabilized vessels will leak less fluid into the tissue, resulting in lower interstitial fluid pressure [23]. Upon implantation, the vascular structures can connect to the vasculature of the patient, thus contributing to implant perfusion and survival [4,24]. Using intravital microscopy, Koike *et al.* showed that vascular networks formed in fibronectin–collagen hydrogels can remain functional and transport blood for 1 year *in vivo* when stabilized by mural precursor cells, even though perfusion of the network was only apparent after 7 days of implantation [25].

These studies are successful in demonstrating the potential of adding a vascular network to engineered tissues, but relying on uncontrolled angiogenesis in engineered tissues results in a random organization of the vascular network. Even though the networks will remodel after implantation, histology shows that the spacing between vascular structures is initially often larger than 200 μm, which means that the random organization is unlikely to be fully able to supply all cells of the engineered tissue with sufficient nutrients shortly after implantation. Apart from that, the random network does not offer clear locations for natural or surgical **anastomosis**, which can result in a delay of the network perfusion.

Glossary

Anastomosis: in the context of angiogenesis, the connection of two vascular structures.
Angioblast: a precursor of endothelial cells, originating from the mesenchyme.
Angiopoietins: a family of growth factors that mainly plays a role in vascular stabilization and destabilization. Generally, Angiopoietin 1 stabilizes vascular networks, while Angiopoietin 2 induces destabilization allowing for vascular remodeling.
Capillary bed: a finely distributed network of capillaries, which are the smallest of the blood vessels in the body, that takes care of the distribution of nutrients over a tissue.
Integrins: transmembrane receptors that play a role in cell–cell and cell–matrix interactions. The presence of specific integrins on a cell surface determines how a cell can respond to stimuli.
Laminins: a class of proteins that are part of the extracellular matrix and that influence cell adhesion, migration, and differentiation.
Mural cells: cells, including vascular smooth muscle cells and pericytes, which are associated with vessels. Mural cells play a role in the stabilization of vascular structures. Apart from that, mural cells have contractile functionalities that can help to regulate the flow of fluid through vessels.
Platelet-derived growth factor (PDGF): a family of growth factors that play a role in cell growth and recruitment. In the context of angiogenesis, PDGF is mainly involved in the recruitment of mural cells.
RGDS: a sequence of peptides, Arg-Gly-Asp-Ser. Within fibronectin and several integrins, this is the sequence that mediates cell attachment.
Sonic Hedgehog (Shh): a protein that plays a role in the patterning of multiple systems during development. Shh is a morphogen and acts by the formation of concentration gradients, where cellular actions depend on the local concentration.
Vascular endothelial growth factor (VEGF): a family of growth factors that play a role in angiogenesis and lymphangiogenesis. As one of the driving growth factors, VEGF is involved in many of the different angiogenic processes.

Patterning of Endothelial Cells in Engineered Tissues

Many studies have focused on the active patterning of vascular networks within engineered tissues to closer resemble the natural organization of a vascular tree. Using novel fabrication technologies, the initial organization of vascular cells can be designed and controlled. This approach offers the clear advantage that the resulting network can be designed such that all cells in the tissue are within 200 μm from a vessel, and provides clear locations for vascular anastomosis.

Vascular plexus: a highly interconnected network of vascular structures, generally without a hierarchical organization. During embryonic development, a vascular plexus is first formed, which later remodels to a more organized network including arteries and veins.

One strategy used to pattern vascular structures is to prepare hollow channels within scaffolds or hydrogel matrices, which can be seeded with vascular cells to form a predesigned pattern of vascular structures. Multiple methods have been reported to create hollow channels in polymeric biomaterial scaffolds, including the use of multi-material 3D fiber deposition [26,27], electrospinning [28], the casting of scaffold material around sacrificial materials [29,30], laser drilling [31], and the use of silicon molds [32,33] to replicate patterns. Even though these approaches have been successful in generating well-organized endothelialized vascular networks, these structures are generally bordered by a dense, impenetrable layer of biomaterial, limiting further vascular remodeling and nutrient transport. Apart from that, the resolution of these methods is generally insufficient to attain a complex network with a highly organized **capillary bed**.

Channels prepared in cell permissive hydrogels offer a more flexible environment, where endothelial cells can sprout into the matrix [34,35], thus further remodeling the initial patterned vascular network. Individual channels [36–38] and interconnected channel networks [34,35,39,40] have been fabricated in hydrogels using sacrificial materials such as gelatin [39] or glass filaments [34] (Figure 1D), structures such as hypodermic needles that can be removed after gelation [37], or polydimethylsiloxane (PDMS) molds [35]. Perfusion of these channels with culture medium results in the active transport of nutrients within engineered tissues, resulting in increased cell survival *in vitro* [41]. A recent study shows that channels as narrow as 20 μm can be seeded successfully with endothelial cells, resulting in millimeter-long perfusable capillaries [42]. This development enables the creation of an engineered tissue with a vascular tree-like network, including a highly organized capillary bed, where all cells are within 200 μm from a vascular structure. Apart from an approach to vascularize large tissue-engineered constructs, the resemblance of these vascular structures with natural vessels makes them a good research platform to test the effect of compounds on vascular structures by investigating, for instance, how readily fluid passes through the vessel wall [35,40] (Figure 1C).

Next to the preparation of channels that can be seeded with endothelial cells and mural cells, vascular structures with a controlled geometry can be obtained by the direct patterning of vascular cells. Structures where vascular cells are patterned in two dimensions (2D) have been prepared using cell sheet technology [43], PDMS molds [44,45], or photopatterning [46]. These approaches all offer good control over the initial organization of the vascular network and the diameter of the vascular structures. By stacking these 2D constructs, more complex, 3D vascular networks can be obtained [43] (Figure 1B). Complex 3D vascular networks can also be prepared directly using technology such as bioprinting [47–49], which allows for the placement of either cellular aggregates [50] (Figure 1E) or biomaterials containing cells [51] at a specific location with a high level of spatial control.

The patterning of endothelial cells, either by preparing channels that are subsequently seeded with vascular cells or by the direct patterning of these cells, enables the creation of complex vascular networks over multiple diameter scales. A study by Chaturvedi *et al.* using patterned vascular structures shows that after implantation, vascular cords ranging in diameter from 25 to 250 μm can anastomose to the mouse vasculature and become functional, perfused vessels

[44]. However, vessels remodeled *in vivo*, resulting in vessels with a diameter in the 10–15 μm range for all starting diameters. This study shows that even though the initial organization and diameter of vascular structures can be controlled, vascular remodeling will change this organization, either during *in vitro* culture or after implantation. Therefore, without further cues to control vascular remodeling, a carefully designed vascular network may not be sufficient to offer long-term functionality.

Guiding Organization of Endothelial Cells in Engineered Tissues

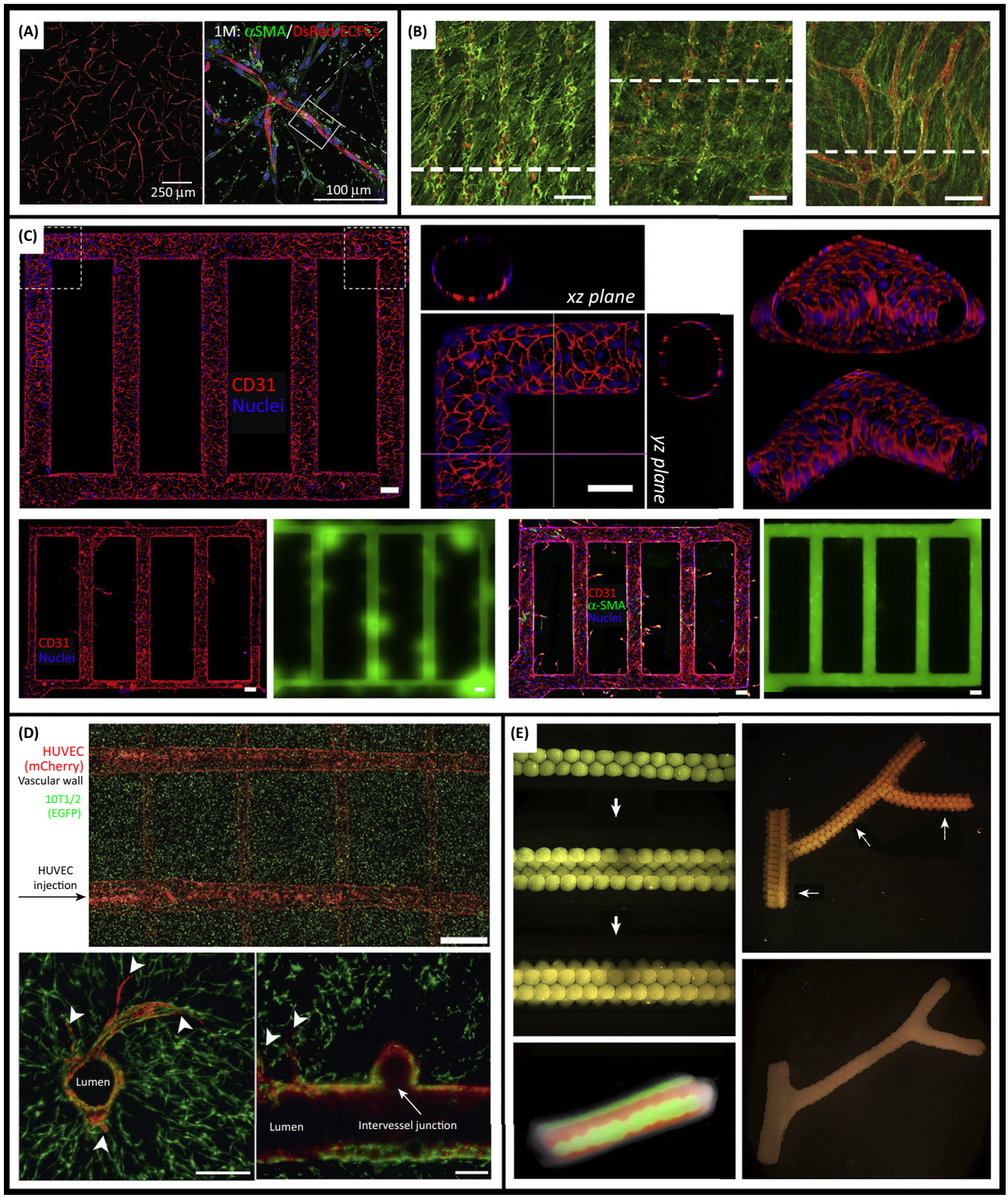
An alternative approach to control the architecture of vascular structures in engineered tissues is to include local cues to guide vascular organization and remodeling. The adaptation of local microenvironments offers the possibility to engineer a complex, predictable vascular organization, starting from an initial random distribution of vascular cells. Even though this approach is less straightforward than the direct patterning of cells, guided morphogenesis may result in a better control of vascular organization in the long term due to an inherent control over the remodeling process. However, owing to the complexity of all environmental factors that are involved in vascular organization and remodeling, an exact prediction and design of the resulting vascular network geometry will be challenging.

Growth Factors and Chemical Functionalities

As has been mentioned earlier, vascular organization and angiogenesis are controlled by growth factors. As such, many approaches to control vascular organization in engineered tissues are based on the local availability of these compounds. Since **vascular endothelial growth factor** (**VEGF**) is the factor that is involved in most angiogenic processes, it has often been the factor of choice to control vascular organization in a tissue engineering setting. It has been shown that in order to control vascular organization, it is not so much the availability of this factor but the presence of gradients that controls vascular migration [52]. By creating distinct patterns of VEGF onto scaffolds or within hydrogels, gradients will be instituted, resulting in spatially driven endothelial cell elongation and branching [53,54] (Figure 2A).

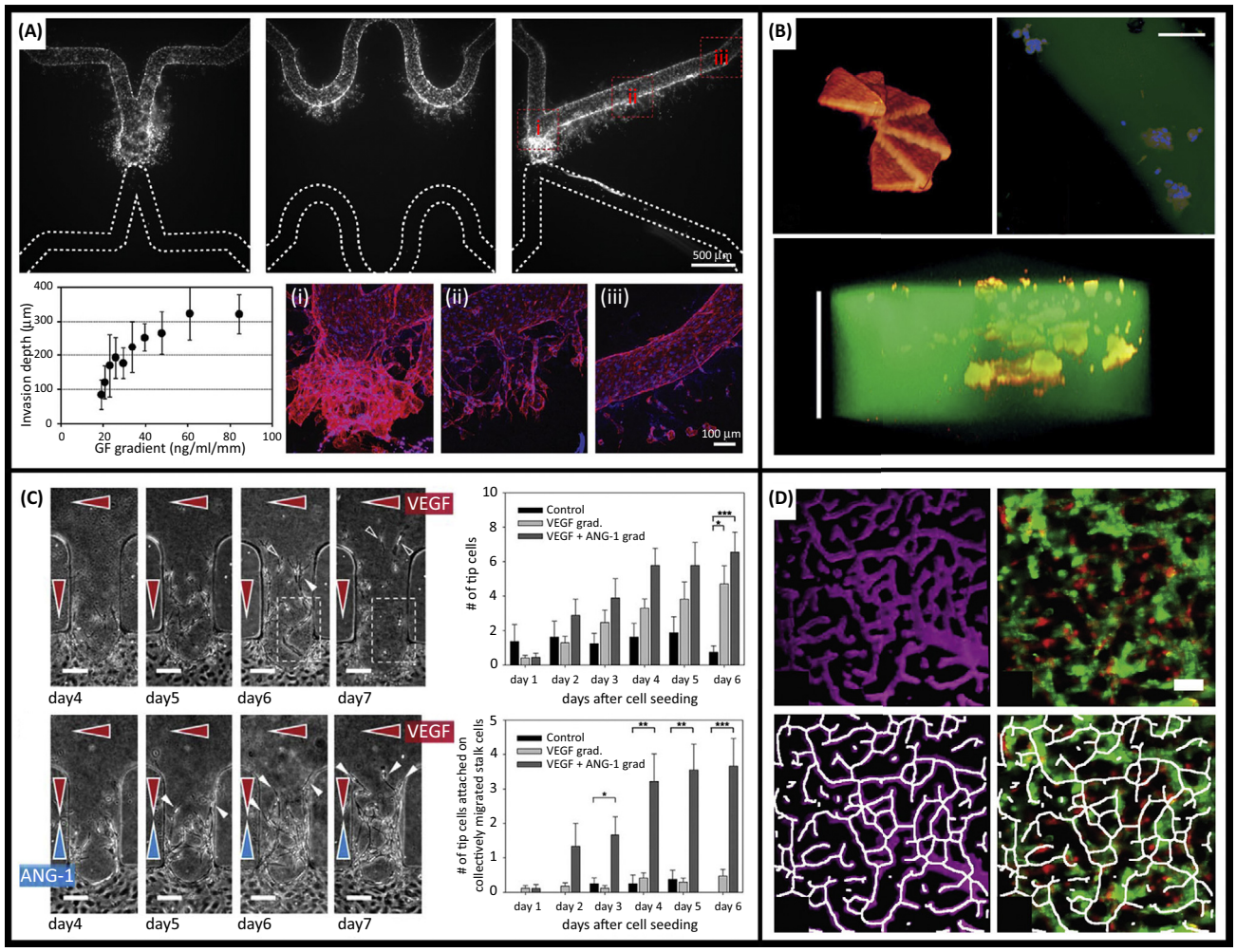
Angiogenesis and vascular organization are processes that consist of multiple phases. After the initiation of vascular network formation, a maturation process where endothelial cells are stabilized and mural cells are recruited is important for vascular function. To control both processes, patterning of a single growth factor will be insufficient. As such, researchers have combined the inclusion of VEGF with other factors governing maturation and mural cell recruitment such as **platelet-derived growth factor** (**PDGF**) [55,56] and **Angiopoietin** 1 (Ang1) [57,58], resulting in an increase in vascular structure formation and maturation compared with VEGF alone (Figure 2C).

A major challenge in the use of growth factors to control vascular organization in engineered tissues is the correct patterning of the multiple involved factors in space and time. To cope with this challenge, several approaches have been investigated where an indirect chemical signal is used. Since *in vivo* angiogenesis is largely governed by oxygen levels, oxygen gradients have been included in engineered tissues [59,60], resulting in local differences in the expression of VEGF by fibroblasts present in the tissue [59]. Similarly, researchers have included the morphogen **Sonic Hedgehog** (**Shh**) to co-cultures of endothelial cells and MSCs for a bone tissue engineering application [61]. This approach results in an increase of vascular structure formation and maturation, by modulating the expression of multiple angiogenic genes including VEGF, angiopoietins, **laminins**, and **integrins**. This result shows that indirect factors have the potential to tune the angiogenic environment of an engineered tissue, by stimulating the resident cells to secrete angiogenic factors. The advantage of this approach is that the factor secretion will be biologically regulated similar to *in vivo* angiogenic processes, where the cells will secrete factors based on the current need.



Trends in Biotechnology

Figure 1. Fabrication of Vascular Networks. Many approaches have been explored to pattern vascular cells. Panel (A) shows co-cultures of HUVECs and MSCs, where endothelial cells spontaneously organize into vascular networks (red) that are stabilized by mural cells (green) originating from the MSC. Panel (B) shows the



Trends in Biotechnology

Figure 2. Guiding Vascular Organization via Chemical Signals. Chemical signals can be used to guide and control the organization of vascular structures. Panel (A) shows three situations where channels have been microfabricated in collagen hydrogels (top). The upper channel is seeded with HUVECs, while the lower channel is perfused with VEGF. By varying the distance between the channels, the local growth factor gradients vary, resulting in differences in endothelial sprouting and invasion (bottom). Panel (C) shows the effect of VEGF gradients on vascular invasion into a collagen gel either in the presence or absence of Ang1. When the cells are stimulated with both factors, more sprouts are formed, and the tip cells of the sprouts stay better attached to the stalk cells. This clearly indicates that the delivery of a single growth factor is often not enough to stimulate the formation of a well-organized vascular network. Apart from controlling the local availability of growth factors, other chemical functionalities can be patterned to control vascular organization. Panel (B) shows the patterning of the cell binding motive RGDS into bio-inert PEG hydrogels using two-photon laser photolithography (top left). Since cells can only attach to the RGDS patterns, cell invasion is limited to these domains (top right and bottom). Panel (D) shows that by patterning RGDS domains in an organization resembling the vasculature of a mouse cerebral cortex (left), this approach can be used to replicate a natural vascular organization by controlling the invasion of HUVECs (green) and fibroblasts (red) (right). Panels (A), (B), (C), and (D) have been adapted, with permission, from [54], [63], [58], and [62], respectively. Abbreviations: HUVECs, human umbilical vein endothelial cells; VEGF, vascular endothelial growth factor; Ang1, Angiopoietin 1; RGDS, Arg-Gly-Asp-Ser; PEG, polyethylene glycol.

organization of HUVECs in 2D by patterning both cell-adhesive and non-cell-adhesive regions on a 2D surface (left). By stacking multiple layers, a 3D network is acquired (middle) that further remodels during culture (right). Panel (C) shows a strategy where channels are prepared in a collagen hydrogel, which are subsequently seeded with vascular cells. This results in a perfusable endothelial lined network (top). Depending on culture conditions and the presence of mural cells, vascular structures will sprout into the hydrogel, and will adapt their barrier function as illustrated using FITC–dextran perfusion (bottom). This approach can be extended to 3D by printing a sacrificial lattice network that is subsequently embedded in hydrogel. After removal of the sacrificial structure and seeding with vascular cells, a 3D perfusable network is acquired as shown in (D). Finally, vascular cells can be patterned directly in 3D using bioprinting. By printing either hydrogels containing cells or cellular aggregates as shown in (E), complex and well-controlled vascular organizations can be achieved. Panels (A), (B), (C), (D), and (E) have been adapted, with permission, from [19], [43], [35], [34], and [50], respectively. Abbreviations: HUVECs, human umbilical vein endothelial cells; MSCs, mesenchymal stem cells.

Apart from using diffusible factors, chemical functionalities have been included in cellular environments to control vascular organization. Using two-photon laser photolithography, complex 3D patterns of chemical functionalities can be included in hydrogels [62,63]. Hahn *et al.* patterned the cell binding motive Arg-Gly-Asp-Ser (**RGDS**) in bio-inert polyethylene glycol (PEG) hydrogels functionalized with the peptide sequence GGPGQGILQGGK, which makes the PEG degradable to matrix metalloproteinase [63]. These enzymes are secreted by invading endothelial cells to degrade the extracellular matrix. The study shows that invading cells are limited to the regions containing the RGDS pattern, thus resulting in the patterning of cellular ingrowth (Figure 2B). Similarly, by locally incorporating RGDS in PEG hydrogels, Culver *et al.* could control the organization of human umbilical vein endothelial cells (HUVECs) and mural precursor cells to resemble the prescanned vasculature from the cerebral cortex of a mouse [62] (Figure 2D). The patterning of chemical functionalities thus allows for the creation of a vascular network with a designable organization, even when the vascular cells are allowed to organize themselves.

Mechanobiology

Cells respond to the mechanical properties of their environment. MSCs, for instance, adapt their differentiation profile to the local stiffness [64]. Several studies have shown that vascular organization is also dependent on the mechanical properties of the matrix in which the endothelial cells reside. A study by Santos *et al.* demonstrates that HUVECs grown on collagen-coated polyacrylamide hydrogels of low (3 kPa) and high (30 kPa) stiffness display similar proliferation and gene transcription levels, but show a lower expression of the functional VEGF receptor-2 protein on the stiffer substrate [65], which means that the extent to which endothelial cells are responsive to angiogenetic processes depends on the mechanical environment. In a different study, Mason *et al.* used a system where the stiffness of a collagen hydrogel can be changed without significant changes to the collagen density and architecture. Increasing the stiffness from 175 Pa to 515 Pa results in a dramatic increase in the length and number of angiogenic sprouts growing from multicellular spheroids [66] (Figure 3A). In another study, Shamloo and Heilshorn point out that the matrix density and mechanical properties do not only have a direct effect on endothelial cells but can also alter the response of endothelial cells to angiogenic growth factors such as VEGF. The number of sprouts, the sprout aspect ratios, and the direction of sprouts in relation to a VEGF gradient all depend on the density of the collagen matrix [67].

Several studies have been reported where gradients and patterns of different mechanical properties are used to direct vascular organization within matrices. In a study by He *et al.*, a stiffness gradient in an RGD-functionalized PEG hydrogel was formed using microfluidic mixing. This results in morphological differences of HUVECs seeded on the hydrogel, with cells maintaining a round morphology in the softer region, and spreading out in the stiffer region [68]. In a different study, Turturro *et al.* report a gradient RGD-functionalized PEG hydrogel prepared using perfusion-based frontal photopolymerization, with the elastic modulus decreasing from 3.2 to 0.62 kPa over a span of 10 mm [69]. The gradient results in a decrease in the overall amount of vascular invasion from co-culture aggregates of endothelial cells and smooth muscle cells, but in a clear increase in the anisotropy of the vascular structures, with an alignment parallel to the direction of the gradient (Figure 3B).

Cellular responses to differences in matrix mechanical properties are mediated by the tension that cells can exert on the matrix upon cellular contraction [70]. Apart from locally changing the mechanical properties of a matrix, this phenomenon provides another opportunity to control vascular organization in engineered tissues. Using a Matrigel assay in PDMS microwells of different shapes including circles, squares, triangles, and stars, Sun *et al.* show that the HUVEC network near the boundary of the shapes has significantly higher densities and shorter mean cord length compared with the center regions [71]. Finite element analysis points out that

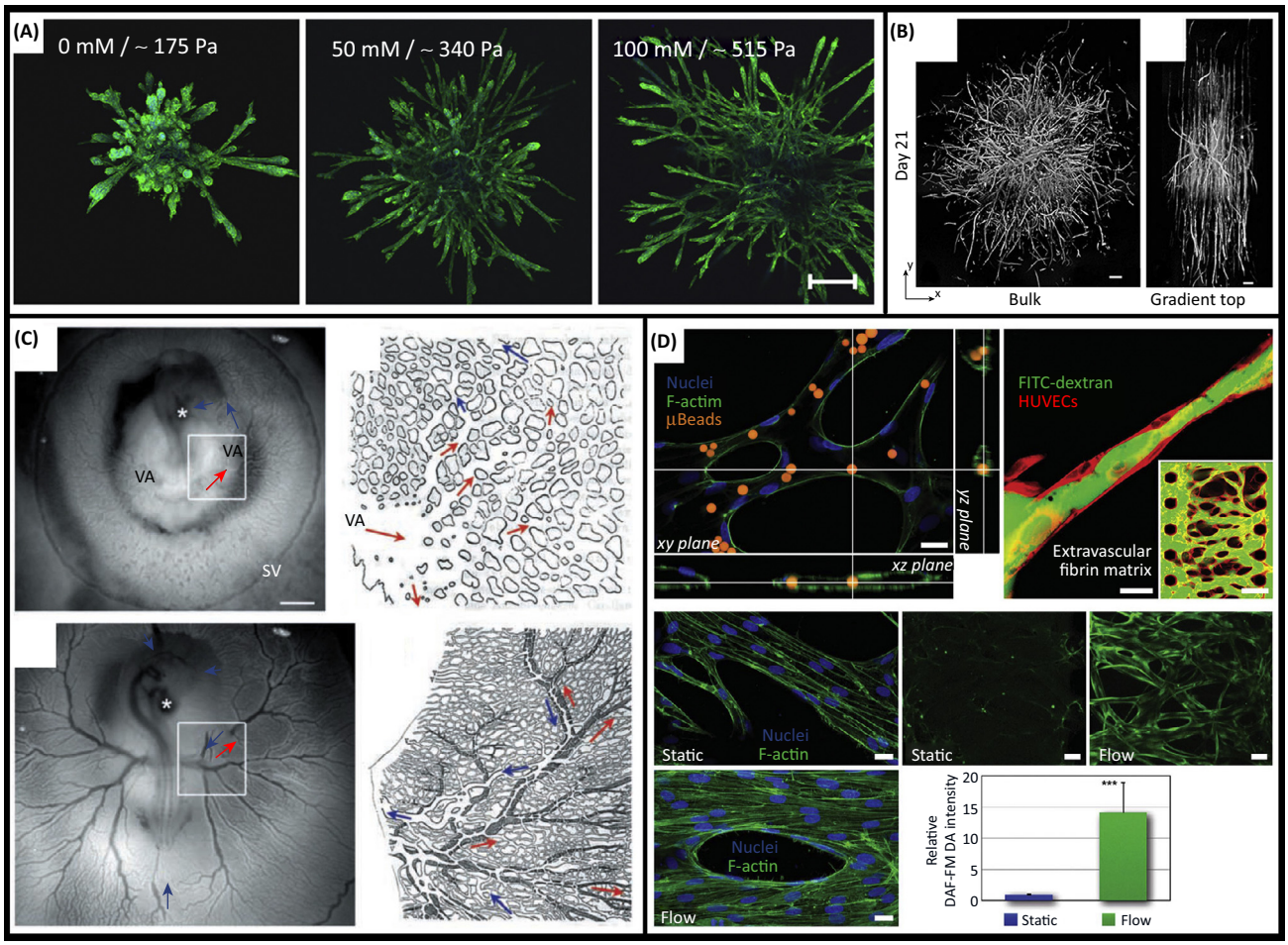


Figure 3. Guiding Vascular Organization via Mechanical Signals. Vascular organization can be guided using mechanical signals. Panel (A) shows a study where the mechanical properties of collagen hydrogels were adapted independently of collagen concentration. Changes in the compressive modulus of the hydrogel resulted in a clear effect on endothelial sprouting into the matrix, with stiffer gels resulting in an increase in the number and length of the sprouts. Panel (B) shows a similar approach where a gradient of RGD-functionalized PEG hydrogel is prepared using perfusion-based frontal photopolymerization, with the elastic modulus decreasing from 3.2 to 0.62 kPa over a span of 10 mm. Compared to a hydrogel with a constant bulk stiffness (left), the gradient hydrogel shows a clear increase in the anisotropy of vascular structure formation, with an alignment parallel to the direction of the gradient (right). Panel (C) demonstrates the importance of fluid flow during embryonic development. Just after the onset of perfusion, the vasculature in a chicken embryo yolk sac is poorly organized (top). There are no separate vessels carrying blood away from the heart (red arrows) or back toward the heart (blue arrows). Triggered by fluid flow, the same embryo shows an advanced state of vascular organization just 26 h later. A clear distinction between arteries (red arrows) and veins (blue arrows) can now be made. Panel (D) shows an example of the use of fluid flow to affect the organization of an engineered vascular network. A perfusable network was formed in a microfluidic device, as evidenced by the perfusion of fluorescent microbeads (top left) and FITC–dextran (top right). Compared to static conditions, endothelial cells respond to luminal fluid flow with cytoskeletal reorganization (bottom left) and an increase in nitric oxide (NO) synthesis (bottom right). Panels (A), (B), (C), and (D) have been adapted, with permission, from [66], [69], [73], and [74], respectively. Abbreviations: RGD, Arg-Gly-Asp; PEG, polyethylene glycol.

physical confinement can tune gel displacement due to cellular contraction, resulting in variations in cellular tension. Similarly, Rivron *et al.* used a shaped microwell system to make biomaterial-free MSC–HUVEC co-culture microtissues of different shapes including circles, squares, and triangles [72]. Local tissue compaction depends on the distance from the microtissue periphery, and the angles of the different shapes, resulting in patterns of vascular structures perpendicular to the strain direction in regions of high deformations. This corresponds with the institution of a long-range VEGF gradient in the interstitial cells, indicating that local tension can shape the angiogenic microenvironment in tissues.

Fluid flow is an important factor for vascular organization and remodeling. During embryonic development of a chick embryo, the vascular network remodels from a largely random **vascular plexus** to an organized vascular tree within 26 h after the onset of perfusion [73] (Figure 3C). Researchers have used flow to guide vascular organization [74–76] and maturation [77] (Figure 3D). Apart from a direct response of vascular cells to fluid flow shear stresses, interstitial flow results in the institution of gradients of growth factors such as VEGF, which further affects vascular organization [78].

Combined, these studies on the effect of mechanical signals show that vascular organization can be controlled. However, it should be noted that it is often not possible to adjust the mechanical environment within an engineered tissue with a high spatial resolution, which means that these strategies by themselves will most likely not enable the creation of a vascular network with designable features on the capillary scale. As such, mechanobiology should be regarded as a complementary tool to better control vascular organization, or as a method to stabilize an already organized vascular network.

Concluding Remarks and Future Perspectives

When regarding vascular networks for engineered tissues, it is important to realize that quality is more important than quantity. It is not about the number of vascular structures in a given volume of tissue but about the amount of blood that is perfused through the vascular network and the distribution of this blood over the tissue volume. Therefore, it is important that the vascular network is well organized and matured. In studies where angiogenesis is overstimulated resulting in excessive amounts of vessels, tracer perfusion experiments show that the vessels are poorly perfused [79]. Studies that stimulate vascular maturation and stabilization by contrast result in reduced vascular branching and density, but enhanced vessel diameter and perfusion [80]. The optimal vascular network will need to be highly organized, including venules, capillaries, and arterioles, to supply all cells with sufficient nutrients. However, organization is not the only characteristic that determines the success of engineered vessels. When assessing the quality of an organized vascular network, it is important to also take into account functional parameters such as perfusability and barrier function, something that is currently often lacking in studies that focus on the patterning of vascular networks. The presence of macrovascular structures that can be microsurgically anastomosed to the patient is desirable for direct perfusion after implantation. In cases where microsurgical anastomosis is not achievable, both the angiogenic activity of the implanted vascular structures and the host tissue can be optimized to enhance inosculation of the two vascular networks [81].

It is clear that approaches that focus on the active patterning of vascular cells within engineered tissues provide the highest level of control over the initial organization of vascular structures, and therefore have the potential to result in the most naturally organized vascular networks at the initial stage of tissue culture. However, given the activity and mobility of endothelial cells, these networks will remodel during *in vitro* culture and after implantation of the engineered tissue. When there are no additional cues to guide this remodeling process, it is likely that what starts out as a well-organized network will soon revert to a random organization of the vascular structures. As such, simply patterning vascular cells in engineered tissues may not be sufficient to ensure a good vascular organization in the long term.

Even though the requirements for vascular networks to be included in engineered tissues are generally well defined, it is not yet clear how all of these requirements can be fulfilled (see Outstanding Questions). As is often the case, an optimal protocol to add a well-organized vascular network will likely combine multiple approaches delineated earlier. Patterning of endothelial cells will provide a good starting situation, but one or more methods to control

Outstanding Questions

What exact combination of cell types will result in the optimal vascular network within engineered tissues? It is clear that endothelial cells are needed to form the linings of the vascular structures and to prevent coagulation upon perfusion with blood. However, whether the source of these cells should be macrovascular, microvascular, progenitor-based, or a combination of multiple sources to optimize vascular organization is not known. Additionally, mural cells or mural cell precursors will be needed to stabilize the vascular network.

What is the optimal balance between vascular organization in engineered tissues before implantation and vascular remodeling after implantation? A certain initial degree of organization is needed to supply all cells with nutrients, but vascular remodeling after implantation should be possible to adapt to the postimplantation environment.

How can a preformed vascular network be anastomosed to the vasculature of the patient in prevascularized tissue engineering? Microsurgical anastomosis is preferable because it will result in instantaneous perfusion, but this is generally not achievable due to insufficient organization and mechanical properties of the engineered vascular network. In this case, both the angiogenic activity of the implanted vascular structures and the host tissue should be optimized to enhance the natural inosculation of the two vascular networks.

The addition of a vascular network to an engineered tissue is important to ensure nutrient availability and cell survival. How does the inclusion of additional cell types, often accompanied with changes in the culture media or growth factors used, affect the development of the base tissue? Even though these effects are often positive, it is an important factor to take into account for individual applications.

vascular remodeling and maturation will need to be included to ensure long-term functionality. Finally, it is important to realize that vascular networks will generally be engineered within a base tissue such as muscle or bone. Strategies that are designed to direct vascular organization, such as growth factor localization or the patterning of mechanical signals, will often have an (unwanted) effect on the development of this tissue as well. As such, the strategies depicted in this review may not be useable in all situations, thus making it unlikely that the future will provide us with one single optimal method to add a vascular network to engineered tissues.

Acknowledgments

J.R. was supported by the People Programme (Marie Curie Actions) under REA Grant Agreement no. 622294 (PreVascIn).

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