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Integrated Approaches to Spatiotemporally Directing Angiogenesis in Host and Engineered Tissues

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Abstract

The field of tissue engineering has turned towards biomimicry to solve the problem of tissue oxygenation and nutrient/waste exchange through the development of vasculature. Induction of angiogenesis and subsequent development of a vascular bed in engineered tissues is actively being pursued through combinations of physical and chemical cues, notably through the presentation of topographical and growth factor cues. Presenting angiogenic signals in a spatiotemporal fashion is beginning to generate improved vascular networks, which will allow for the creation of large and dense engineered tissues. This review provides a brief background on the cells, mechanisms, and molecules driving vascular development (including angiogenesis), followed by how biomaterials and growth factors can be used to direct vessel formation and maturation. Techniques to accomplish spatiotemporal control of vascularization include incorporation or encapsulation of growth factors, topographical engineering, and 3D bioprinting. The vascularization of engineered tissues and their application in angiogenic therapy *in vivo* is reviewed herein with an emphasis on the most densely vascularized tissue of the human body, the heart.

Graphical abstract

Keywords

Angiogenesis; Biomaterials; Vascularization; Growth Factors; Cardiac Tissue; Tissue Engineering

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Disclosures

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1.0 Introduction

The regenerative potential of the human body after injury or disease is variable across different organs and tissues. In any healing scenario, there is a need for local microvasculature so that cells and proteins can be transported to the site of the injury to initiate and support the wound-healing response [1]. Furthermore, a hierarchical vascular bed is necessary to efficiently deliver blood to tissues, which necessitates a balance of capillaries and larger arteriolar vessels. The wound healing process is particularly paramount in organs like the brain and heart that have little functional regenerative potential, but where functional recapitulation of the damaged tissue is necessary for the continued health of the host. However, even with therapeutic intervention it is not yet possible to achieve this. For example, the high metabolic demand of cardiac tissue necessitates a high density, perfusable vascular network for sufficient nutrient and oxygen flux throughout the tissue. Consequently, the human heart has a dense vessel network of 2000-3000 capillaries/mm² and ~108 arterioles/mm², which supports the terminally differentiated, non-proliferative cardiomyocytes that endow contractile function [2–5]. Major coronary events such as a myocardial infarction (MI) can cause localized cell death if the tissue is not quickly supplied with adequate oxygen and nutrients, followed by fibrous scar tissue formation over the infarct area [6]. Post-MI complications such as reduced ejection fraction, ventricular hypertrophy, and arrhythmias can arise and lead to progressive heart failure wherein the heart is unable to pump enough blood to the rest of the body [7]. Cardiac tissue engineering seeks to regenerate the contractility of the heart that is lost following MI, but efforts up to this point have been unable to achieve full functional restoration, which may be partially attributed to the fact that currently implantable engineered constructs evaluated *in vivo* develop microvascular densities ~7-fold lower than that of the native heart [8]. The only suitable therapy for end-stage heart failure patients is a heart transplant, but a lack of available donors and numerous complications make this a limited option for clinicians and patients [3]. Indeed, the shortage of donor organs is a ubiquitous issue across many conditions, and tissue engineering solutions hold promise to alleviate this problem through the development of therapies to restore tissue function.

In tissue engineering, scaffolds made of degradable biomaterials are commonly used to facilitate the creation, growth, implantation, and integration of cells with the host [7,9]. However, there are a significant number of considerations involved in creating these tissues. One such challenge is the generation of thick, complex engineered tissues with sufficient microvasculature to support oxygen and nutrient transport between cells. The diffusional limit of oxygen restricts cells to being in close proximity to a capillary in order to acquire an adequate flux of oxygen, and the highly metabolic cardiomyocytes of the heart must be within 100-200 µm of a capillary for this reason [10–12]. The presence of a developed vascular network would have significant downstream effects on the success of the whole engineered tissue, namely enabling the development of more dense construct that better approximates the native tissue, allowing for superior inosculation with host vasculature upon implantation, and even having implications in remodeling and maturation, such as electrical syncytium formation in cardiac tissue [9,10]. Thus, one of the major current goals of tissue

engineering and regenerative medicine is the vascularization of 3D tissue constructs to mimic the networks of host tissue.

Complex physical and chemical mechanisms act on endothelial cells (ECs) to initiate *in vivo* angiogenesis followed by further processes to remodel and mature vasculature, which tissue engineers seek to emulate *in vitro* and *in vivo* through several methods [13]. Of interest in this review are techniques involving the manipulation of biomaterials to act as topographical signals to direct angiogenesis, and exposure to select growth factors (GFs) to similarly influence network formation [3,10,14]. Excellent reviews on biomaterial formulation and production for tissue engineering are found elsewhere [3,9,15–17]. However, a major obstacle and yet unmet need in the field is developing angiogenic systems of sufficient complexity such that dense and perfused microvascular networks form. Recent studies have attempted to introduce more sophisticated uses of physical and chemical cues to selectively induce angiogenesis in engineered tissues, including *in vitro* prevascularization and encouraging penetration of host vasculature into implants via *in situ* tissue engineering methods. This review will summarize recent methods of spatiotemporally controlling angiogenesis in engineered tissues using biomaterials and combinatorial biological cues, including growth factors. Techniques will be drawn from all forms of vascular tissue engineering with the purpose of understanding, inspiring, and applying design considerations for spatiotemporal control across all of tissue engineering and within the subfield of cardiac tissue engineering.

2.0 Vessel Development and Physiology

The formation of vascular networks in the body begins early during embryonic development, and dynamic changes to the network occur throughout life in response to both stimulatory and inhibitory signals. Neovascularization arises through both vasculogenesis and angiogenesis, in which EC precursors differentiate into arterial, venous, or lymphatic endothelium. While angiogenesis is more commonly utilized in tissue engineering applications, it is crucial to fully understand the physiological processes that dictate vessel formation and remodeling to discern both the inspirations for studies as well as their current limitations. Vascularization is also integral in the overall wound-healing process, wherein neovessel formation is necessary to facilitate the transport of cells and proteins to the site of injury in order to repair the damaged tissue. Readers are directed to Barrientos et al. for a comprehensive review of the mechanisms of wound healing [1].

2.1 Vasculogenesis

Vasculogenesis is the *de novo* formation of blood vessels, most commonly via angioblast differentiation in prenatal development [10,18]. Hemangioblasts originating from the lateral plate mesoderm are directed to aggregate and form “blood islands”, or aggregates of cells, comprised of hematopoietic stem cells surrounded by angioblasts. Endothelial progenitor cells (EPCs) arising from the angioblasts differentiate into ECs and the aggregates fuse into a primitive vascular plexus, generating lumens throughout the body that are perfused following the onset of the heartbeat [19]. The driving mechanisms of embryonic vasculogenesis are difficult to determine, and there exist several hypotheses and

computational models suggesting that vascular development and arrangement occurs via cell motility guided by the extracellular matrix (ECM), paracrine and autocrine-mediated chemotaxis, and cellular contact signaling [20,21]. Postnatal vasculogenesis is less well understood, but is thought to be dictated by EPCs residing in various locations around the body, such as within stem cell niches of the vascular basement membrane, in the circulation, or in the bone marrow that are mobilized in response to stimuli like growth factors and hypoxic stress [22]. These immature cell types are clearly crucial for *de novo* vessel formation, and studies have pursued methods such as the use of angioblast-like cells and co-culture to initiate vasculogenic processes in *in vitro* and *in vivo* settings [23,24]. The use of developmental precursor cells such as pluripotent stem cell-derived angioblasts is promising as an *in vitro* model of vasculogenesis and for translational applications [25].

2.2 Angiogenesis

Angiogenesis is the major determinant for postnatal vascularization in which new capillaries are generated from existing vasculature through sprouting and intussusception processes [13,26]. Sprouting occurs in response to events like inflammation or ischemia that cause the release of stimulatory autocrine and paracrine cytokines, such as the very potent vascular endothelial growth factor (VEGF). Subsequent degradation of the local basement membrane and migration of vessel ECs, led by a specialized tip cell, results in leaky vasculature. Buildup and polymerization of plasma-derived proteins such as fibrinogen and fibronectin from the blood causes the formation of a provisional ECM that guides EC extension into the surrounding environment [27,28]. Proliferation of stalk cells behind the tip cell and recruitment of mural cell populations allow for elongation, stability, and lumen formation of the sprout [13,26,29]. The process of intussusception involves the remodeling of existing vasculature via the protrusion and fusion of opposite vessel walls within a lumen, effectively splitting an existing vessel into two and creating a branched structure. In comparison to sprouting, intussusceptive angiogenesis is significantly faster in expanding capillary networks as it doesn't rely on cellular division, but rather reorganization of existing ECs [29]. Stress triggers like hypoxia and injury induce angiogenesis to form immature microvascular networks through both sprouting and intussusception processes, followed by pericyte invasion and subsequent cytokine-mediated cellular recruitment to stabilize and remodel the vessel walls and basement membrane [13,27,29]. The network of premature vessels is then pruned and remodeled to establish an efficiently perfused, hierarchical vascular bed that meets the oxygenation needs of the tissue. Vessel ablation in this manner is mediated primarily by hemodynamic cues from local vasculature, in which ECs migrate and integrate with nearby perfused vessels or otherwise undergo vascular regression via apoptosis due to lack of pro-survival stimuli such as shear flow and growth factor gradients [30]. As discussed below, many tissue engineering applications aim to induce an angiogenic response from host endothelial cells that takes advantage of the body's natural capacity to vascularize tissue through angiogenesis when regenerative strategies are employed in specific wound beds, such as an infarcted heart.

2.3 Arteriogenesis

In contrast to the capillary-forming process of angiogenesis, arteriogenesis is the compensatory remodeling of existing collateral blood vessels into functional arterial vessels

in response to physical stimuli like flow-related stresses due to large pressure differentials between upstream arteries and downstream branches [31]. This process effectively increases vessel diameter and wall thickness through EC activation of downstream cellular pathways such as proliferation, and recruitment of supportive cell types for further autocrine and paracrine signaling. In instances where vascular networks are implanted, compensatory arteriogenesis does not occur until inosculation with the host network is achieved, as the absence of a flow stimulus in vasculature instead promotes vessel regression [30]. Caution is advised in how the tissue engineering community uses the term “arteriogenesis,” as published literature has defined it as the appearance of new artery-sized vessels (e.g., >50 μm), although this is not the accepted definition historically in the field of vascular biology [32]. Arteriogenesis does not induce new vessel formation, but can act independent of angiogenesis through related pathways [33,34]. It is an important post-anastomotic maturation process that operates in conjunction with vascular pruning to direct efficient vascular adaptations based on a tissue’s oxygenation needs [34]. For example, there is evidence that suggests that existing coronary arteries can undergo arteriogenesis in response to a cellular implant after MI [35]. Indeed, this remodeling mechanism is an excellent target of opportunity to ensure sufficient blood flow to implanted engineered tissues or to remodel bypass routes for increased blood flow such as in the case of peripheral artery disease or around a stenotic site.

2.4 Lymphovasculogenesis and Lymphangiogenesis

The vasculature of the lymphatic system is a uniquely blind-ended vascular tract that is mainly derived from the venous lineage of endothelial cells during embryonic development (sometimes referred to as lymphovasculogenesis), and can be identified by lymphatic-specific markers such as PROX-1 and VEGFR3 [36,37]. The lymphatic endothelial cells (LECs) that form lymph vessels are structured with loose intercellular junctions and a discontinuous basement membrane to facilitate the functions of the lymphatic system, which ranges from supporting the immune response to introducing interstitial fluid back into the circulatory system [38]. In the context of wound healing, lymphangiogenesis occurs in lymphatic capillaries after injury, wherein recruited inflammatory cells like macrophages release growth factors that stimulate lymphatic vascular growth, further facilitating transport of immune cells and cytokines to the site of damage as well as promoting interstitial drainage [37]. Lymphangiogenesis thus acts in concert with angiogenesis, yet there are far fewer studies pursuing or even considering lymphatic tissue engineering applications despite the equal importance of the former [39]. For example, Klotz et al. demonstrated that MI induction in mice causes upregulation of pro-lymphangiogenic genes in the surrounding heart tissue, which aids and may even expedite the resolution of the inflammatory response [36,40]. Güc et al. demonstrated that an engineered VEGF-C variant that binds fibrin induced local lymphangiogenesis in a subcutaneous rodent model, and observed increased granulation tissue thickness and ECM deposition indicative of improved wound healing [41]. While not a focus of this review, it is important to understand how lymphangiogenesis interfaces with angiogenesis in wound repair, and how this process can be effectively integrated into current vascular tissue engineering efforts.

3.0 Cells in Vascular Tissue Engineering Research

The development of vascular endothelium and its specialized arterial and venous lineages are a central focus in vascular tissue engineering efforts and of interest to this review. The differences between individual cell types, what phenomena are trying to be recapitulated, and how relevant those results are to *in vivo* processes must be considered when choosing an EC lineage for vascularization studies. Furthermore, the interactions between ECs and other cell types must be carefully dissected and understood as well, either for co-culture studies or *in vivo* interactions with host cells.

3.1 Endothelial Cell Types

In vascular biology and tissue engineering research, ECs are commonly identified by staining for highly expressed markers such as platelet endothelial cell adhesion molecule-1 (PECAM-1, more commonly known as CD31), RECA-1 (rat endothelial cell antigen-1, the rodent equivalent of CD31), or von Willebrand factor (vWF) [42]. Human umbilical vein endothelial cells (HUVECs) remain one of the more popular endothelial cell lines to use due to their low cost, simple isolation, and high angiogenic potential [43]. However, these are not the most physiologically relevant cell type to use, as angiogenic efforts often focus on microvascular bed development which is mediated by different, organ-specific EC lineages. Alternatively, studies have used primary aortic, vein, lymphatic, and organ-specific microvascular ECs from both humans and rats, donor human samples, and most recently ECs derived from human embryonic or induced pluripotent stem cells (hESC-ECs and hiPSC-ECs) [39,44–46]. Rodent cells, while acceptable for *in vitro* and *in vivo* studies, are not translationally feasible given their xenogenic origin and thus incompatibility with the human immune system. Furthermore, species-specific differences, like the differential response of human and rodent aortic smooth muscle cells (SMCs) to the anti-inflammatory molecule dexamethasone, limits the application of research findings in translational, clinical studies [47].

hiPSC-ECs provide a target of opportunity for translational application in that autologous therapies may be viable in the future [2]. Differentiation protocols to generate hiPSC-ECs previously relied on stromal cell co-culture, embryoid body, and small molecule monolayer strategies [45]. However, these methods are devoid of physical stimuli like flow which help specify particular EC fates, and often have low yields which necessitates time-consuming and expensive purification processes like fluorescence-activated cell sorting. It is suggested that genetic background has a bearing on the differentiation potential of stem cells across many lineages, including hiPSC-ECs [48]. Thus, lineages must be properly characterized before use whether differentiated or commercially sourced, and in the former case the differentiation protocol must be optimized for that specific lineage. More recently, attempts to generate hiPSC-ECs have used 3D matrices and/or induced flow to encourage hiPSC-EC differentiation for higher yields. The following articles are suggested for further reading on the differentiation, characterization, and application of hiPSC-ECs [45,49].

EPCs are unspecialized, but determined vascular cells which have become a recent subject of interest in recapitulating embryonic vasculogenic development processes *in vitro* and *in vivo*. There is an important debate around what hemangioblast lineages constitute “true”

EPCs, as these cells can be sourced from various organs, have variable differentiation efficiency into endothelial cells, and thus contribute differentially to vessel development [38,50,51]. EPCs can also arise from the differentiation of other cell types, such as the transdifferentiation of macrophages into lymphatic EPCs [52]. Peters et al. describes the requirements of EPCs to be expression of EC-associated markers (CD31, CD105, CD144, CD146), as well as a lack of certain hematopoietic markers (CD14, CD45, CD115), and readers are further referred to this review for an in depth review of the underlying biology of EPCs [38]. The main criticisms behind use of these cells is their relatively unknown characterization and thus unpredictable effectiveness in culture, and the inability to easily source them commercially; many studies rely on human donor cells which are expensive and time-consuming to harvest.

In vascular tissue engineering and angiogenesis research more broadly, there are overarching concerns with the use of ECs and their related lineages *in vitro* and *in vivo*. Most endothelium in the body is naturally quiescent, and only takes on a proliferative phenotype in response to injury [43]. In this active state, ECs undergo a change in surface marker expression and can lose organ- and function-specific characteristics. Furthermore, the specific characteristics and responses of ECs are dependent on the tissue they originate from [53,54]. This is a major challenge when developing studies involving ECs, as the need for proliferative and tubulogenic cells to form a vascular bed conflicts with the desire for those cells to recapitulate the structure and function of a native tissue. Furthermore, characteristics such as fenestrated, continuous, and discontinuous endothelium are unique and crucial to the proper function of organs, but experimental design to develop a capillary phenotype other than continuous endothelium is not considered in vascular tissue engineering studies, with “leakages” often being attributed to unstable, immature vessels [55]. However, these characteristics are specified in a paracrine fashion by local tissue, suggesting that specific phenotypes could be induced by surrounding host tissue after implantation [54]. Lastly, endothelial cell types tend to suffer from inactivation of proliferative and tubulogenic capacity at higher passages in culture [43].

Vascular endothelium is supported by cell types that are recruited at various steps of vascular development, homeostasis, and remodeling. SMCs are present around all vessels except capillaries and contract circumferentially to regulate transport of blood around the body, and can thus be used to identify arterioles by staining for α -smooth muscle actin (α -SMA) [27]. Pericytes maintain the basement membrane of capillaries and provide a number of supportive roles for ECs depending on the organ, but notably mediate paracrine signaling, hemostasis, and vessel stabilization [56]. Multipotent lineages such as bone marrow- or adipose-derived mesenchymal stem cells (MSCs) can also be recruited and differentiate into vascular mural cell types [57]. Leukocytes like macrophages and monocytes provide cues for vascular remodeling such as matrix metalloproteinases (MMPs) and pro-angiogenic growth factors.

3.2 Co-culture with Endothelial Cells

Co-culture of ECs with these supportive cell types is a natural option given the multitude of cell types necessary to develop and maintain vasculature. This enables further specialization

of an engineered tissue for specific applications, but requires consideration of the cell-cell signaling interactions of different lineages and their dependence on ratios of different cell types, as well as culture conditions such as substrates, matrix proteins, and media exposure *in vitro* [58]. For example, SMCs have been cultured with ECs to encourage the formation of arteriolar vessels [59]. Supportive stromal cell types are necessary for long-term (>1 year) stabilization of EC networks by inducing a paracrine-mediated quiescent phenotype in the vascular cells [60,61]. Paracrine signaling between ECs and supporting cell types also confers effects on gene expression, such as the upregulation of pro-angiogenic cytokines which affect vascular morphology [62,63]. Optimal ratios of cell types for co-culture is less clear, as there does not appear to be a consensus due to the vast number of parameters to control, even within applications of a specific tissue type. Metabolic and proliferative potential, angiogenic potential of the ECs, lineage differences, paracrine interactions, culture media, and cell-biomaterial interactions are among the many potentially confounding variables that complicate the determination of definitive co-culture ratios [58]. However, it is known that high amounts of endothelial cells relative to other cell types typically induces vessel regression due to the redundancy of vascular structures [30]. In applications regarding the heart, cardiomyocytes have been cultured with ECs or SMCs for cardiac tissue engineering applications and shown beneficial effects on cardiac tissue metrics [64,65]. Tri-cultures of cardiomyocytes, ECs, and supportive stromal cells have been performed, but most studies are relegated to two cell populations due to the additional biological complexity that arises when >2 cell populations exist in a system, which in certain cases produced less favorable results than co-culture [66–68]. The additional biological complexity of co-culture systems enables a greater and more physiologically relevant understanding of cellular interactions both with other cells and their microenvironment, which is critical in implementing vascularization efforts in specific tissue engineering applications.

4.0 Factors and Signaling Pathways in Angiogenesis

Angiogenesis is dependent on cell-cell and cell-matrix interactions, meaning that careful orchestration of the appropriate signaling factors and pathways is required to induce neovascularization in engineered tissues. The most well-known molecules used to encourage vascularization are angiogenic growth factors, morphogenetic proteins that induce and support angiogenic processes such as proliferation, differentiation, and migration of cells through a variety of unique signal transduction pathways [69]. Indeed, the use of “traditional” angiogenic factors and their recombinant variants is ubiquitous in the literature in a variety of applications, and research has focused heavily on improving the stability, expression, delivery, and retention of single factors to target tissues [69]. The natural complexity of angiogenesis stems from the multiple isoforms of growth factors and cytokines that interact with target receptors in time-dependent and spatially-controlled ways to elicit a specific response [70]. The nuances of these signaling pathways is a great challenge for translational application of angiogenic proteins as therapeutics. Temporal exposure of growth factors must be carefully considered, such as a controlled release system can be designed to maintain a local concentration that elicits a prolonged physiological response. However, inhibition, sequestration, or downregulated production of growth factors and their downstream pathways is just as important to prevent uncontrolled growth and

potentially harmful consequences [70]. Thus, therapeutic angiogenesis must be precisely regulated by limiting it to the intended therapeutic areas for an appropriate time frame. There are even studies showing that controlled inhibition can assist in angiogenesis, such as through anti-growth factor drugs like anti-VEGF or by specific inhibition of proteins like glycogen synthase kinase 3 β in post-MI hearts to activate the Wnt signaling pathway [55,71–73]. Despite being utilized less in vascular tissue engineering, these inhibitory compounds are as important as their angiogenic-inducing analogs [42]. A few common growth factor families that are important in angiogenic cascades and utilized in vascular regeneration will be briefly discussed to better understand their role in developing mature vascular networks.

4.1 Vascular Endothelial Growth Factor (VEGF)

The VEGF family of angiogenic growth factors is considered the gold standard, playing a significant role in both vasculogenesis and angiogenesis [19,74]. These homodimeric polypeptides consist of six main isoforms of VEGF (VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E, and VEGF-F), as well as placental growth factor (PlGF), and the associated receptors VEGFR-1, VEGFR-2, VEGFR-3, Neuropilin-1 (NRP-1), and NRP-2 [55,74,75]. VEGF-A was the first factor of the family discovered and is presently the most well-known and widely used factor, as it plays a major role in mediating vascular EC survival, proliferation, and migration [13]. VEGF-A and its spliced variants can bind both VEGFR-1 and VEGFR-2, although binding of the latter receptor is far better understood and mediates pathways with the strongest angiogenic responses [76]. Binding to VEGFR-2 causes dimerization of the receptor and enables phosphorylation at multiple tyrosine domains, which dictates further downstream signaling cascades to orchestrate angiogenesis. VEGF-B assists in the maintenance and maturation of formed vessels, and interestingly a recent study implicated VEGF-B as a non-angiogenic cardioprotective factor through mitigation of oxidative stress-induced apoptosis [73,77,78]. VEGF-C and VEGF-D can bind VEGFR3 (primarily), VEGFR2, NRP-1, and NRP2, causing downstream upregulation of lymphangiogenesis to support the wound-healing process [75,76]. PlGF is a key player in vascular development during embryogenesis, and additionally displays synergistic roles with other VEGF isoforms in wound healing, ischemia, and other stressed conditions [1,13,55,79]. VEGF-E and VEGF-F are special angiogenic proteins; the former is encoded by the Orf virus and only binds VEGFR-2, and the latter encompasses a subfamily of VEGF-like molecules present in snake venom which bind mainly VEGFR-1 and promote high vascular permeability [79]. VEGF-A remains the most commonly used angiogenic growth factor within the VEGF family in vascular engineering applications due to its ubiquitous presence in initiating angiogenic processes and its potent effects, with the other isoforms often relegated to more niche applications. However, despite this pervasive presence, studies have demonstrated that VEGF alone is limited in its ability to induce the formation of functional, dense, and persistent vessel networks [70,80]. Disorganized and often leaky vessels result from the sole delivery of VEGF, which can be indicative of unstable, immature vessels that require additional modulation from other factors or cells [81].

4.2 Fibroblast Growth Factor (FGF)

The FGF family consists of 22 growth factors and 4 associated receptors with their alternative splicing variants, which mediate a wide variety of functions from angiogenesis to neural development [82,83]. Despite the name, some of the molecules in this family are not considered traditional growth factors, but they all share the common ability to bind to FGF receptor (FGFR) isoforms [83]. Of significant relevance to angiogenesis are FGF-1 (acidic FGF, or aFGF) and FGF-2 (basic FGF, or bFGF), although other isoforms have also been implicated in angiogenic pathways [83,84]. Binding to the appropriate receptor (such as FGF-1 to FGFR-1 or FGFR-2) in the presence of heparin-sulfate proteoglycans in ECM and on the surface of cells causes tyrosine phosphorylation and subsequent signal transduction, such as through binding of the cytoplasmic effectors FGF receptor substrate 2 α or phospholipase C γ [55,83]. FGF promotes tubulogenesis and migration of ECs and EPCs, and can assist in signaling for both ECM proteolysis and synthesis of collagen, fibronectin, and proteoglycans, as well as enhancing VEGF production, suggesting synergistic actions for angiogenesis [84–86]. Further, both VEGF and FGF provide cues for initiating angiogenesis by inducing EC proliferation and migration, but act through different pathways [55]. For example, a study by Lee et al. compared the effects of VEGF and bFGF in subcutaneously implanted alginate hydrogels and found that both growth factors were individually able to induce granulation tissue and vessel formation [87]. However, FGF signaling was unable to increase blood vessel density to levels as high as in VEGF-loaded hydrogels. Studies have also posited that FGF isoforms assist in cardiac remodeling and endow cardioprotective properties through angiogenesis and lesser-understood paracrine pathways [83].

4.3 Platelet Derived Growth Factor (PDGF)

Closely related to VEGF is the PDGF family of growth factors which encompasses PDGF-A, PDGF-B, PDGF-C, PDGF-D, the heterodimer PDGF-AB, and the receptors PDGFR- α and PDGFR- β [88,89]. Homodimer PDGF isoforms remain inactive until dimerization and receptor binding occurs, allowing for receptor dimerization, auto-phosphorylation and subsequent downstream signaling dependent on the variety of dimer complexes formed, phosphorylated domains, and associated adapter molecules involved [89]. While normally secreted by circulating platelets in the bloodstream, other cell types have been shown to release specific isoforms of the factor. PDGF-AA, BB, and CC are the most well-known supporting factors for angiogenesis. ECs, fibroblasts, macrophages, and other vascular support cells can release this growth factor after neovessels are established, generating a chemotactic gradient that further attracts pericytes, leukocytes, fibroblasts, and vascular SMCs to stabilize, remodel, and mature vessels [55,90–92]. PDGF isoforms exhibit VEGF-independent stimulation of angiogenic pathways, such as the necessity of PDGF-B in recruiting SMCs/pericytes to immature vessels for stabilization and remodeling [92,93]. Absence of this factor has been found to result in a maintenance of the proliferative EC phenotype which is not conducive to maturation, or even vessel regression in the event of arrested VEGF signaling [91,93].

4.4 Other Growth Factors and Cytokines Contributing to Angiogenesis

Many other cytokines participate in angiogenesis in addition to the dominant effects of those described above. Some are not considered traditional growth factors at all, but cytokines and other proteins that produce downstream effects on angiogenesis in tissues which can be dependent on specific conditions, including location, cell type, and timing. For example, the angiopoietin (Ang) family of growth factors are critically involved in embryonic vascular development via the Tie pathway, and are also necessary for sprouting angiogenesis, vessel wall remodeling, EC survival, and mural cell recruitment [84,90,94,95]. Transforming growth factor- β (TGF- β) is released by ECs as part of the inflammatory response to recruit macrophages, and further can help mediate the differentiation of vascular SMCs and contribute to EC proliferation, migration, and tubulogenesis to form and maintain granulation tissue [84,91]. Sonic hedgehog (SHH) is a morphogen that is present in both embryonic development as well as postnatal angiogenesis, and has been shown to mediate expression of angiogenic growth factors like VEGF-A and Ang isoforms, promote angiogenic cytokine production from stromal populations, and enhance recruitment of EPCs to remodel microvascular networks [96–101]. SHH is upregulated in the ischemic heart, and has been shown to act in a cardioprotective manner via paracrine-mediated growth factor upregulation, arrested cardiomyocyte apoptosis, and neovascularization [102,103]. MMPs initiate ECM remodeling events that are required for EC sprouting and neovessel intussusception [13,84]. MMP-14 (also called MT1-MMP) in particular is a membrane-bound MMP that has been shown to play a crucial role in facilitating angiogenesis by degrading the ECM for cell migration and activating membrane-bound growth factors [104]. MMP-9 and MMP-12 have been noted to play a role in angiogenesis and vessel stabilization as well, such as in creation of angiostatin to inhibit vessel formation, although many studies examining these proteins are found in the context of inhibiting tumor angiogenesis and metastasis via MMP inhibition [84,105].

Specific events can induce a variable angiogenic response as well, as is the case of factors that provide some level of compensatory cardioprotective effect after a heart attack. When myocardial ischemia occurs, hepatocyte growth factor (HGF) is induced in the myocardium, and nerve growth factor (NGF) and insulin-like growth factor-1 (IGF-1) are secreted to mitigate cardiac damage after infarction and assist in activating angiogenic pathways [13,106–108]. Neovascularization-related growth factors play an important role in ischemic heart disease (reviewed by Henning, 2016) including stromal cell-derived factor-1 (SDF-1), which has been shown to attenuate cardiomyocyte apoptosis and stimulate angiogenesis in both *in vitro* angiogenic assays and *in vivo* rat models of MI [74,109,110]. Other types of exogenously delivered molecules can be used to induce vascularization in contexts like cardiac repair. For instance, the drug ONO1301, an agonist for Prostaglandin I₂, was shown to induce expression of VEGF, SDF, and HGF in the myocardium and acted in a cardioprotective role by increasing local vascularization and overall cardiac performance in a hamster model of dilated cardiomyopathy [108]. Growth factor fragments and peptide sequences derived from larger molecules involved in angiogenesis pathways have demonstrated improved function versus full-length molecules due to modified binding affinities and protein stability [111–114]. For example, a peptide from the Notch ligand Jagged1 improved the vascular bed in an *in vivo* model of cardiac failure and recruited

cardioprotective cytokines through downstream pathway activation [115]. Adipose-derived microvascular fragments isolated from rodent fat pads have been found to induce significant angiogenesis and lymphangiogenesis when implanted subcutaneously, and may be a translationally relevant methodology for implant prevascularization or direct transplantation [116,117].

The vast number of molecules that participate in angiogenesis via different pathways prompts the notion that preferential molecule choice will be required to stimulate the intended angiogenic phenotype. For example, Benest et al. demonstrated *in vivo* that VEGF signaling induced several short, highly branched vessels, while Ang-1 signaling produced longer but less branched vessels as well as higher pericyte recruitment [118]. At the same time, there may be some leeway in angiogenic growth factor selection if another molecule can sufficiently supplant the original factor's angiogenic functions, and surrounding host tissue can be left to provide the necessary maturation and remodeling signals. Grigorescu et al. demonstrated in their study of angiogenic properties of EPCs that the depletion of VEGF-A alone did not significantly interrupt angiogenic processes in culture, but simultaneous knockout of both VEGF-A and SDF-1 resulted in severely stunted angiogenesis [119]. This calls into question the assumption that VEGF is the primary initiator of angiogenesis and encourages discourse around where interventions should target along angiogenic signaling cascades to produce the intended therapeutic effect. Exposure to multiple factors to robustly stimulate this cascade in key pathways may be enough to develop a new, permanent vasculature in tailored regenerative medicine applications. There are still many unknown intermediate mechanisms, such as the interplay of CD31 in the angiogenesis pathway, and often *in vitro* isolation studies are unable to capture the complexity of growth factor response *in vivo* [42]. What has been made clear is that these signaling molecules elicit multi-pathway effects and induce paracrine activity [14,108]. Thus, angiogenic systems must be designed knowing as much as possible about systemic effects across the regulatory network, and be inclusive of the waterfall effects of the biochemical stimuli used.

4.5 The Necessity of Combinatorial Growth Factor Use

The study of single-use growth factor release has revealed the great complexity of angiogenic signaling responses. The vast research into the actions of growth factors has made it clear that the use of a single growth factor is not sufficient to create a stable vascular structure, as many growth factors work either in tandem or sequentially [120]. For example, it has been shown that VEGF-C promotes PDGF-BB expression in ischemic tissues [13]. A study by Stone et al. found that endogenous VEGF-A alone cannot properly induce both angiogenesis and arteriogenesis, and that additional modulation with exogenous Ang-1 to act as a maturation agent could produce more physiologically relevant vascular networks [80]. Brudno et al. further demonstrated that simultaneous exposure of VEGF-A and Ang2 additively induced angiogenesis *in vitro* and *in vivo* [94]. This response was inhibited by synchronous release of PDGF-BB and Ang1, but delayed release of the latter two factors did not interfere with angiogenesis but instead promoted vessel maturation and remodeling. As a final example, bone morphogenetic proteins (BMPs) have been shown to promote angiogenesis by stimulating osteoblasts to produce VEGF-A [121]. Thus, understanding these relationships within the organ of interest is crucial to designing angiogenic tissue

engineering systems. Figure 1 demonstrates the temporal complexity and overlap of multiple prominent pathways in angiogenesis and its relation to the wound healing cascade. Implementing this notion of combinatorial growth factor use in experimental design has enabled a more thorough examination of multi-pathway effects and paracrine signaling in angiogenesis, which can then be leveraged to generate more robust vascular beds in engineered tissues by creatively presenting cues that initiate these interactions. This beneficial shift in direction has been accompanied by the popularity of engineering biomaterials for tissue engineering applications, providing the field with the tools necessary to develop and examine more complex systems for spatiotemporal administration of multiple angiogenic growth factors.

5.0 Techniques for Controlling Angiogenesis with Biomaterials

The use of biomaterials has expanded in the past decade with the development of innovative techniques to support the growth of tissues both *in vitro* and *in vivo* and provide cells with well-defined spatiotemporal cues to initiate angiogenesis [10]. The use of synthetic and natural materials imposes constraints dependent on the specific application of the biomaterial. These materials must be chosen carefully, as there will inevitably be interactions when placed into a physiological environment, whether that be through cell-material interactions *in vitro* or an implant *in vivo*. Cells that require adhesions should be able to adhere and interact with the matrix and neighboring cells, whereas cells not requiring adhesions should be able to survive and function within the microenvironment of the biomaterial. In short, the material in question must be biocompatible and tailored to the specific engineered tissue [3,9]. Material selection is thus a significant component of tissue engineering, and certainly there is no shortage of studies examining optimal material choices and even developing composites and entirely new materials with which to engineer replacement tissues [122–125]. Of interest to the subject of this review, however, is the manipulation of these materials and their interactions with angiogenic growth factors to control angiogenesis and neovascularization in engineered tissues.

5.1 Matrix Incorporation

Incorporation of angiogenic growth factors within a scaffold matrix is a common method of presenting bioactive agents to cells. Direct interactions between proteins and materials provides growth factors with some level of protection from natural processes in the body that could inhibit activity, such as cellular inactivation or enzymatic digestion [44,126]. Angiogenic growth factor release kinetics are mediated by factors including concentration, size, diffusion rate, porosity, scaffold degradation rate, and extent of polymer crosslinking. Material formulations and modifications can enable release by specific stimuli like pH and temperature change or biochemical activation, which lends increased spatiotemporal control to the system [14,27,125]. Retention of factors in the scaffold encourages cellular responses originating from cells seeded into the scaffold as well as ingrowth from host tissue in *in vivo* settings. Growth factor incorporation is commonly achieved through covalent binding interactions of the cytokine with the matrix or binding domain modification, but can also be achieved through techniques like adsorption (adhesion of substances to a surface) and ionic

complexation (formation of polyion structures through combinations of various ions and molecules) [27,125].

Tuning EC responses by covalently bound growth factor incorporation demonstrates how both understanding the cell biology and materials chemistry is critical for advancing angiogenic therapies. For example, Chiu et al. showed that covalent immobilization of VEGF-165 (a recombinant variant of VEGF-A) and/or Ang-1 on collagen scaffolds using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) chemistry resulted in increased proliferation and tubulogenesis of seeded ECs *in vitro* after 7 days in comparison to controls containing solubilized growth factors [127]. Stepwise angiogenic growth factor immobilization after EDC activation in a phosphate buffered saline (PBS) reaction buffer also resulted in higher protein incorporation than simultaneous processing in PBS, DI water, or 2-(N-morpholino)ethanesulfonic acid (MES) buffers. In a related study by the same group, Odedra et al. created radial gradients of immobilized VEGF-165 in EDC-activated cylindrical collagen scaffolds using point source, flow, and source-sink methods [128]. Enhanced D4T EC (an embryoid body-derived mouse lineage) migration from the periphery of the 12 mm diameter, 2.5 mm thick scaffolds created a cell distribution mimicking the angiogenic growth factor gradient, although a corresponding enhanced proliferation was not observed. This study demonstrates that mimicking the chemotactic gradients generated by angiogenic growth factors is a useful tool for organizing and directing ECs *in vitro*.

Introducing heparin or heparin-binding domains into a biomaterial allows for increased specificity to sequester heparin-binding growth factors such as VEGF, PDGF, and bFGF in the material matrix, and even utilize it to sequester endogenous factors in a reservoir when implanted *in vivo*. Wu et al. bound heparin to decellularized liver scaffolds using an end-point attachment technique (terminal reductive amination of the sugar chain and attachment with an amine linker), followed by incubation of the scaffolds overnight with VEGF-165 to allow for adsorption [129,130]. Significant proliferation of HUVECs *in vitro* (~65% in comparison to 20-25% for controls, $p < 0.05$) was present in the growth-factor bound scaffolds as measured by Ki67 expression. One area of consideration is uniform binding of heparin to a scaffold surface, as heterogeneous distribution of heparin means that there will also be a heterogeneous distribution of growth factor throughout the matrix. Techniques like the toluidine blue metachromatic assay can be used to stain and thus visually confirm the presence of a heparin coating [131]. Quade et al. utilized a bolus injection strategy reminiscent of Odedra et al. in which VEGF was incorporated in a single central biopolymer depot within a collagen scaffold, and the kinetics of release and resulting migration on human dermal microvascular ECs were examined *in vitro* over 28 days [46]. Heparin, alginate, hyaluronic acid, alginate-heparin, and alginate-hyaluronic acid biopolymers were all tested, with the authors finding that the alginate and alginate-heparin depots could best retard burst release of VEGF within the first four days and allow for more sustained release in subsequent days. Migration assays revealed that the increase in cellular proliferation was correlated with the release of VEGF in each biopolymer group, with higher burst release materials resulting in increased proliferation at early time points. In contrast, the heparin and alginate-heparin groups demonstrated higher proliferation rates at later time points. Migration assays revealed that the alginate-heparin group enabled the highest migration

towards the central depot, with a >2-fold increase in the number of cells found 2000-2500 μm deep in comparison to controls.

As an example of dual growth factor incorporation, Lakshmanan et al. demonstrated the incorporation of VEGF and bFGF in a scaffold blend made of poly(L-lactide-co-caprolactone) (PLCL) and poly(2-ethyl-2-oxazoline) (PEOz) by dissolving the factors in PBS and adding it to the prepolymer solution prior to electrospinning [132]. Release of the growth factors from the matrix *in vitro* resulted in HUVEC proliferation and migration, and gene expression analysis confirmed the presence of angiogenic gene upregulation in the form of VEGFR, PDGFR, and FGFR isoform expression. However, the authors reported an encapsulation efficiency of <30% across different combinations of their growth factors and materials, and a significant burst release likely due to the lack of strong binding interactions between the growth factors and biomaterials.

Despite being a widespread method of modulating growth factor retention and release, matrix incorporation has inherent drawbacks. Angiogenic growth factors in a matrix can fail to generate a truly guided vessel network because random distribution and immobilization within the matrix can generate imperfect chemotactic gradients upon release, resulting in less well-defined spatial control of angiogenesis [10]. While biological vascular development is not necessarily structured and organized, tissue engineers may seek to have greater spatial control of angiogenic processes in their systems. There are studies that address this issue, such as a study by Yuen et al. wherein the use of stimulatory VEGF and inhibitory anti-VEGF signals restricted angiogenesis of human dermal microvascular ECs to 1 mm thick zones within a scaffold using a layer-by-layer incorporation strategy, although specific vessel geometries could not be controlled [71]. This dual incorporation method attenuated the initial net VEGF burst release due to the antagonistic effect of the two molecules, allowing for a temporally stable release profile over 30 days *in vitro* in the specified angiogenic zones. A final issue is that properties like loading and release rates of angiogenic growth factors are directly tied to the parent scaffold, meaning altering the properties and kinetics of growth factors in a system necessitates adjusting the scaffold properties [126]. These problems indicate the need for more sophisticated modulation of angiogenesis within a scaffold matrix through spatiotemporal interactions of growth factors and biomaterials with constituent cells in the scaffold, which has motivated the development of encapsulation methods to increase the control over material, protein, and/or cell localization.

5.2 Encapsulation

The encapsulation of angiogenic growth factors is another method of controlling growth factor release similar to incorporation, but the key difference is that the protein is incorporated in a separate, often micro- or nano-sized carrier composed of a material different from that of the bulk scaffold. This effectively decouples the scaffold properties from the properties and kinetics of the growth factor release, and provides an additional layer of protection to maintain angiogenic growth factor bioactivity [126]. The carriers themselves are typically spherical in shape and can be created by way of solvent extraction/evaporation, coacervation (phase separation), or spray-drying [133]. Solvent extraction/

evaporation consists of dispersing a bioactive molecule in the particle precursor solvent, emulsifying the solvent in an immiscible phase, then extraction of the solvent to obtain microparticles. Coacervation is the separation of a coating material's liquid phase from a polymer-rich solution containing the molecule of interest, followed by solidification of the coating material around the core polymers. Spray drying is formation of microparticles by spraying an amphipathic prepolymer solution loaded with a bioactive compound through a heated atomizer to form micelles. Encapsulation of bioactive compounds is achieved through various methods such as sequestration inside a material, property-based interactions based on chemical properties such as solubility and hydrophobicity, and covalent chemical binding, and can be accomplished either during or after carrier formation [27,125,134]. The overall process is often referred to as micro- or nanoencapsulation depending on the size scale of the synthesized carriers. The loaded particles can then be embedded in a scaffold or delivered separately to the target site, then released via diffusion. Sequestration of proteins in microspheres provides an opportunity for spatiotemporal control of angiogenic growth factor bioactivity beyond that capable with incorporation, which can aid in generating chemotactic gradients to facilitate angiogenesis and cell recruitment [135,136].

Many of the same strategies in growth factor incorporation can be applied to microsphere encapsulation. For example, Roberts et al. demonstrated heparin-mediated loading and release of VEGF and bFGF from poly(vinyl alcohol) (PVA)-heparin hydrogels with decreased burst release when compared to control PVA gels [135]. This dual but independent angiogenic growth factor release resulted in a 4.2-fold increase in the proliferation of HUVECs over 3 days in comparison to cells cultured only in endothelial cell media. In a study by Akar et al., 10 mm bilayer disk scaffolds composed of a 3.7 mm thick layer of PEG-fibrin under a 0.3 mm layer of PEG-poly(lactic acid) (PEG-PLLA) with PDGF-BB-loaded poly(lactic-co-glycolic acid) (PLGA) microspheres seeded into the thin layer were implanted subcutaneously in rats next to skeletal muscle [137]. The release and diffusion of the growth factor generated a chemotactic gradient that caused dose-dependent invasion of cells and increased blood vessel density in the PEG-fibrin scaffold over six weeks.

Encapsulation techniques enable greater flexibility in designing complex growth factor release systems. For example, Lai et al. demonstrated the independent release of four growth factors from a layered electrospun scaffold made of collagen and hyaluronic acid with embedded gelatin nanoparticles [120]. As the construct degraded, bFGF and epidermal growth factor (EGF) were released from the scaffold as well as VEGF and PDGF from the nanoparticles over the course of a month, significantly increasing HUVEC proliferation, tubulogenesis, and branching *in vitro* in comparison to controls. *In vivo* models showed that the growth factors resulted in approximately 148 capillaries/mm² (control = 90 capillaries/mm²) and significantly higher CD31-positive and α -SMA-positive vessels in the wound area by week 4 as determined by quantitative histological staining, which is indicative of stabilized vasculature. In an example of multiphase growth factor release, Izadifar et al. developed a model for nanoparticle release kinetics, and validated it experimentally with bilayer nanoparticles made of PLLA and PLGA which released VEGF and bFGF followed by PDGF from the nanoparticle core after 5 days [138]. The authors further found that nanoparticles with a smooth polymer surface reduced surface area and

abated the burst release. In another study, Dashnyam et al. promoted *in vitro* tubulogenesis in HUVECs using mesoporous silica nanoparticles that released silicon ions and VEGF [139]. The bioactive silicon ions had been shown to induce angiogenesis in ECs following studies investigating the paracrine effects of bioactive glasses on angiogenesis signaling *in vitro* and *in vivo* [140]. Interestingly, gene and protein expression analysis indicated that the silicon molecules upregulated expression of hypoxia inducing factor 1 α (HIF1 α) ($p < 0.05$), causing downstream angiogenic signaling that resulted in increased bFGF protein expression as determined by western blot analysis [139].

Recent innovations in the field have begun to ameliorate what were once significant disadvantages of growth factor encapsulation. Encapsulation method, carrier size, material selection, and protein to carrier ratios are among the considerations that affect encapsulation efficiency and thus directly impact downstream properties in the system like release kinetics [133]. Burst release is common due to the high ratio of surface area to volume of the encapsulation vehicles but can be prevented by developing multi-layer particles or modifying the particle characteristics [125,138]. Quantification of release profiles has been problematic as assumptions of *in vitro* release do not necessarily translate to *in vivo* outcomes, as demonstrated in the study by Izadifar et al. who were unable to determine the benefits of co-release of three growth factors at once opposed to sequential release [14,138]. This can be approached through the use of model proteins and development of analytical programs to predict loading efficiency and release kinetics [136,138], yet tracking actual protein release *in vivo* is the gold standard and can be achieved using unique protein tags such as biotinylation [141]. Understanding the properties of the biomaterials and the proteins or other biologicals themselves used in microencapsulation methods is key to designing an effective delivery system.

5.3 Topographical Engineering

Vascular beds can alternatively be generated with specific and detailed geometries through topographical engineering, which is the manipulation of materials or chemicals to act as regulatory cues for the directed assembly of multicellular structures, often at the hundreds of microns scale and often containing micro- and nanoscale topographical features. In the context of vascularization, this generally entails physical or chemical methods of patterning vessels by restricting EC attachment through methods like specific adherence and alignment to predefined strips of material or fibers, or organization on or within patent channels [142].

Lithography is the largest subgroup within topographical engineering which can generate specific geometries using microfabrication techniques. Soft lithography is commonly used to induce these physical cues through the creation of an elastomeric mold with defined topographies such as oriented channels. These techniques often involve the creation of intermediate molds, typically made of silicon, acrylic, or glass, and which allow for high throughput replication of the final mold, often made of polydimethylsiloxane (PDMS), and lower overall production costs. For example, Zorlutuna et al. utilized X-ray interference lithography to generate nanopatterned tubular collagen scaffolds which were then seeded with human vascular SMCs and human internal thoracic artery ECs [143]. The topography of the scaffold assisted in orienting the SMCs circumferentially around the lumens and ECs

in the interior lumen, all while maintaining the proliferative capacity of each cell type. In another study by Zheng et al., soft lithography was used to create a microfluidic device made of collagen gel with continuous channels that generated patent 3D microvessels when perfused with HUVECs in growth medium for 7 days followed by pro-angiogenic media (containing exogenous VEGF, bFGF, and phorbol12-myristate-13-acetate) for another 7 days [144]. In the presence of human brain vascular pericytes, the constructs demonstrated differential sprouting angiogenesis into the collagen bulk, with half of the constructs sprouting an average of 75.4 μm into the bulk, and other samples not sprouting, and even retracting from the walls. The authors posited several hypotheses for these conflicting results, including variable density of cells and different conditions when gelling the collagen. Lithographic microfluidics are also useful in characterization of cell lines, as demonstrated by Kurokawa et al. who developed a PDMS microfluidic device composed of sequential tissue chambers flanked on either side with fibronectin-coated fluid channels that could be used to characterize the physiological functionality of their iPSC-ECs [145]. In this study, the authors first differentiated mCherry-transfected iPSCs into ECs, then sorted and seeded the CD31+ cells with fibrin into the main chambers and channel flow was set to generate a shear stress of 4 dyn/cm^2 . Over the course of 14 days, the ECs aligned in the direction of flow and formed vessels 60 μm in diameter on average which inosculated with the adjacent fluid channels, as determined by perfusion of FITC-dextran.

Other forms of topographical modification can also be performed, such as stamping films of self-assembling monolayers as in microcontact printing, or circulating chemicals and cells as in microfluidics [146,147]. Chemical stamping induces patterning, either by preferential adsorption of a peptide to a substrate or surface patterning to mediate cellular behavior [142]. Photolithography and electron beam lithography enable the use of UV-curable materials by applying a mask and photoresist layer to materials in order to fabricate specific topographies on a substrate with resolutions as low as a 1 μm [142,147]. In a study aimed at inducing angiogenesis through other means than exogenous growth factors, Lei et al. stamped SVVYGLR peptide strips (found adjacent to the RGD sequence in thrombin-cleaved osteopontin, and reported to activate ECs *in vitro* and induce angiogenesis *in vivo*) onto a polyethylene terephthalate film using photolithography, then seeded HUVECs to the substrate [148]. Cells began to align within 4 hours of seeding and underwent morphogenesis into lumen shapes, with greater alignment and shape change occurring in lower-width peptide strips of 10 and 50 μm as opposed to 100 μm .

Topographical engineering has been utilized to generate vessel-like conduits in cardiac tissues engineered *in vitro*. Thomson et al. developed a porous fibrin scaffold from a micro-template of parallel-aligned 60 μm polycarbonate fibers bundled in a poly(methyl methacrylate) (PMMA) shell and spaces infiltrated with 27 μm diameter PMMA beads [68]. Once the fibrin polymerized around the shell template, the polycarbonate fibers and beads were sacrificed to generate a porous microstructure with micro-channels spanning the length of the scaffold. With subsequent centrifuge-assisted loading of a tri-culture of primary neonatal rat ventricular myocytes (NRVMs), neonatal rat cardiac fibroblasts, and ECs (HUVECs or rat aortic ECs), the constructs supported *in vitro* cellular organization into the scaffold micro-channels, with the ECs forming aligned lumen structures, the NRVMs aligning and spontaneously beating, and ECM deposition by the fibroblasts. In another

study, Vollert et al. created vessel channels by embedding alginate fibers in a fibrin cardiac scaffold with a murine cell mixture of cardiomyocytes, fibroblasts, SMCs, and ECs, sacrificing the alginate, then perfusing the generated lumens over 15-25 days [149]. The average luminal diameter was estimated to be 100 μm , which expanded to approximately 500 μm during perfusion, and histological analysis indicated that ECs from the migrated to the lumen walls. Recently Zhang et al. developed a synthetic microvascular chip coined 'Angiochip' for loading cells into bulk spaces and ECs into patterned lumens that could be surgically anastomosed to host vasculature *in vivo* for immediate blood perfusion of the implant [150]. Soft lithography was used to shape layers of poly(octamethylene maleate (anhydride) citrate) (POMaC) prepolymer, which were then UV-cured and adhered to a glass substrate. The resulting patterned chip had internal and perfusable branching networks with channels as small as 50-100 μm and with 25-50 μm thick walls, designed with pores throughout the POMaC layers to facilitate molecule diffusion and exchange. *In vitro* experiments demonstrated that lumens could be endothelialized with HUVECs and the parenchymal spaces filled with other cell types, such as cardiomyocytes or hepatocytes, demonstrating that Angiochip could be a versatile platform for engineering patterned, vascularized tissue *in vitro*.

Topographical engineering is amongst the broadest of categories for manipulating biomaterials to induce angiogenesis. There are a multitude of techniques available to generate intricate geometries for vascularization, but each technique also has drawbacks. Soft lithographic techniques have inherent height and size-scale limitations, and some materials and processing methods can be toxic to cells or unfavorably affect substrate surfaces [142,147]. Protein and peptide-based techniques suffer from protein stability and short half-lives, and sacrificial materials can leave toxic degradation products if not properly removed [10]. Microfluidic devices are often designed with non-implantable materials, making them incompatible for *in vivo* studies. There is great potential for this subfield to spawn a new generation of vascularized constructs due to the freedom afforded to it, but current implementations are often fabricated with *in vitro* modeling in mind, rather than *in vivo* implantation. As a result, there is often conflict between the disadvantages of topographical engineering techniques and the criteria for implantation [11].

5.4 3D Bioprinting

The advent of 3D printing inspired the regenerative medicine community to adapt the technology to address biomedical problems. Coined "3D bioprinting," this technique allows for the bottom-up production of 3D constructs with complex geometries and micron-scale resolution through the deposition of cells, materials, and biological factors, as well as fabrication techniques for patterning vasculature [10,151,152]. Geometric features can be modeled using computer-aided design (CAD) methodologies, enabling quick and precise alteration of variables to affect properties such as scaffold size and engineered macro-porosity via patterned channels or a lattice. As a new fabrication tool, 3D bioprinting processes are continuing to be developed, and this review will focus on the main principles that define different 3D bioprinting strategies and their current applications in vascular tissue engineering.

Inkjet bioprinting is a non-contact, layer-by-layer method wherein a piezoelectric or thermal actuator is used to disperse bioink droplets onto a substrate [151]. This is an inexpensive method of bioprinting because regular inkjet printers can be easily modified to be compatible with bioprinting. However, nozzle extrusion generates shear stresses in addition to pressure or thermal stress from the respective actuator types, which can cause damage to extruded cells and uneven dispersion [153,154]. Lee et al. demonstrated the use of inkjet bioprinting to create 3D polycaprolactone (PCL) lattice scaffolds that supported the growth of a tri-culture of primary rat hepatocytes, HUVECs, and human lung fibroblasts to study liver tissue engineering in a mechanically robust 3D environment [155]. Seeding the tri-culture in a collagen solution into alternating canals in the bioprinted scaffold allowed for the formation of a dense, interconnected capillary-like network that spanned 36.9% of the $10.2 \times 10.2 \times 1.2$ mm hydrogel within 14 days.

Similar to inkjet printing is pressure-based bioprinters, which act by inducing pneumatic pulses that extrude solutions onto a substrate [10]. Using an air pressure-based 3D bioprinting process, Kolesky et al. were able to simultaneously extrude a cell-laden (HUVECs and human MSCs) fibrinogen-gelatin solution alongside a vascular ink made of Pluronic and thrombin into a 3D perfusable chip to create bone tissue with thicknesses greater than 1 cm [156]. ECM-native proteins were then gelled on top of the inks and the vascular ink was evacuated, forming a perfusable channel network in the gel which was endothelialized by the vascular cells. Perfusion of an osteogenic differentiation media containing BMP-2 enhanced mature osteogenic markers such as mineral deposition in the vascularized tissues, but not in the avascular controls [156]. In a study combining both 3D bioprinting and encapsulation techniques, Poldervaart et al. demonstrated the release of VEGF from gelatin microparticles in a pressure-based, 3D-bioprinted Matrigel-alginate lattice scaffold for localized angiogenesis [157]. *In vitro* studies with human late outgrowth EPCs showed peak migration between 50-100 ng/mL of VEGF, and *in vivo* studies with subcutaneously implanted scaffolds in mice showed that despite quick scaffold degradation, the localized release of VEGF (25 $\mu\text{g/mL}$ in 5 mg microparticles in scaffolds) into the surrounding tissue resulted in the formation of an average of 18 erythrocyte-perfused vessels/100 μm^2 in the encapsulated VEGF group at one week as determined by Goldner's trichrome stains. In comparison, the control and free (not encapsulated) VEGF groups had approximately 5 vessels/100 μm^2 ($p < 0.01$) [157].

Inkjet and pressure-based bioprinting techniques have also been used to directly fabricate tubes to act as vasculature, such as in Zhang et al. who demonstrated the ability to create, perfuse, and embed chitosan-alginate tubes with diameters as low as ~ 200 μm and variable pathways in alginate hydrogels using a pressure-assisted bioprinter [158]. In another study, Miller et al. used a thermal bioprinter to create a sacrificial glass lattice made of sucrose, glucose, and dextran, which was then embedded into either a Matrigel, alginate, agarose, fibrin, or PEG-based gel [159]. The lattice was then sacrificed, generating channels with diameters as low as 150 μm and perfused with HUVECs, generating a confluent vascular wall within 1 day. In a similar approach derived from microfluidics, DiVito et al. utilized a hydrodynamic shaping device to directly fabricate 125 μm vessels composed of either HUVECs or a combination of HUVECS, SMCs, and vascular pericytes in a complex matrix of PEG, methacrylated gelatin, fibronectin, hyaluronic acid, and collagen [59]. These

capillary and arteriole mimics developed a basement membrane within 12 days in culture as quantified by immunofluorescence staining of collagen IV and laminin. When embedded in Matrigel or gelatin hydrogels containing human dermal fibroblasts, fabricated channels showed prolific sprouting angiogenesis into the gel and remained viable during perfusion with pressures of up to 2500 Pa and shear stresses up to 25 dyn/cm².

A final class of 3D bioprinting is laser-based bioprinting, a nozzle-free method that is performed by pulsing a laser on a bioink coated with an energy-absorbing layer (often a metal like titanium), and collecting the resulting droplets on a substrate below [154,160]. The absence of a nozzle eschews the issues of clogging and shear stresses on cells, and the technique can even deposit individual cells [152]. However it is extremely expensive, relatively low throughput, and prone to contamination from the metallic film [153]. It has however shown success in patterning vascular networks, such as in a study in which Wu et al. were able to direct the growth of HUVECs and SMCs into branch and stem structures using laser-assisted bioprinting [161]. The deposited HUVECs formed interconnected lumens at droplet spacings of 50 and 100 μm within 20 hours, and deposition of SMCs near these lumens appeared to encourage both SMC proliferation and stabilization of the HUVEC lumens. Of interest to note in this subclass of bioprinting is the multiphoton excitation-based technique of laser bioprinting, which uses similar principles to multi-photon microscopy to print biological materials into 3D scaffolds [160]. This technique enables resolutions as high as 3 μm , but currently cannot support the printing of cells and limits scaffold size to the millimeter length scale. Gao et al. demonstrated the use of this technique to print gelatin methacrylate into a 2.4×2.4 mm scaffold with a submicron topography that mimicked the submicron features of fibronectin distribution in adult murine myocardium, using a 100×15 μm grid of patterned scaffold for cellular alignment [162]. The scaffolds were seeded with 50,000 hiPSC-cardiomyocytes, hiPSC-SMCs, and hiPSC-ECs (all differentiated from a human cardiac lineage stem cell) and implanted in a mouse MI model, and by 4 weeks the implants improved MI recovery shown by reduced scar size and increased fractional shortening (from 35% to 45% at 4 weeks), likely through reduced apoptosis and increased vascular area density (from 400/mm² for acellular scaffold implants up to 1200/mm² for cell-laden scaffolds) in the infarct border zone.

3D bioprinting is relatively new and underdeveloped, thus its adoption is limited to those groups able to develop the technology or utilize commercially available bioprinters. However, continued innovations and technical developments in the coming decade are certain to increase the popularity and adoption of bioprinting, such as advances in instrumentation and biomaterials to better accommodate cells for printing at higher resolution and in customized microenvironments with reasonable cost and time. Current studies have demonstrated progress in creating vascularized constructs using 3D bioprinting, and the compatibility of multiple cell types means that this technique is feasible for generating vascularized cardiac tissues as well, such as a heart valve and myocardium [150,153]. Combinatorial approaches to vascularization utilizing multiple techniques such as bioprinting and encapsulation in one construct have also been demonstrated and show promise in providing hierarchical structure to bioprinted tissues [157,163]. Yet there is still significant work to be done to create large, complex tissues with deeply penetrating microvascular networks for organ engineering [154].

6.0 Beyond *In Vitro* Analysis of Combinatorial Angiogenesis Strategies

Developing a perfusable angiogenic network *in vivo* presents a significantly greater challenge than *in vitro*, owing to the great multitude of known and unknown biological processes that impact the effectiveness of a therapeutic. However, 2D *in vitro* cultures cannot accurately mimic the complex 3D *in vivo* environment, whether that be the topography, paracrine cues, or host cell recruitment [164]. Further studies are thus necessary to understand how these controlled systems behave in more dynamic environments. Table 1 provides a succinct summary of the studies discussed below, with additional articles included that help illustrate the progress of *in vivo* vascularization efforts.

6.1 *Ex Vivo* Models of Angiogenesis

Ex vivo assays are commonly used to bridge the gap between *in vitro* and *in vivo* studies as they are often relatively cheap and provide a more physiologically relevant environment than *in vitro* studies. The chicken chorioallantoic membrane (CAM) assay is a standard model for determining vascular growth due to the relative ease of manipulating the model and visually assessing results. The CAM acts as a respiratory organ for chicken embryos that grows and matures rapidly, quickly generating a densely packed vascular plexus within 7 days [165]. Intervention via the addition of reagents allows for quick and inexpensive determination of angiogenic efficacy, although it can be difficult to differentiate between new and existing capillary formation. The CAM assay is commonly used as a precursor to trials with traditional small animal models, such as in the study by Chiu et al. that examined collagen scaffolds with immobilized VEGF and Ang-1 [127]. Dashnyam et al. used the CAM assay to create vascular maps and quantitate total length, size and junctions that developed as a result of their silicon/VEGF-encapsulated microspheres [139]. Wu et al. found that heparinized, VEGF-bound liver scaffolds had increased vessel number and density in the CAM assay, and were able to then move forward with an *in vivo* model [129].

Another common assay used to determine angiogenic efficacy is the aortic ring assay, in which an animal aorta is dissected, cut into thin slices, embedded in a collagen or fibrin matrix, and then cultured with a therapeutic of interest and examined over a period of time [166,167]. Slicing the aorta initiates an angiogenic response from the native cells which can be compared to the response from the therapeutic treatment via quantifiable angiogenic metrics such as EC proliferation, migration, tubulogenesis, and branching. The assay is an excellent *ex vivo* procedure for determining sprouting angiogenesis as it is inexpensive, high-throughput, and requires relatively little expertise to perform in comparison to *in vivo* procedures, but fresh tissues must be prepared for each experiment [168]. Further, the common practice of using Matrigel and serum must be carefully considered when interpreting results or may be eliminated by using a completely chemically defined culture system. The microvessels that develop are physiologically similar to those generated *in vivo* and paracrine interactions are more apparent due to the presence of multiple cell types. For example, Izadifar et al. cultured rat aorta slices with their bilayer nanoparticles and showed that co-release of VEGF and bFGF followed by sequential release of PDGF generated microvessels with significantly greater average diameter, sprout length, and number of sprouts versus those generated with VEGF alone ($p<0.05$) [138]. Pericyte recruitment and

anastomosis by day 9 indicated that the vessels had stabilized and matured due to the growth-factor nanoparticles.

6.2. *In Vivo* Models of Angiogenesis

In vivo research models for tissue engineering are paramount for investigation into a scaffold's angiogenic and inosculative efficacy in a physiologically relevant environment. The choice of animal is often dependent on the results of interest. Small animal models such as rodents are often preferred over large animal models due to ease in handling, gestation period, costs, and particularly the ability to genetically engineer strains for studying diseases [169,170]. Small animal models are often used for a general understanding of the host response to the implant, whether that be immune response, vascularization, degradation, or other factors that can be dependent on the specific location of the implant [171]. In studying organ-specific phenomena, it becomes more important to select animals with the most relevant physiology to humans. For example, pigs are a common choice of large animal model for preclinical cardiovascular studies due to their similarity in excitation coupling, excitation-relaxation kinetics, and heart rate in comparison to other models [170].

The subcutaneous site is prevalent in the literature for assessing tissue invasion and vascularization because it is a relatively easy operation to perform which provides a suitable wound bed to initiate angiogenesis with multiple implant sites in one animal. Jiang et al. implanted PEG-PLLA scaffolds loaded with bFGF and PDGF-BB subcutaneously in rats and demonstrated sequential growth factor release that encouraged mature vessel formation with SMCs, although vessel density peaked at 1 week after implantation and decreased afterwards, suggesting pruning of the new vascular bed as one might expect in the absence of an oxygen sink [123]. Kang et al. utilized the principles of pressure-assisted 3D bioprinting combined with topographical engineering to develop a multi-cartridge extrusion machine that could fabricate tissue constructs as large as $3.6 \times 3.0 \times 1.6$ cm [172]. Dubbed an integrated tissue-organ printer (ITOP), the machine extruded PCL fibers, sacrificial Pluronic hydrogels, and various cell types to create porous tissue constructs with incorporated channels as small as $500 \times 300 \mu\text{m}^2$ to facilitate nutrient diffusion. In demonstration of the ITOP, constructs were fabricated for human mandible bone, rat calvarial bone, human ear, and mouse muscle tissue reconstructions based on clinical imaging data. In the case of a skeletal muscle reconstruction, Kang et al. showed that printed mouse myoblast constructs implanted subcutaneously in rats along with a section of the common peroneal nerve developed aligned muscle fibers, acetylcholine receptors and neurofilament contacts indicative of nerve integration, electrical activation when stimulated, and vascularization as determined by staining for von-Willebrand factor throughout the implant (density not quantified) after 2 weeks.

To demonstrate the use of physical cues for *in vivo* vascularization, Jang et al. fabricated a 3D-printed lattice scaffold using a bioink composed of decellularized porcine heart ECM and printed on top of a PCL mesh layer [173]. Human cardiac progenitor cells and human turbinate MSCs with exogenous VEGF were additionally printed with the bioink in alternating strips. In a BALB/c mouse subcutaneous model, the striped scaffolds induced the formation of blood vessels with 50 μm diameter and 25 vessels/ mm^2 in density, whereas

scaffolds with mixed cell types and no stripes generated vessels $<22\text{ }\mu\text{m}$ in diameter and a density of 16 vessels/mm^2 . In a rat MI model, fluorescent staining of CD31 at 8 weeks revealed that the MSCs differentiated into endothelial cells and penetrated the infarct area. The patterned scaffolds had $\sim 175\text{ CD31-positive vessels/mm}^2$ versus 110 and 100 in the mixed cell and cardiac progenitor-only scaffolds, respectively. In another example, Fleischer et al. developed a modular, multi-layer scaffold in which each $5 \times 5\text{ mm}$ sheet of electrospun albumin was topographically engineered for a specific function and individually cultured before being glued together and implanted in a rodent MI model [163]. The cardiomyocyte layer was comprised of NRVMs seeded to alternating grooves and ridges spaced 115 and $120\text{ }\mu\text{m}$ apart, respectively, with transversely oriented $40\text{ }\mu\text{m}$ micro-holes placed on the ridges to facilitate nutrient exchange. The vascular layer consisted of $450\text{ }\mu\text{m}$ micro-channels seeded with HUVECs between cage-like structures that housed VEGF-loaded PLGA particles, and the final unique layer consisted entirely of cage structures housing dexamethasone-loaded PLGA particles. These unique layers enabled alignment and electromechanical coupling of cardiomyocytes, vascular patterning within the predefined channels, and extended release of both VEGF and dexamethasone up to 14 days. Analysis of 5 mm thick scaffolds consisting of six cardiomyocyte layers and six vascular layers flanked on either side by dexamethasone layers implanted onto post-MI rat hearts revealed an increased capillary density in the patch (approximately 110 vessels/mm^2 up from $60/\text{mm}^2$, $p<0.007$) and significantly increased area occupied by vessels (from 2% to 4.5%, $p<0.0004$) due to the presence of the VEGF-loaded microparticles.

Other implant locations can be utilized, which can be chosen depending on the application or intended data collection. As a proof-of-concept demonstration of immediate surgical anastomosis *in vivo*, Zhang et al. showed rapid perfusion through surgical anastomosis of their fabricated Angiochip with the rat femoral artery in both artery-to-artery and artery-to-vein configurations [150]. Perfusion occurred immediately regardless of the presence of ECs lining the lumens with apparent pulsatile flow, and after one week there was minimal clotting and angiogenic sprouting was evident surrounding the implant. The mesentery is an example of a highly vascularized membrane that has been used to demonstrate the angiogenic efficacy of therapeutics, such as in Benest et al. (see Section 3.4) [118]. In the same vein, implantation on the greater omentum acts as another pro-angiogenic implant site. Wu et al. illustrated the angiogenic potential of their decellularized liver scaffolds with heparin-bound VEGF using this rodent implant model, which resulted in increased blood vessel formation within the scaffold at 28 days versus controls [129]. The scaffolds with heparin-bound VEGF had higher vessel penetration into scaffolds at approximately 28 vessels/visual field in comparison to 7 vessels/visual field in control liver scaffolds.

While these types of implants are useful in demonstrating *in vivo* effectiveness of a therapeutic, direct implantation onto the organ or tissue of interest is necessary to acquire the most clinically relevant information. In the case of cardiac applications, implantation onto an infarcted heart and subsequent analysis can provide valuable information regarding the therapeutics' ability to modulate coronary perfusion, sprouting angiogenesis, cardiac sparing and remodeling, and heart function post-MI. Dvir et al. implanted alginate scaffolds composed of NRVMs along with 100 ng of VEGF, IGF-1 and SDF-1 on the greater omentum of rats for 7 days as a pre-vascularization medium before transplanting the

scaffolds onto rat hearts 7 days after infarction for 28 days [106]. Analysis showed that the omentum-vascularized tissues were able to successfully engraft on top of existing scar tissue, thus thickening the ventricular wall to values close to control and maintaining fractional shortening as well as fractional area change values in comparison to baseline. The tissues further demonstrated greater electrical activity between the patches and the myocardium and contained networks of 60 vessels/mm² versus 30 vessels/mm² in controls. In another study, Song et al. implanted acrylated hyaluronic acid hydrogels that contained immobilized SDF-1 as well as the angiogenic peptide Ac-SDKP derived from the protein thymosin beta 4 on an rat heart 4 weeks after MI induction to demonstrate the effectiveness of their constructs in a chronic heart failure model [113]. Histological analysis revealed an increase in arteriole number comparable to that of the sham group and a ~141.5% increase in capillary number relative to the infarct group. Heart functionality determined by intraluminal pressure-volume measurements showed significant increases in stroke volume, cardiac output, and ejection fraction, although none of these values reached the respective levels in negative controls. In a slightly larger animal model, Lakshmanan et al. implanted their VEGF- and bFGF-incorporated PLCL-PEOz electrospun scaffolds onto infarcted rabbit hearts and demonstrated preserved left ventricular wall diameter and increased ejection fraction and fractional shortening without causing unwanted surgical complications like adhesions or edemas that may disrupt implant integration with the host [132]. Uniform and α -SMA-positive blood vessels (~20 vessels in the patch region compared to <10 in all other groups) formed in the region of the implanted patch, indicative of angiogenic processes initiated by the growth factors which contributed to the increased viable myocardium and reduced necrotic regions. However, the authors did not report any vascular or cardiomyocyte alignment *in vivo* in response to the presence of the electrospun fibers. Riemenschneider et al. developed fibrin scaffolds seeded with human outgrowth EPCs, human brain pericytes, and an incorporated growth factor cocktail containing stem cell factor, interleukin-3, and SDF-1 α , and cultured the constructs for 8-9 days to develop an aligned vascular bed prior to implantation on infarcted rat hearts for 6 days [8]. The authors reported a total human and rat vessel density of 435 vessels/mm², but only 40% (173 human and 93 rat vessels/mm²) were perfused, and mainly around the border zone. In comparison, patches lacking EPCs contained approximately 100 vessels/mm², all originating from the host. Furthermore, capillary diameter increased from 6 μ m pre-implantation to 8 μ m at explant. The patches were not found to significantly improve functional cardiac output by 6 days despite a noted increase in ejection fraction and fractional shortening across all treatment groups by 10%.

6.3 Analysis of *In Vivo* Progress in Vascularization

Discussed above are just a few of the studies that have demonstrated the progressive steps taken to better understand the interplay of physical and chemical cues that dictate the formation of microvascular networks, with an emphasis on cardiac regeneration. Of interest to note is the difficulty of preserving and assessing the benefits of physical cues *in vivo*, particularly on the micron scale, because geometry can be easily lost during the implantation procedure, over time from scaffold degradation, and during histological processing. Intentional micron- and submicron-level cues for vascularization are thus a significant challenge to maintain *in vivo*, and their necessity is debatable still [71,137]. This is a prime opportunity for applications in topographical engineering to transition into *in vivo*

applications, but this requires a re-envisioning of system design and thoughtful material choice to facilitate incorporation into animal models at the same micron scale. Current state of the art scaffold implants often rely on pro-angiogenic molecules (mainly growth factors), hydrogels, and relatively macro-scale physical cues to promote vascularization and overall cellular orientation.

Methods for characterizing vascularization are often inadequate, and angiogenic analysis is often relegated to spatially- and temporally-vague metrics such as number of vessels present in the scaffold. One basic characterization of vasculature that is often overlooked is the presence of lumens within an endothelial network (as opposed to simple EC aggregates), which can be clearly distinguished as shown by Davis and colleagues [174]. Capillaries and arterioles are differentiated through quantification of either vessel size or staining for vascular SMCs, but venule development is altogether neglected [141]. This is a prime opportunity to utilize other molecular markers to differentiate endothelium, such as NRP1 and NRP2 expression for arterial and venous specification, respectively [54]. Quantification of vascular geometry can also be improved through metrics like branch points, branch angles, branch lengths, vessel alignment, and tortuosity and comparisons to native, healthy tissue vascular geometries. These metrics should stabilize in time to provide evidence for vascular bed maturation and persistent function, and although these types of vascular characterizations are challenging to implement, they need to be more widely adopted *in vivo*. It is a natural next step to analyze normal vessel functions like perfusion and modulation of vascular tone, followed by organ-specific function of implants like filtration via permeability in liver regeneration applications, or barrier function in blood-brain barrier recapitulation.

Temporal metrics to assess vessel stability and regression are critically important but extremely difficult to capture. Indeed, it is a fundamental challenge in vascular tissue engineering to accurately assess vascularization *in vivo* (particularly in deep tissues like the heart) at high resolution with a non-invasive and longitudinal (non-terminal) imaging technique. The dynamic nature of angiogenesis necessitates a dynamic imaging modality or examination at multiple time points, but the current gold standard of histology is a static 2D image often assessed at a single time point and not wholly representative of the underlying processes that occur during wound healing. Techniques that enable the 3D reconstruction and skeletonization of vascular networks are desirable, such as methods to label cardiac vasculature via retrograde coronary perfusion of isolectin or a radiopaque dye [12,35]. However, this is also a terminal procedure, precluding longitudinal studies, whereas an ideal imaging technique should be able to provide live flow imaging at the capillary scale in a non-invasive manner. High spatial and temporal resolution of vascularization *in situ* may be possible using combinations of imaging techniques, such as a study by Liu et al. that utilized optical coherence tomography (OCT) and photoacoustic tomography to noninvasively view the microvasculature in various regions of the skin [175]. Another study by Qin et al. utilized OCT angiography to image coronary vasculature at an axial resolution of 3 μm via *ex vivo* retrograde perfusion of Intralipid solution [176]. The dorsal skinfold chamber is a device that has been used for decades that enables examination of implant vascularization in rodents via non-invasive intravital microscopy, but the device is large, unwieldy, and cannot be used outside of subcutaneous studies [177]. For applications deeper than superficial

imaging, inspiration and innovation is necessary to improve the resolution of techniques like ultrasound and angiography. A major challenge today is the lack of standardization in vascular bed quantification, which complicates comparison between studies and thus communication within the scientific community. The field must determine what the necessary parameters are to measure for a given vascular application, and how to best assess them.

Progress in developing a vascular bed *in vivo* has been aided by improved prevascularization of tissue *in vitro* (as opposed to fabrication and implantation around or at the same time, which can reduce the amount of time an implant must endure hypoxic conditions before vascular ingrowth and anastomosis occurs [178]. Vessel stabilization due to co-culture has enabled studies of *in vitro* prevascularization, such as in Riemenschneider et al. and Fleischer et al. described above [8,163]. In another example, Kreutziger et al. formed scaffold-free patches containing various ratios of hESC-derived cardiomyocytes, HUVECs, and human MSCs, and cultured them for 2 days prior to implantation on uninjured nude rat hearts for 1 week [65]. Upon explant, tri-cell patches supported higher (~ 175 vessels/mm²) vessel formation than other cell formulations with either no stromal support cell, mouse embryonic fibroblasts, or neonatal human dermal fibroblasts (approximately 35, 60, and 125 vessels/mm², respectively). *In situ* prevascularization is another option, where the implant is placed on a highly vascular bed (such as the greater omentum of rodents) prior to transplantation to the site of interest, which has been used with some success in the heart [106]. The arterio-venous (AV) loop model is another example of an *in situ* prevascularization technique that is accomplished by surgically connecting an artery and vein (often the saphenous vein and artery) into a loop fistula and isolating it in a protected chamber within the animal [179]. Constructs housed within the chamber are then vascularized from angiogenic sprouts off the main loop. Both Tee et al. and Morritt et al. have demonstrated the use of this technique to generate vascularized, beating cardiac tissues by seeding NRVMs in Matrigel within the chamber, and the AV loop has even been used in a clinical setting, which indicates translational feasibility [180–182]. Prevascularization may be necessary in the near future, as a study by Utzinger et al. showed that neovessel growth (of rodent microvessel fragments in a 3D collagen gel) averages only 5 μ m/hr, which will not establish vasculature fast enough to prevent necrosis in larger and physiologically dense tissues [183].

Additionally, a multiple studies suggest that prevascularization enables faster functional anastomoses once implanted, with anastomoses occurring within as little as three days when prevascularized, as opposed to at least 8 days without prevascular support [65,184,185]. The exact molecular mechanisms of anastomosis in mammals are not fully known yet, but studies have also found evidence of vessel chimerism, in which engineered tissues with prevascularized human vessels anastomose with host rodent vessels upon implantation [65]. In examining the dorsal longitudinal anastomotic vessel of zebrafish embryos, Herwig et al. determined that anastomosis occurred through two distinct mechanisms [186]. After primordial ECs made contact, junctional proteins were expressed in a ring-like fashion, causing apical polarization and the formation of membrane compartments. The cells then either rearranged themselves into a multicellular tube through the merging of distinct membrane compartment, or generated a unicellular tube via progressive invagination of the

membrane compartments. Whether this is a stochastic process has yet to be determined, and further studies are necessary to understand how this occurs in mammals, and whether mechanisms differ between tissues.

Despite these complications, combinatorial growth factor approaches to vascularization of host tissue and implanted engineered tissues have shown *in vivo* success with incorporation of sophisticated spatiotemporal cues in the experimental design of the therapeutic. Application of these principles in the field of cardiac tissue engineering has demonstrated the ability of implanted tissues to anastomose with host vasculature and improve post-MI cardiac remodeling and function. Yet there is more to be done, as the goal of the field is to create a bed of capillaries with densities that are tailored to the needs of the host and engineered tissues to regenerate the native structure and function, whether it is myocardium, brain, liver, or another organ. To reach that goal, novel technologies and customized biomaterials must continue to be pursued, as well as a deeper understanding of material-cell interactions, physical and chemical cues to drive cellular signaling, and the host inflammatory and wound healing response after injury, disease, and therapeutic intervention.

7.0 Conclusions

As the development of replacement tissues has become a reality and feasible therapeutic option, research has turned towards understanding the mechanisms behind vascularization as a necessity for building organs from biomaterials, cells, and signaling molecules. Replicating the complex microvasculature of an engineered tissue in a way such that it can perfuse the entire implant and inosculate with host tissue has proven to be a complex undertaking. It is now understood that single growth factor systems may initiate angiogenesis, but are too simple to mimic the complete vascularization process, and as a result, vascularization research has turned to understanding the interplay of physical and biological cues involved in establishing a mature, hierarchical, and perfused vessel network. *In vitro* work has embraced spatiotemporal mediation of these cues, and a deeper understanding of these processes through *in vivo* application is currently unfolding. Ongoing work is required to identify pathways that promote increased capillary density, appropriate hierarchical vessel remodeling, and permanence of new vessels.

As the field of neovascularization advances, the broader issue of building tissues with sufficient architecture and signaling cues to be able to unite with the biology of the target host tissue to develop a sufficient vasculature must be considered. The field has relied on powerful growth factors like VEGF and bFGF, which have shown to be insufficient clinically when administered alone without spatial or temporal control, and is now slowly adopting an approach that recognizes that there are nuanced inter-related pathways for vascular development depending on the tissue type and disease context [187]. Identifying an optimal cocktail of growth factors and the appropriate spatiotemporal presentation of them via a biomaterial scaffold is essential in creating a highly vascularized network within dense engineered tissues, and this must be tailored to the application. An ongoing challenge is to design systems that are going to be adopted broadly for translational applications. Thus, using materials that are already FDA-approved is appealing to fast-track clinical use, but is not suited for all applications. Development of novel materials or new methods that utilize

currently available materials will be required as technologies advance for designing capillaries (with <10 microns diameter). Technologies that allow for precise, nanoscale fabrication of vessel architecture could be invaluable in spatially controlling vascular bed formation. Prospective studies should examine the ability of engineered vessel networks to handle physiological characteristics of organ-specific vasculature, such as flow rates, homeostasis, permeability, vascular tone, and immune response upon implantation in the host. These studies should further seek to push the boundaries of current characterization practices to develop techniques that provide temporal vascularization information with high spatial fidelity. The continued development of these parallel and complex challenges will surely advance the field towards the generation of mature vessel networks in host tissue and engineered tissues to restore organ function.

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Statement of Significance

Vascularization is vital to wound healing and tissue regeneration, and development of hierarchical networks enables efficient nutrient transfer. In tissue engineering, vascularization is necessary to support physiologically dense engineered tissues, and thus the field seeks to induce vascular formation using biomaterials and chemical signals to provide appropriate, pro-angiogenic signals for cells. This review critically examines the materials and techniques used to generate scaffolds with spatiotemporal cues to direct vascularization in engineered and host tissues *in vitro* and *in vivo*. Assessment of the field's progress is intended to inspire vascular applications across all forms of tissue engineering with a specific focus on highlighting the nuances of cardiac tissue engineering for the greater regenerative medicine community.

Figure 1.

Temporally controlled, overlapping, parallel, and repetitive signaling events (abridged) in angiogenesis and its role in the wound healing response. Examples of activated factors are listed below each stage of wound healing. Ang: Angiopoietin; bFGF: Basic fibroblast growth factor; EGF: Epidermal growth factor; bFGF: Basic fibroblastic growth factor; IL-1: Interleukin-1; MMPs: Matrix metalloproteinases; PDGF: Platelet-derived growth factor; SHH: Sonic hedgehog; TGF- β : Transforming growth factor beta; TNF: Tumor necrosis factor; VEGF: Vascular endothelial growth factor.

Figure 2.

Spatiotemporal control of angiogenesis can be achieved through the presentation of chemical and physical cues. This can be categorized into four distinct categories, which is further differentiated by the techniques utilized to present spatiotemporal cues within each category. Combinations of these techniques are often utilized to precisely engineer vessel network formation using chemical and physical cues integrated in one system. Of interest to note is the similarity in techniques used to present chemical cues in the matrix incorporation and encapsulation methods, the distinction being one related to material choice and design.

Table 1

Studies applying spatiotemporal control of physical and chemical cues in an *in vivo* environment.

Study	Model	Biomaterial(s)	<i>In Vitro</i> Study Cell Type(s)	<i>In Vivo</i> Study Cell Type(s)	Angiogenesis-Inducing Molecule(s)	Technique(s)	Results
Chiu et al. (2010)	CAM (Chicken)	Scaffold: Collagen	HSV Cells (Murine heart endothelium)	N/A	VEGF-165, Ang-1	Incorporation - covalent EDC	Increased tubule formation and vessel penetration into scaffold compared to independently immobilized growth factors.
Dashnyam et al. (2016)	CAM (Chicken)	Carrier: Silica	HUVECs	N/A	Si, VEGF-165	Encapsulation	Si release caused HIF1 α upregulation, resulting in downstream expression of angiogenic molecules including bFGF, eNOS, and VEGF. Stimulated EC migration, sprouting, and tubule networking.
Wu et al. (2016)	CAM (Chicken)	Scaffold: Decellularized liver	HUVECs	N/A	VEGF-165	Incorporation - heparin-binding	Higher vessel density, as determined by quantifying number of branch points.
Izadifar et al. (2016)	Aortic ring (Rat)	Scaffold: Fibrin; Carrier: PLGA core, PLLA shell	None	N/A	VEGF, bFGF, PDGF	Encapsulation	Bi-layer sequential release of VEGF and bFGF followed by PDGF from core. Increased microvessel sprout density by day 8, pericyte recruitment by day 5.
Lai et al. (2014)*	Diabetic skin wound (Rat)	Scaffold: Collagen-hyaluronic acid; Carrier: Feltanin	HUVECs	HUVECs	bFGF, EGF; VEGF, PDGF-BB	Incorporation - electrospinning; encapsulation	Complete wound closure by week 6, greater density of matured vessels and collagen deposition.
Brudno et al (2013)*	Subcutaneous implant (Mouse)	Scaffold: Fibrin (<i>in vitro</i>), PLG (<i>in vivo</i>); Carrier: PLG (<i>in vivo</i>)	HUVECs, Placental pericytes	None	VEGF, Ang-2, Ang-1, PDGF-BB	Incorporation; encapsulation	Simultaneous release of all four factors resulted in lower vessel density than VEGF+Ang-2 alone, or VEGF+Ang-1 followed by delayed release of PDGF and Ang1. Delayed release of PDGF and/or Ang-1

Study	Model	Biomaterial(s)	<i>In Vitro</i> Study Cell Type(s)	<i>In Vivo</i> Study Cell Type(s)	Angiogenesis- Inducing Molecule(s)	Technique(s)	Results
							resulted in more mature vessels measured by α-SMA staining.
Güç et al. (2017) [*]	Subcutaneous implant (Rat)	Scaffold: Collagen-fibrin	Human dermal LECs	None	FB-VEGF-C	Incorporation	Increased lymphangiogenesis without interfering with angiogenic sprouts. Lymphangiogenic effects were localized with no downstream remodeling to collecting vessels, and unaltered fluid clearance.
Poldervaart et al. (2014) ^{*,†}	Subcutaneous implant (Mouse)	Scaffold: Matrigel-alginate; Carrier: Gelatin	Human cord blood EPCs	Human cord blood EPCs	VEGF	3D bioprinting - pressure-assisted; Encapsulation	Increased neovessel formation in sustained release group, but immature vessels due to negative α-SMA staining. Slower VEGF release encouraged vessel formation.
Jiang et al. (2013)	Subcutaneous implant (Rat)	Scaffold: PEG-PLLA-DA & PEG-DA, Fibrin; Carrier: PLGA	HUVECs	HUVECs	FGF-1; PDGF-BB	Incorporation; Encapsulation	Sequential release of FGF-1 followed by PDGF-BB to promote mature vascular network formation and scaffold ingrowth.
Akar et al. (2015) ^{*,†}	Subcutaneous implant (Rat)	Scaffold: PEG-DA-fibrin, PEG-PLLA-DA; Carrier: PLGA	None	None	PDGF-BB	Encapsulation	Formation of a chemotactic gradient that resulted in increased tissue invasion and blood vessel density at higher magnitudes.
Park et al. (2015) ^{*,**}	Subcutaneous implant (Mouse)	Scaffold: PCL, collagen, gelatin-alginate	hDPSCs	hDPSCs	BMP-2, VEGF	3D printing - pressure-assisted	Temporal and spatially-distinct release of BMP-2 and VEGF allowing vascularization towards the center of the scaffold. Vasculogenesis from DPSCs in hypoxic areas and angiogenesis from host tissue. Accelerated bone regeneration.

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Study	Model	Biomaterial(s)	In Vitro Study Cell Type(s)	In Vivo Study Cell Type(s)	Angiogenesis-Inducing Molecule(s)	Technique(s)	Results
Kang et al. (2016)	Mandible, calvarial bone, ear cartilage, skeletal muscle (Rat)	Scaffold: PCL, Pluronic	hAFSCs, rabbit ear chondrocytes, mouse C2C12 myoblasts	hAFSCs, rabbit ear chondrocytes, mouse C2C12 myoblasts	N/A	3D bioprinting - pressure-assisted, topographical engineering - sacrificial materials	Multi-cartridge extrusion for patterned scaffolds with integrated porous lattice channels to facilitate nutrient exchange. Tissue formation demonstrated with vessel formation and no necrosis.
Jang et al. (2017)	Subcutaneous implant (Mouse)	Scaffold: Decellularized porcine ECM, PCL mesh	hCPCs, hMSCs	hCPCs, hMSCs	VEGF	3D Bioprinting - pressure-assisted	hCPCs and hMSCs differentiated into cardiac and endothelial cells. Increased vascularization penetrating the scaffold. Increased ejection fraction, reduced fibrosis.
Fleischer et al. (2017)	Subcutaneous implant (Rat)	Scaffold: Albumin; Carrier: PLGA	NRVMs, HUVECs	NRVMs, HUVECs	VEGF	Encapsulation	Generation of 5mm thick scaffolds with electrical coupling in z direction. Increased capillary density, increased area occupied by vessels, anastomosis of predefined channels
Zhang et al. (2016)	Femoral vessel ligation (Rat)	Scaffold: POMaC	HUVECs, hMSCs, hESCs	RVECs, NRVMs	N/A	Topographical engineering - soft lithography, photolithography	Enabled artery bypass and artery-to-vein anastomosis, instant and pulsatile perfusion. Angiogenesis and minimal clotting one week after surgery.
Yuen et al. (2010)*	Ischemic hind limb (Rat)	Scaffold: PLG	hDVECs	hDVECs	VEGF-165, anti-VEGF	Incorporation	Temporally stable release profile, spatially sharp angiogenic sections, and moderate necrosis prevention.
Wu et al. (2016)	Greater omentum (Rat)	Scaffold: Decellularized liver	HUVECs	HUVECs	VEGF-165	Incorporation - heparin-binding	Higher number, distribution, and diameter of blood vessels in and around implant.
Dvir et al. (2009)	Greater omentum, then MI - LAD permanent occlusion (Rat)	Scaffold: Alginate-sulfate	None	NRVMs	IGF-1, SDF-1, VEGF	Incorporation	Cardiac patches populated with dense vasculature when on the omentum. After

Study	Model	Biomaterial(s)	<i>In Vitro</i> Study Cell Type(s)	<i>In Vivo</i> Study Cell Type(s)	Angiogenesis- Inducing Molecule(s)	Technique(s)	Results
Sonnenberg et al. (2015)*	MI - LAD IR (Rat)	Scaffold: Porcine pericardium- derived ECM hydrogel	RASMCs, NRVMs	None	HGF fragment	Incorporation	Increase in fractional area change, arteriole density, and neovascularization while mitigating changes in LV dimensions.
Boopathy et al. (2015)*	MI - LAD IR (Rat)	Scaffold: Self-assembling peptide hydrogel	None	None	Jagged1 peptide mimic	Incorporation	Improved LV morphology, angiogenesis in implant area, EC proliferation, and stem cell recruitment. Reduced fibrosis.
Gao et al. (2017)	MI - LAD permanent ligation (Mouse)	Scaffold: Methacrylated gelatin	hiPSC-CMs, hiPSC-ECs, hiPSC-SMCs	hiPSC-CMs, hiPSC-ECs, hiPSC-SMCs	N/A	3d bioprinting - multiphoton excitation	Increased vascular area density, reduced apoptosis, improved ejection fraction, fractional shortening, and reduced infarct size.
Song et al. (2014)	MI - LAD permanent ligation (Rat)	Scaffold: Acrylated hyaluronic acid	hMSCs	None	SDF-1, Ac-SDKP	Incorporation	Reduced infarct size and improved stroke volume, ejection fraction, cardiac output. Higher number of mature arterioles and capillaries.
Lakshmanan et al. (2016)	MI - LAD, DCx, PDA permanent ligation (Rabbit)	Scaffold: PLCL/PEOz	HUVECs	None	VEGF, bFGF	Incorporation - electrospinning	Improved cardiac function based on M- mode echocardiographic measurements of LV dimensions and performance. Increased viable myocardium, vessel penetration and sprouting capillary formation.
Riemenschneider et al. (2016)	MI - LAD permanent ligation (rat)	Scaffold: Fibrin	human blood outgrowth EPCs, human brain vascular pericytes	human blood outgrowth EPCs, human brain vascular pericytes	SCF, IL-3, SDF-1α	Incorporation	High capillary density within 6 days in aligned scaffold implants with 40% perfused, slight

Study	Model	Biomaterial(s)	<i>In Vitro</i> Study Cell Type(s)	<i>In Vivo</i> Study Cell Type(s)	Angiogenesis- Inducing Molecule(s)	Technique(s)	Results
Cheng et al. (2016)**	MI - LAD permanent ligation (Rat)	Carriers: PLGA-PNIPAM	HUVECs, NRVMs	None	NaB	Encapsulation	Stimulated EC proliferation by promoting mRNA expression of VEGF-A, HGF, bFGF, and PDGF. Reduced generation of reactive oxygen species and cellular autophagy. Increased LVEF. Increased infarct size.
Rodness et al. (2016)**	MI - LAD permanent ligation (Rat)	Scaffold: Chitosan sheet; Carrier: Alginate	HSVECs	None	VEGF-164 (recombinant rat)	Encapsulation - fused microparticles	Higher capillary density and mature arteriolar vessels in patch. Sc ars were thicker but Improved fractional shortening. smaller in area and length.
Awada et al. (2017)**	MI - LAD permanent ligation (Rat)	Scaffold: Fibrin; Carrier: PEAD, heparin	None	None	TIMP-3, bFGF, SDF-1α	Incorporation, encapsulation	Reduced fibrosis due to TIMP-3 release, improved reduced inflammation, cardiomyocyte survival, enhanced angiogenesis and SMA+ vessels by 8 weeks.

Articles are arranged primarily by model, then appearance in section 5. Articles marked with * are referenced only outside of section 5, and those marked with ** are not discussed in this review but are provided for additional examples and further readings. Ang-1: angiopoietin 1; bFGF: basic fibroblast growth factor; BMP2: bone morphogenetic protein 2; CM: cardiomyocyte; CAM: chorioallantoic membrane assay; DCx: distal circumflex artery; DPSC: dental pulp stem cell; EC: endothelial cell; ECM: extra-cellular matrix; EDC: 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride chemistry; EGF: endothelial growth factor; FB-VEGF-C: fibrin-binding vascular endothelial growth factor C variant; FGF-1: fibroblast growth factor 1; hAFSCs: human amniotic fluid-derived stem cells; hCPCs: human cardiac progenitor cells; hDPSCs: human dental pulp stem cells; hDVECs: human dermal vascular endothelial cells; HGF: hepatocyte growth factor; hiPSC: human induced pluripotent stem cell; hMSCs: mesenchymal stem cells; HUVECs: human umbilical vein endothelial cells; HIF1α: hypoxia-inducible factor 1α; HSVECs: human saphenous vein endothelial cells; IGF-1: insulin-like growth factor 1; IR: ischemia-reperfusion; LAD: left anterior descending artery; LECs: lymphatic endothelial cells; LV: left ventricle; LVEF: left ventricle ejection fraction; NaB: sodium butyrate; NRVMs: neonatal rat ventricular myocytes; PCL: polycaprolactone; PDA: distal posterior descending artery; PDGF: platelet-derived growth factor; PEAD: poly(ethylene arginylaspartate diglyceride); PEG: polyethylene glycol; PEG-DA: polyethylene glycol diacrylate; PEOz: poly(2-ethyl-2-oxazoline) ; PLCL: poly(L-lactide-co-caprolactone); PLG: copolymer of D,L-lactide and glycolide; PLGA: poly(lactic-co-glycolic acid); PLLA: poly(lactic acid); PNIPAM: poly(N-isopropylacrylamide); POMaC: poly(octamethylene maleate (anhydride) citrate); RASMCs: rat aortic smooth muscle cells; RVECs: rat vein endothelial cells; SDF-1: stromal cell-derived factor 1; α-SMA: α-smooth muscle actin; SMC: smooth muscle cell; TIMP-3: tissue inhibitors of metalloproteinases-3; VEGF: vascular endothelial growth factor; Si: silicon.