Review

Vascularization in tissue engineering

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Tissue engineering has been an active field of research for several decades now. However, the amount of clinical applications in the field of tissue engineering is still limited. One of the current limitations of tissue engineering is its inability to provide sufficient blood supply in the initial phase after implantation. Insufficient vascularization can lead to improper cell integration or cell death in tissue-engineered constructs. This review will discuss the advantages and limitations of recent strategies aimed at enhancing the vascularization of tissue-engineered constructs. We will illustrate that combining the efforts of different research lines might be necessary to obtain optimal results in the field.

Introduction

Most tissues in the body rely on blood vessels to supply the individual cells with nutrients and oxygen. For a tissue to grow beyond 100–200 μm (the diffusion limit of oxygen), new blood-vessel formation is required [1], and this is also true for tissue-engineered constructs. During *in vitro* culture, larger tissue-engineered constructs can be supplied with nutrients, for instance in perfusion bioreactors [2,3]. However, after implantation of tissue constructs, the supply of oxygen and nutrients to the implant is often limited by diffusion processes that can only supply cells in a proximity of 100–200 μm from the next capillary. In order for implanted tissues of greater size to survive, the tissue has to be vascularized, which means that a capillary network capable of delivering nutrients to the cells is formed within the tissue. After implantation, blood vessels from the host generally invade the tissue to form such a network, in part in response to signals that are secreted by the implanted cells as a reaction to hypoxia. However, this spontaneous vascular ingrowth is often limited to several tenths of micrometers per day [4], meaning that the time needed for complete vascularization of an implant of several millimeters is in the order of weeks. During this time, insufficient vascularization can lead to nutrient deficiencies and/or hypoxia deeper in the tissue. Moreover, nutrient and oxygen gradients will be present in the outer regions of the tissue, which could result in non-uniform cell differentiation and integration and thus decreased tissue function [5].

Because the speed of vascularization after implantation is a major problem in tissue engineering, the successful use of tissue-engineered constructs is currently limited to thin or avascular tissues, such as skin or cartilage, for which postimplantation neovascularization from the host is sufficient to meet the demand for oxygen and nutrients [6]. To succeed in the application of tissue engineering for bigger tissues, such as bone and muscle, the problem of vascularization has to be solved [7].

Vascularization in tissue engineering

After implantation of tissue-engineered constructs, a spontaneous vascularization of the implant is usually seen (Box 1). This is in part due to an inflammatory wound-healing response, which is induced by the surgical procedure. Furthermore, the seeded cells often create a hypoxic state in the implant, which stimulates the endogenous release of angiogenic growth factors [8]. However, this induced vessel ingrowth is often too slow to provide adequate nutrient transport to the cells in the interior of the transplanted tissue. Therefore, additional strategies for enhancing vascularization are essential to ensure the survival of large tissue-engineered grafts.

Several strategies for enhancing vascularization are currently under investigation. These include scaffold design, the inclusion of angiogenic factors, *in vivo* prevascularization and *in vitro* prevascularization (see Figure 1). Although all these strategies can in principle enhance

Glossary

Angiogenesis: new blood-vessel formation by the growth and sprouting of existing blood vessels.
Hypoxia: a state in which the oxygen concentration is lower than the physiological level.
Matrix metalloproteinases (MMPs): enzymes capable of degrading multiple extracellular matrix proteins. MMPs are secreted by migrating endothelial cells in order to break down the extracellular matrix that surrounds vessels and thus to allow for vessel growth.
Mural cells: the combined term for smooth muscle cells and pericytes.
Perfusion bioreactor: a bioreactor in which medium is perfused through a construct. This allows the active delivery of nutrients and oxygen to the cells in the interior of the construct.
Pericytes: the cells that surround endothelial cells in capillaries. The role of pericytes is similar to the role of smooth muscle cells in bigger vessels.
Prevascular network: in this article this is defined as an engineered vascular network that lacks the organization of, for instance, a vascular tree. As such, a prevascular network is comparable to the primitive vascular network that is formed during vasculogenesis.
Smooth muscle cells: the cells that surround endothelial cells in all blood vessels, except capillaries. Smooth muscle cells stabilize the vessels and play a role in the expansion and contraction of the vessels.
Vascular anastomosis: the process by which two vessels are functionally connected to each other.
Vascular axis: a macrovascular structure that is used for the transport of blood to and from a certain location. A vascular axis is often associated with a vascular tree, which distributes blood within a tissue.
Vascular tree: the typical organization of a vascular network within a tissue. A blood-supplying artery branches into smaller vessels (arterioles) that subsequently branch into capillaries. These combine again into venules that combine into a vein that transports the blood away from the tissue.
Vasculogenesis: the *de novo* formation of blood vessels by endothelial progenitor cells.
Vessel maturation: the process in which vessels are stabilized by the association with mural cells and the synthesis of extracellular matrix. Vessel maturation is generally accompanied by an inhibition of vessel growth.

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Box 1. Vascularization

Blood vessels are part of the circulatory system. They transport blood, and thus nutrients and waste products, to and from almost every part of the body. Three distinct structures can be distinguished in the vascular system. These are the (i) macrovessels (arteries and veins), which branch out into (ii) microvessels (arterioles and venules) and finally into (iii) capillaries. The capillaries facilitate the actual distribution of nutrients to the tissues in the body.

During blood-vessel formation, three processes can be distinguished; vasculogenesis, angiogenesis and arteriogenesis [52]. Vasculogenesis is the *de novo* vessel-forming process, which takes place during early embryonic development. Endothelial cells differentiate from their precursors and proliferate within a previously avascular tissue to form a primitive capillary network [53]. Vasculogenesis is followed by angiogenesis, when the initial vascular network is remodeled into more complex networks [54]. During this process, endothelial cells are activated and begin to degrade their surrounding matrix by the release of matrix metalloproteinases (MMPs). After this, the endothelial cells migrate into the gaps, resulting in the formation of capillary buds and sprouts. Endothelial cells that are located behind the migrating endothelium proliferate, thereby elongating the newly developing blood vessel [8]. Arteriogenesis is the process of structural enlargement and remodeling of preexisting small arterioles into larger vessels (Figure I) [55]. For a long time, it was generally accepted that new vessel formation in adults was limited to angiogenesis and arteriogenesis. However, more recent data suggest that the basis for native as well as for therapeutic neovascularization also includes postnatal vasculogenesis processes. It has been established that bone-marrow-derived endothelial progenitor cells, which are present in the peripheral blood, are augmented in response to certain cytokines and/or tissue ischemia and home into sites of neovascularization, where they are incorporated [56–58].

Vessel maturation is an important process in blood-vessel formation. Differentiated pericytes and smooth muscle cells stabilize vessel structures and suppress endothelial cell growth [59]. Vessel growth that is not accompanied by vessel maturation results in disorganized, leaky and hemorrhagic blood vessels that are prone to regression [6]. Because maturation is accompanied by a suppression of endothelial cell growth, the timing of maturation is crucial when designing vascularization strategies. If maturation starts too early, the vascular network will not be extensive enough to supply the entire tissue with nutrients. Conversely, if maturation starts too late, vessels are likely to regress and thus will not be able to establish a physiological circulation of blood.

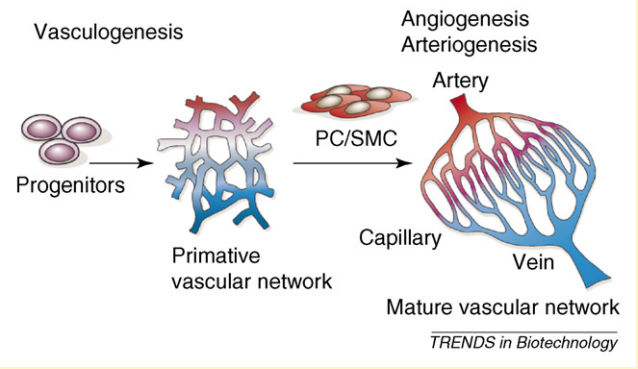

Figure I. Vascular tree development. During vasculogenesis, endothelial progenitor cells give rise to a primitive vascular network. In the next stages, termed angiogenesis and arteriogenesis, the network expands, and pericytes (PCs) and smooth muscle cells (SMCs) are recruited for the stabilization of the vessels. Finally, a mature organized vascular network emerges. Adapted, with permission, from Ref. [60].

vascularization after implantation, the degree to which these strategies are capable of enhancing vascularization varies, and this is further illustrated in Figure 2. The first two approaches, scaffold design and angiogenic factor delivery, both rely on the ingrowth of host vessels into the entire implanted construct. Therefore, although these strategies are able to increase the rate of vascularization, it would still take several days to weeks for the center of the implant to become perfused. By contrast, *in vivo* prevascularization can in principle result in the instantaneous perfusion of a construct after implantation at the final site because the construct is microsurgically connected to the host vasculature. However, before implantation at the final site, a pre-implantation period is necessary, during which time the implant has to rely on spontaneous angiogenesis from the surrounding vessels into the construct. Therefore, nutrient limitations are likely to occur during this stage. *In vitro* prevascularization does not result in the instantaneous perfusion of a construct because vessels have to grow from the host into the construct until they reach the vascular network formed *in vitro*. The invading vessels can then anastomose to the present vasculature, resulting in the perfusion of the entire construct with blood. Compared to scaffold design and angiogenic factor delivery, this method can dramatically decrease the time that is needed to vascularize the implant because host vessels do not have to grow into the entire construct but only into its outer regions, that is, until the ingrowing vessels meet the preformed vascular network.

Scaffold design

The architecture and design of a scaffold has a profound effect on the rate of vascularization after implantation. First, the pore size of the scaffold is a critical determinant of blood-vessel ingrowth. Druecke *et al.* showed that vessel ingrowth was significantly faster in scaffolds with pores greater than 250 μm than in those with smaller pores [9]. However, it is not only the pore size that is important for vascularization: the interconnectivity of the pores is also significant because cell migration, and thus vascularization, will be inhibited if pores are not interconnected, even if the scaffold porosity is high [10–11].

Conventional scaffold fabrication techniques include, amongst others, gas foaming, phase separation, freeze drying and particulate leaching [12]. These fabrication techniques have been widely used to produce 3D scaffolds for tissue-engineering applications. Although the shape and the size of the pores can be varied by changing the parameters of these techniques, the resulting organization of the pores is random. This can lead to pore pathways that are only partially connected and that follow contorted routes, which could impede the supply of nutrients and the ingrowth of tissue and vessels into the scaffold. Because they offer better control and the ability to actively design the porosity and interconnectivity of scaffolds, solid free-form fabrication systems are nowadays at the center of attention [13,14]. These versatile systems are capable of producing complex scaffolds with well-defined architecture and optimized pore interconnectivity. In addition, distinct regions, which might be of benefit for the recreation of zonal tissues like cartilage, can easily be created within a single scaffold.

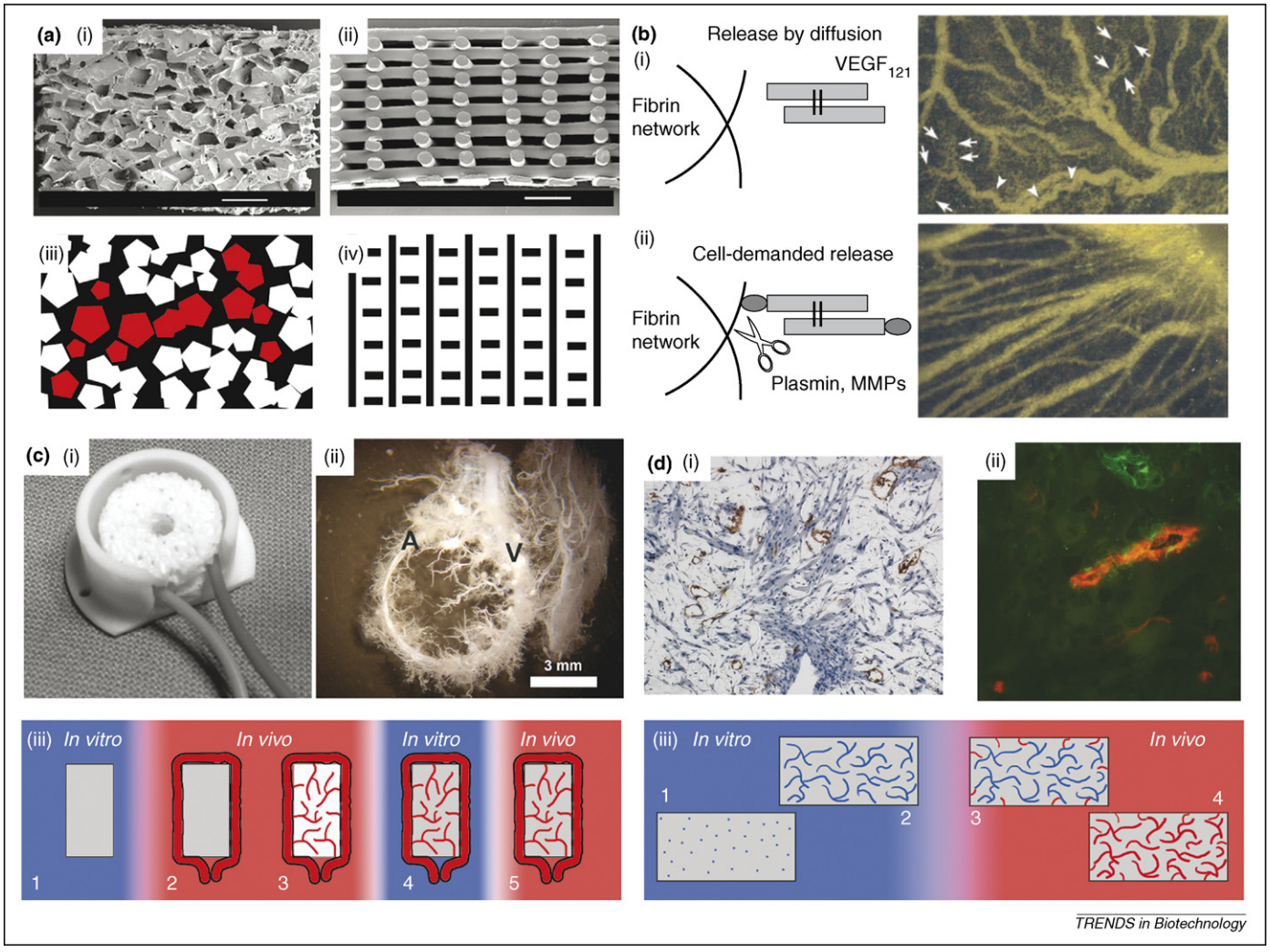

Figure 1. Different strategies for improving vascularization in tissue engineering. (**a**) Scaffold design. Panel (i) shows a scaffold that was prepared with compression molding and salt leaching. The scaffold in panel (ii) was obtained by 3D fiber deposition. Panels (iii) and (iv) schematically illustrate the scaffold geometries of (i) and (ii), respectively. Note that in the irregular scaffold (iii), some pores (depicted in red) cannot be reached from the outside, so vascular ingrowth will be prevented in these pores. Partly adapted, with permission, from Ref. [51]. (**b**) Growth factor delivery. Fibrin gel matrices were placed on a chicken chorioallantoic membrane (a membrane of the chicken egg). Panel (i) shows the effects of freely diffusible $VEGF_{121}$, which resulted in the formation of vessels with a disturbed morphology. Many of the newly formed vessel branches were characterized by malformed, corkscrew-like structures (indicated by the arrowheads). Furthermore, many of those branches appeared to abruptly drain into zones of irregular capillary enlargement and growth (indicated by the arrows). In panel (ii), $VEGF_{121}$ was released enzymatically by MMPs in a cell-demanded release. Note that upon cell-demanded release, a more regular organization of the vascular structures can be observed. Adapted, with permission, from Ref. [32]. (**c**) *In vivo* prevascularization. An artery (A) and a vein (V) were joined via a loop, which was then placed around a bone-tissue-engineered scaffold and implanted. Panel (i) shows the construct before implantation with plastic tubing instead of the arteriovenous loop for illustration. Panel (ii) shows the highly vascularized construct that was obtained eight weeks after implantation. Panel (iii) schematically depicts *in vivo* prevascularization. 1: Tissue construct preparation *in vitro*. 2: Implantation at the prevascularization site, supplied by a vascular axis. 3: Formation of a microvascular network by vessel ingrowth from the vascular axis. 4: Explantation of the prevascularized construct with the vascular axis. 5: Implantation of the construct at the defect site and surgical connection of the vascular axis to the vasculature. Partly adapted, with permission, from Ref. [34]. (**d**) *In vitro* prevascularization. Mouse myoblast cells (C_2C_{12}) were combined with human umbilical vein endothelial cells (HUVECs) and mouse embryonic fibroblasts (MEFs) and seeded on a scaffold, resulting in the formation of a 3D prevascular network. After implantation, the network anastomosed to the mouse vasculature. The prevascular network that is formed *in* vitro is shown in (i). This picture shows a cross section of the scaffold after *in vitro* culture in which endothelial cells are stained brown and muscle cells are stained blue. Note the presence of cross sections of tubular structures in brown, which shows that the endothelial cells have organized into vascular structures. The anastomosis of the prevascular network after implantation is illustrated in (ii), which shows a cross section of the scaffold after implantation. The vascular network that was formed *in vitro* is stained in green and all vessels that were perfused with blood at the time of explantation are stained red. The double staining demonstrates that the preformed vessels connected to the host vasculature and were perfused with blood. Panel (iii) schematically depicts *in vitro* vascularization. 1: A tissue construct containing endothelial cells is prepared *in vitro*. 2: The endothelial cells organize into a vascular network (blue). 3: The tissue construct is implanted and host vessels (red) grow into the construct. 4: When the host vessels reach the precultured vascular network, the vessels connect and the entire construct becomes perfused. Partly adapted, with permission, from Ref. [39].

An example of a solid free-form fabrication system for the production of tissue-engineering scaffolds is rapid prototyping or fiber deposition technology. With this technique, molten polymers, hydrogels or biomaterial pastes are extruded in the form of a fiber and, based on a CAD (computer-aided design) pattern, deposited on a stage to form a layer of the scaffold. An entire 3D scaffold can be prepared via layer-by-layer assembly. The technique is compatible with different materials, including polymers [15], metals [16], ceramics [17] and even gels with encapsulated cells [18].

Apart from solid free-form fabrication systems for the creation of regular scaffolds that favor tissue and vessel ingrowth, other strategies for enhancing vascularization have been explored. For instance, Gafni *et al.* designed a system in which a highly degradable biomaterial was used to create a filamentous scaffold. This scaffold was then seeded with endothelial cells *in vitro*, resulting in a

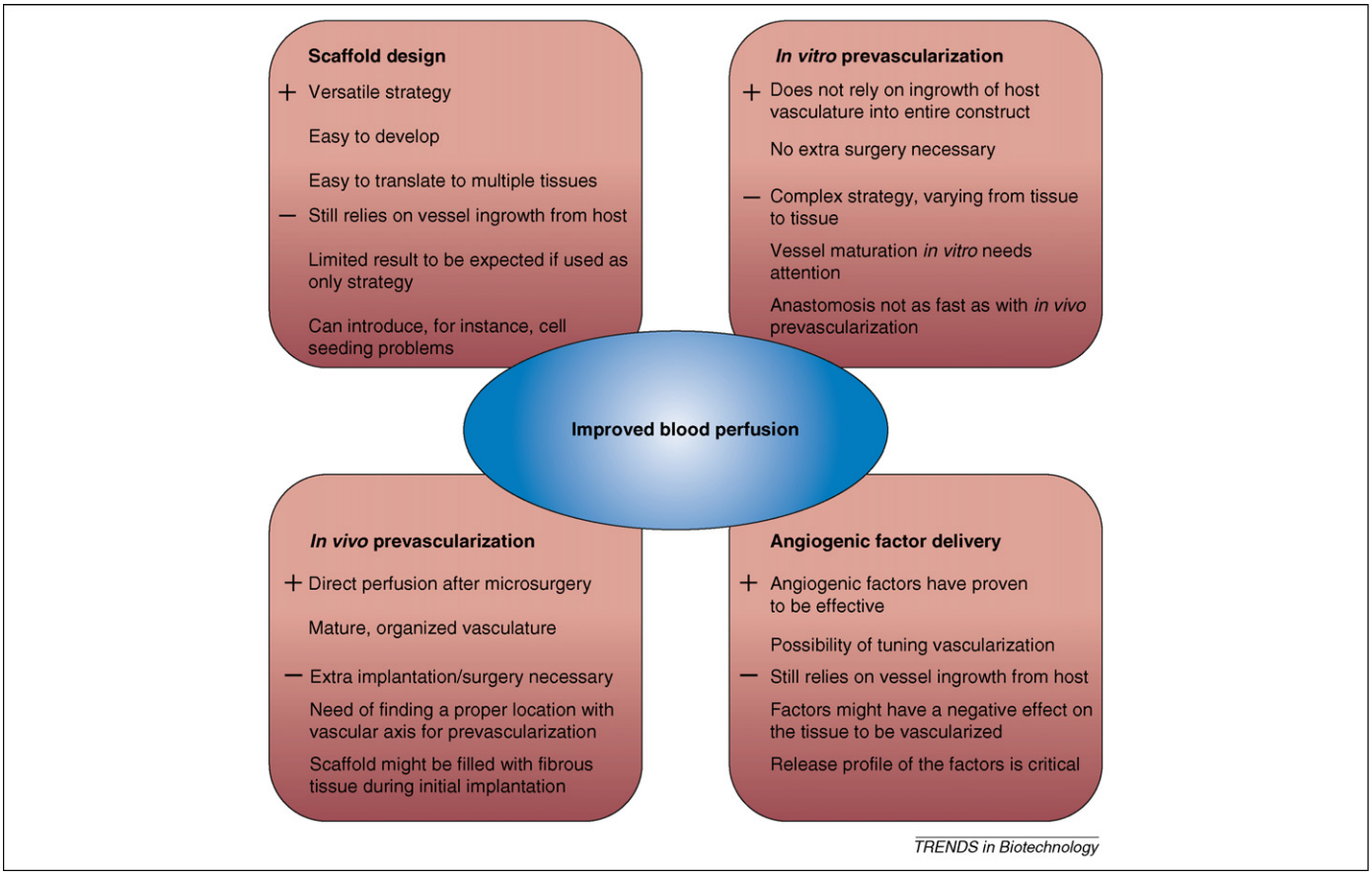

Figure 2. Overview over the advantages (denoted by +) and disadvantages (denoted by −) of the different strategies for vascularization in tissue engineering. The major goal is the improvement of blood perfusion and not merely an increase in the number of vessels, because nonperfused vessels do not contribute to the supply of nutrients. Although all depicted strategies can improve vascularization, a combination of the different strategies might be necessary to achieve sufficient perfusion for the prevention of nutrient limitations.

monolayer of endothelial cells on the filaments. After implantation, the filaments degraded but tubular structures of endothelial cells remained. After two weeks of implantation, it was observed that these tubular structures had become perfused vessels, illustrating that a vascular network can be directly designed using free-form fabrication techniques [19].

Angiogenic factor delivery

It is well established that the addition of angiogenic factors to tissue-engineered constructs can enhance their vascularization after implantation [20]. Angiogenic factors can be used to stimulate different stages of blood-vessel formation to increase the vascularization of a tissue-engineered graft. First, the formation of new vessels can be increased by growth factors, such as vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) [21], that stimulate the mobilization and recruitment of endothelial (progenitor) cells and therefore accelerate the onset of angiogenesis. Although the delivery of these growth factors generally results in increased angiogenesis, the resulting vessels are often disorganized, leaky and hemorrhagic [6]. Moreover, the factor dosage must be tightly controlled because excess amounts of VEGF have been shown to cause severe vascular leakage and hypotension [22]. Furthermore, it is important that newly formed vessels are stabilized. Except for the smallest capillaries, this is usually accompanied by a recruitment of smooth muscle cells or pericytes to the vessels and the subsequent production of an extracellular matrix. Growth factors that are important for the stabilization of new vessels include platelet-derived growth factor (PDGF), transforming growth factor β (TGF-β) and angiopoietin 1 (Ang1). PDGF is responsible for the recruitment of smooth muscle cells and pericytes, and TGF-β has been shown to be important for the production of extracellular matrix and the correct interaction between endothelial cells and mural cells [21,23]. Because both the formation and subsequent stabilization of new vessels are important for the creation of a functional vascular network within a tissue-engineered graft, the delivery of two sets of factors that are able to stimulate new blood-vessel formation and maturation might be necessary for optimal blood perfusion. Indeed, the delivery of both VEGF and PDGF has been shown to result in the formation of a high number of mature vessels in implanted scaffolds [20,24], indicating that this promising approach can be used to positively influence implant vascularization.

In addition to the delivery of factors that directly stimulate vessel formation or maturation, more indirect approaches have been studied as well. These encompass the delivery of other factors, such as sonic hedgehog homolog (SHH) [25], hypoxia-inducible factor 1 (HIF-1) [26] or bone morphogenetic protein (BMP)-2, -4 or -6 [27], all of which stimulate cells close to the vascularization site to produce angiogenic factors. This indirect approach has

several advantages over the direct delivery of angiogenic growth factors. First, the secretion of angiogenic factors by cells is often regulated and thereby ensures that the concentration of angiogenic factors is in the physiological range and can be adapted according to the requirements of different stages of vessel formation. Second, the production of angiogenic factors results in the formation of growth factor microgradients, which have been shown to be important for capillary morphogenesis [28]. Third and lastly, the stimulation with indirect factors often results in the secretion of several angiogenic factors that are able to regulate both vessel formation and maturation. For example, SHH was able to induce interstitial mesenchymal cells to secrete several factors, including VEGF and angiopoietins-1 and -2, and this resulted in the formation of highly organized, mature vessels [25].

Several strategies for the delivery of both direct and indirect angiogenic factors have been developed. These include the addition of recombinant proteins [29] and genes [30] to biomaterials and the use of cell transplants that are genetically engineered to overexpress specific factors [31]. The addition of recombinant proteins to biomaterials is the easiest method and thus has been most widely studied. The delivery of growth factors with biomaterial matrices is either driven by passive diffusion or can be coupled to the rate of biomaterial degradation. Both processes usually occur independently from each other and are often not in tune with the actual healing process [22], therefore resulting in a growth factor release profile that cannot be adjusted or fine tuned. The degree of release can be varied to some extent by altering the amount of growth factor added to the matrix. The kinetics of factor release can be influenced by varying the degradation rate of the material, which depends both on its chemical composition and its geometry. However, these limited measures are often insufficient to synchronize the growth factor levels with actual cellular demands. A novel approach to solving this problem utilizes a specific chemical linkage of growth factors to a gel matrix. The endothelial cells of ingrowing blood vessels secrete matrix metalloproteinases (MMPs) that are able to degrade the matrix to allow the vessels to penetrate the tissue. By degrading the matrix, the cells thus release the growth factors locally in response to cellular demand. It has been shown that the neovasculature that was induced by cell-demanded release of growth factors showed a higher degree of organization than neovasculatures that arose from an uncontrolled growth factor release [32,33].

In vivo prevascularization

Another promising strategy for enhancing vascularization in tissue engineering is *in vivo* prevascularization. This method, also referred to as tissue prefabrication, involves two distinct stages. In the first stage, a tissue-engineered construct is implanted into a region with an artery (or vascular axis) suitable for microsurgical transfer. This can mean that the tissue-engineered graft is either wrapped in an axially vascularized tissue, such as muscle, or that an artery is implanted into the graft. Although this ensures that a transplantable macrovessel is present in or around the graft, the graft is not yet supplied with a capillary network at this stage. A vascularization period of several weeks at this initial implant site will result in the formation of a microvascular network in the engineered construct, which is supplied with blood by the vascular axis (see Figure 1c) [34]. After this initial stage, the tissue-engineered construct is harvested together with the microvascular network and the supplying artery and then reimplanted at the defect site. At this site, the vascular axis is connected to the local vasculature using microsurgical vascular-anastomosis techniques, which results in instantaneous perfusion of the entire construct [35]. The advantage of this technique is that after implantation at the final site, the construct becomes immediately perfused by surgical anastomosis. However, its drawbacks are that two separate surgeries (one to implant the construct at the vascularization site and one to implant the construct at the final defect site) are necessary. In addition, a vascular axis has to be removed from the initial implantation site and, furthermore, cells might have to be reseeded before implantation at the final defect site because nutrient limitations are still likely during the vascularization period at the initial implantation site.

In vitro prevascularization

A strategy for improving vascularization that has gained interest recently is *in vitro* prevascularization. Tissues that have been studied include skin [36–38], skeletal muscle [39], bone [40–43] and cardiac muscle [44,45] (Table 1). This strategy is based on the observation that endothelial cells are able to form prevascular structures when they are cultured under the right conditions and environment *in vitro*. During *in vitro* prevascularization, endothelial cells are added to other tissues *in vitro*, which results in the formation of a prevascular network within this tissue. After implantation, this network can then anastomose spontaneously to the ingrowing vasculature of the host and supply the construct with nutrients. With this approach, host blood vessels do not need to grow into the entire construct, but only into the outer regions of the construct until the prevascular structures are reached. Even though this reduces the time needed for complete vascularization from weeks to days, perfusion is not as fast as with the previous strategy because the vascular network is not microsurgically connected after implantation. However, future developments in this field might aim to include the creation of a vascular axis within the *in vitro* construct that could be surgically connected to the host vasculature.

For prevascularized tissue engineering, endothelial cells are combined with other cell types to attain a tissue or tissue precursor together with a prevascular network (for instance, muscle cells and endothelial cells for prevascularized muscle). It is therefore important to find culture conditions that are suitable for both the organization of the vascular network as well as the development of the tissue that is being engineered. This implies that the use of angiogenic growth factors has to be minimized because they might negatively influence cells other than the target cell type that are present in the treated tissue. In bone-tissue engineering for instance, the use of VEGF could result in endothelial instead of osteogenic differentiation of the mesenchymal stem cells (MSCs) that are

Table 1. Published examples that used *in vitro* prevascularization as a strategy for improving vascularization in engineered tissues

Tissue	Cells used	(Implantation) result	Refs
Bladder[a]	Porcine smooth muscle cells and urothelial cells + porcine endothelial progenitor cells from peripheral blood	Non-endothelialized constructs showed implant thrombosis within 30 min after implantation, whereas endothelialized constructs showed no thrombosis after 3 h	[47]
Bone	Human mesenchymal stem cells from bone marrow + human umbilical vein endothelial cells	Vascular structures were still present after two weeks of implantation. However, no perfusion of the implanted structures was observed, indicating that they were not functional	[41]
Bone	Human bone marrow derived fibroblasts + human bone marrow endothelial cell line (HBMEC-60)	No implantation study performed. The paper points out that biomaterial composition and surface has an effect on the differentiation and organization of the co-cultures *in vitro*	[40]
Bone	Human primary osteoblasts or human osteoblast-like cell line (MG-63) + human dermal microvascular endothelial cells	No implantation study performed. The paper explores the use of various 3D bone biomaterials	[42]
Bone	Human primary osteoblasts or human osteoblast-like cell line (MG-63) + outgrowth endothelial cells from human peripheral blood or human umbilical vein endothelial cells	No implantation study performed. The paper illustrates that outgrowth endothelial cells show superior performance with regard to the formation of a vascular network *in vitro*	[43]
Cardiac muscle	Rat cardiomyocytes + human umbilical vein endothelial cells	Perfusion of the implant after 60 h of implantation was reported, but the origin of the perfused vessels (host- or implant-derived) was not determined	[45]
Cardiac muscle	Human embryonic stem-cell-derived cardiomyocytes + human umbilical vein endothelial cells; human embryonic stem-cell-derived endothelial cells + embryonic fibroblasts	No implantation study performed. The paper is innovative because the authors demonstrate the formation of a prevascularized tissue from a single cell source: human embryonic stem cells	[44]
Skeletal muscle	Mouse myoblast cell line (C_2C_{12}) + human umbilical vein endothelial cells; human embryonic stem-cell-derived endothelial cells + mouse embryonic fibroblasts	Implant-derived vessels showed anastomosis to the host vasculature. 40% of implant-derived vessels were perfused after two weeks. Prevascularized structures resulted in increased implant perfusion and survival	[39]
Skin	Human keratinocytes + human dermal fibroblasts + human umbilical vein endothelial cells	No implantation study performed. However, this was the first paper to illustrate the possibility of *in vitro* prevascularization	[36]
Skin	Human keratinocytes + human dermal fibroblasts + human umbilical vein endothelial cells	Prevascularized constructs were perfused with blood more quickly then non-prevascularized constructs. Perfused implant-derived vessels could be detected after four days	[37]
Skin	Human keratinocytes + human endothelial progenitor cells from cord blood, peripheral blood or human umbilical vein endothelial cells	Prevascularized constructs resulted in increased vascularization of the implant. Implant-derived vessels were coated with mural cells. No vessel perfusion data was presented	[38]

[a]This example differs in that it did not rely on the organization of endothelial cells into vascular structures. Here, decellularized native vascular structures were reseeded with endothelial cells followed by microsurgical anastomosis of the host vasculature.

typically used as bone precursor cells [46]. Strikingly, it has been demonstrated that endothelial cells can organize within a tissue without the addition of angiogenic factors [36,38,39,41,45]. This is an important finding because it might allow for the formation of a prevascular network without disturbing the development of the surrounding tissue.

The efficacy of *in vitro* prevascularization has been shown by studies that demonstrated that the prevascular networks formed *in vitro* can connect to the host vascular system after implantation [37,39]. Tremblay *et al.* reported that the prevascular network in a skin construct could anastomose to the host vascular system within four days, whereas vascularization of a non-prevascularized graft took as long as 14 days [37]. Moreover, Levenberg *et al.* reported that prevascularization of a skeletal muscle construct *in vitro* significantly enhanced construct vascularization, perfusion and survival after implantation [39]. In bone-tissue engineering, however, the *in vivo* success of *in vitro* prevascularization has so far been limited. We have shown that prevascular structures obtained from co-cultures of human umbilical vein endothelial cells (HUVECs) and human MSCs (hMSCs) were stable and organized into a more mature network after implantation [41]. However, anastomosis to the host vasculature was limited, indicating that *in vitro* prevascularization might not be successful in enhancing vascularization in all tissues.

A different strategy for *in vitro* prevascularized tissue engineering that does not rely on the spontaneous organization of endothelial cells has been reported by Schultheiss *et al.* for bladder tissue [47]. In this study, a segment of a porcine small bowel that contained a vascular network, supplied by a vascular axis, was decellularized. The matrix was subsequently reseeded with porcine smooth muscle cells and urothelial cells, whereas the vascular network was reseeded with porcine endothelial progenitor cells. This resulted in a prevascularized construct that could be microsurgically connected to the host vasculature. After implantation, the prevascularized construct was successfully perfused with blood, whereas the non-prevascularized construct was blocked by blood clots within 30 min. This demonstrated the feasibility of reseeding endothelial cells in a decellularized vascular network as an alternative means of prevascularizing an engineered tissue.

One crucial aspect of *in vitro* prevascularized tissue engineering is the source of the endothelial cells that are used for the formation of the prevascular network. Current developments in the field of endothelial progenitor cells, which can be easily isolated from blood, indicate their great potential in forming prevascular networks [48]. A detailed discussion of the nature of endothelial progenitor cells is outside the scope of this review, but two recent reviews can be consulted for more information [49,50].

Conclusions and future perspectives

Vascularization remains one of the main obstacles that needs to be overcome before large tissue-engineered constructs can be applied in clinical applications. Multiple strategies for improving vascularization in the field of tissue engineering have been developed. These can be divided into four groups: scaffold design, angiogenic factor delivery, *in vivo* prevascularization and *in vitro* prevascularization. However, at present it is still uncertain which will prove to be the best method for successful *in vivo* applications.

When only the speed of vascularization of a tissue-engineered construct after implantation at a defect site is taken into consideration, *in vivo* prevascularization is the most promising strategy because vascularization is instantaneous thanks to surgical anastomosis. In terms of speed, *in vivo* prevascularization is followed by *in vitro* prevascularization, angiogenic factor delivery and scaffold design, respectively. However, even with *in vivo* prevascularization, a construct will not be completely vascularized if the scaffold design does not allow for vascular ingrowth. Moreover, vascularization speed is not the only factor that will determine the success of a tissue-engineering strategy. Practicality in the clinic is another important aspect to be taken into account. In this regard, *in vivo* prevascularization poses the clear disadvantage that it requires two separate surgeries, and *in vitro* prevascularization is associated with a complex *in vitro* culture period that might not be easy to perform in a standard hospital situation.

Unfortunately, at present there is no convincing evidence that any of the described strategies will be sufficient to sustain tissue-engineered constructs that are larger than several millimeters after implantation. To increase the chances of success, researchers should not focus solely on any one of these strategies but should instead investigate the integration of several strategies with the aim of combining their strong points and eliminating their weaknesses. Apart from that, research should not only focus on the formation of blood vessels but also on the functionality and maturation of the newly formed vessels. This means that histology alone is not sufficient to determine the success of an experiment and that functional tests to assess vessel perfusion and stability will have to be implemented. In the end, it is not the overall number of vessels that is important but the number of functional vessels and the amount of blood they can carry.

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