Chapter 2
Mechanical Regulation of Vascularization in Three-Dimensional Engineered Tissues

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2.1 Introduction

Three-dimensional (3D) tissue engineering generally involves the design of 3D polymeric scaffolds in combination with one or more cell types, to form implantable, tissue-like devices. Such engineered tissues can be used to replace autologous tissue conventionally used to repair substantial tissue damage and can serve as tissue-scale models to study normal and diseased biological processes (e.g., drug screening tests). Vascularization of engineered tissue constructs is a challenge of great significance in the field of regenerative medicine. Without a stable and perfusable blood vessel network providing oxygen and nutrients, cells cannot survive tissue dimension growth beyond several hundreds of microns, due to diffusion limitations. Several approaches have been applied to promote the vascularization of engineered implants, including pre-vascularization of implants relying on self-assembly of endothelial cells and mural cells to vessels. The mechanisms by which endothelial cells (ECs) self-assemble into vascular networks are quite diverse. Current scientific understanding generally separates these processes into vasculogenesis and angiogenesis, where vasculogenesis refers to the de novo creation of vascular networks from endothelial progenitor cells (angioblasts), as seen in the embryo's primitive vascular plexus. Angiogenesis refers to the expansion of existing vascular networks into new blood vessels, via mechanisms such as sprouting, intussusception (vessel splitting), and vessel fusion. During angiogenesis, ECs exert mechanical forces on their environment while invading, proliferating and migrating. These forces are generated by the contraction of several cytoskeletal proteins such as actin, microtubules, and actomyosin and are affected by environmental cues. Additionally, EC lining blood vessel walls are continuously exposed to mechanical

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stimuli, in the form of fluid shear stress generated by flowing blood, and tension generated by external contractile forces. Various in vitro models, consisting of ECs cultured in 3D scaffolds composed of different biomaterials, have been established to study the effect of mechanical stimuli on vascularization processes. Some of these models are designed to manipulate the mechanical properties of the 3D matrix such as matrix stiffness and boundary conditions, while others are designed to actively apply fluid shear stress and external tensile forces using designated bioreactors. This chapter will summarize the main insights gained regarding the impact of internal and external mechanical cues on the formation of vascular networks in 3D culture systems (Fig. 2.1). Internal mechanical forces refer to cell-induced forces regulated by the cellular environment, while external forces are those that are actively applied on the engineered tissue by external sources.

2.2 Internal Forces

2.2.1 Cellular Forces

Mechanical interactions between cells and the extracellular matrix (ECM) play a central role in regulation of cell division, motility, and differentiation [10, 32, 38]. Cells, including ECs, modify the mechanical and structural properties of their surrounding ECM by exerting contractile forces. Endothelial invasion and sprouting involve three-dimensional (3D) matrix deformation, as demonstrated by fluorescent particle displacement in gel [28, 49], anisotropic fibrillar structure of the ECM [30], and local ECM stiffness [25]. These matrix alterations subsequently trigger feedback responses which dictate vascular network morphogenesis. For example, endothelial sprouts exert mechanical forces that reorganize the matrix to support tubelike endothelial structures and branching point formation. It has also been shown that cell contractile forces regulate sprouting directionality. Korff et al. demonstrated that forces induced by sprouting vessels led to long-range deformation of the underlying collagen gel. Interestingly, sprouts of nearby EC spheroid followed the direction of tension-aligned fibers generated by the ECs [26]. Cell-cell mechanical communication has also been demonstrated in an experimental model in which EC sprouting correlated with substrate deformations generated by neighboring cells in a compliant polyacrylamide gel (Fig. 2.2a) [43].

2.2.2 Matrix Stiffness

The role of ECM stiffness in regulating cellular morphology, differentiation, traction force generation, focal adhesion, and cell migration dynamics is well studied [4, 10, 41, 42, 61]. Modification of the fibrin or collagen gel density has been a common approach to manipulate stiffness of 3D matrices in vitro. Studies

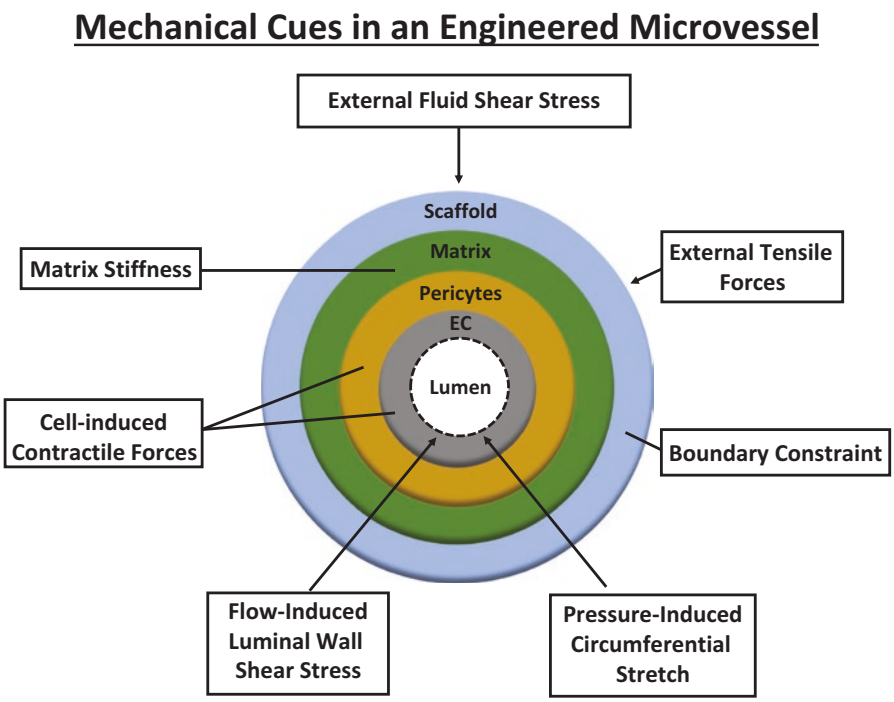

Fig. 2.1 Diagram of mechanical cues that regulate vascularization in 3D engineered tissue

demonstrate that a less dense fibrin matrix is essential for the formation of capillary-like networks and induces longer endothelial sprouts [24, 53]. Additionally, when co-cultured with supportive cells (fibroblasts) in 3D engineered construct, ECs self-assembled into more enhanced vascular networks on matrices with lower fibrin concentrations [31]. The same tendency was observed in studies utilizing collagen gels, where lower collagen density resulted in a more developed vascular network [9]. In another study, the best vascularization results were achieved with relatively intermediate collagen concentrations [48]. However, the impact of matrix stiffness per se is still questionable, since manipulation of gel density also alters chemical properties of the gel such as adhesion ligand concentrations, structure, and diffusion coefficients, all of which may directly or indirectly influence the residing cells. For example, limited diffusion of secreted growth factors was suggested as one reason for reduced sprouting in more concentrated gels [16]. Modification of collagen gel stiffness by glycation, pH adjustments, and the addition of stiffness-tunable hydrogels are some of the approaches also used today to independently study the impact of matrix stiffness on vessel networks. When adjusting gel stiffness by pH, ECs formed thicker and deeper vascular networks in rigid gels, as opposed to dense and thin networks on flexible gels [60]. Moreover, when stiffness was tuned by polyacrylamide hydrogel-coated collagen within a relevant physiological stiffness range (3–30 kPa), the expression of important pro-angiogenesis mediators (i.e., VEGFR-2,

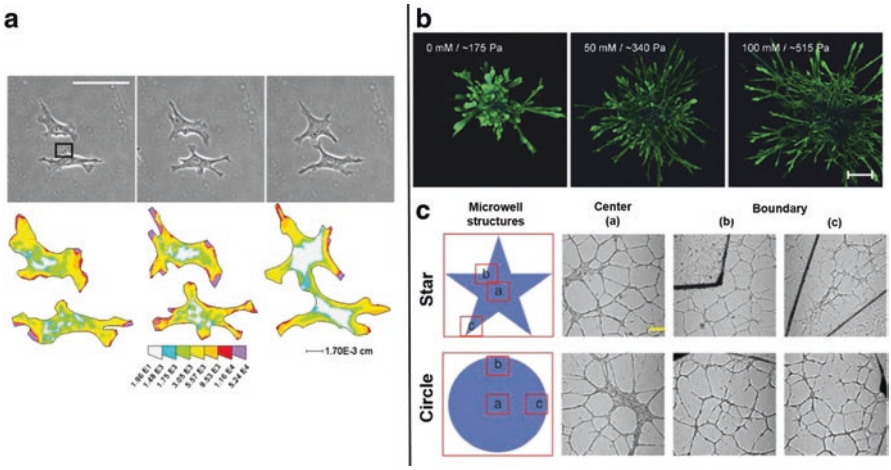

Fig. 2.2 Mechanical regulation of cell-induced forces affected by matrix stiffness and boundaries in vascular organization. (**a**) Cell-cell mechanical communication demonstrated by traction stress distribution. Phase images and corresponding traction maps of ECs coming into contact show guided communication via substrate deformations in a 5500 Pa gel conjugated with 1.0 mg/mL RGD peptide (adapted, with permission, from Reinhart-King et al. [43]). Bar, 100 μm. Color map is in units of dyn/cm^2. (**b**) Endothelial spheroids were cultured in nonenzymatically glycated collagen gels, which enabled assessment of the impact of gel stiffness on EC sprouting, independently of matrix density. Changes in the compressive modulus of the hydrogel resulted in an increase in the number and length of sprouts in the higher stiff matrix (adapted, with permission, from Mason et al. [36]). Bar, 200 μm. (**c**) The effect of mechanical boundaries on capillary-like structure formation using polydimethylsiloxane (PDMS) fences. Bright-field images of capillary-like structures on circular and star-shaped matrigels (adapted, with permission, from Sun et al. [50]). Bar, 300 μm

caveolin-1, and β-catenin) was significantly decreased on stiffer substrates [47]. Although glycated collagen culture models have shown that sprouting angiogenesis was delayed in stiff collagen gels [12], nonenzymatic glycation approach has shown altered tendency. When collagen stiffness was tuned by nonenzymatic glycation, the numbers and lengths of angiogenic sprouts were dramatically increased in a stiffer matrix (from 175 Pa to 515 Pa) (Fig. 2.2b) [36]. So despite many studies showing that vascular organization is dependent on the mechanical properties of the supporting matrix, the regulatory effect of stiffness on 3D vascular arrangement is still a source of controversy.

2.2.3 Boundary Constraint

Cells induce contractile forces that lead to the compaction of the surrounding matrix. Cells cultured in a gel with no rigid boundary (free gel) exert and sense less tension forces, compared to cells cultured in a constrained gel where the gel deformability is limited. Consequently, vascular network formation in a gel is demonstrated

to decrease when boundary limitation is high, corresponding to higher matrix stiffness conditions. In several studies, differential boundary conditions were applied by constraining the gel to predetermined boundary geometries such as a rectangle, triangle, square, star, or circle (Fig. 2.2c) [50, 52]. Sun et al. demonstrated that vascular networks situated along the boundary of the shapes were significantly denser and had a shorter mean cord length, compared with the central regions. The local strain field experienced by microvessels was predicted by computational finite element models simulating the contraction of gels constrained by various boundary conditions [50]. This boundary effect was then eliminated using a Rho-associated protein kinase inhibitor, which demonstrated the correlation with cell traction force [50]. These findings demonstrate that boundary conditions and, thus, the effective stiffness of the matrix provide an alternative means of controlling vascular organization in engineered tissues without modifying matrix chemical properties.

2.3 External Forces

2.3.1 Tensile Forces

During physiological growth, blood vessels remodel and grow in response to tensile stress and the resulting strain within the vessel wall [34]. In recent years, there have been growing attempts to include tensile stress, primarily applied by the use of bioreactors, as a mechanical stimulator when engineering blood vessels. Recent experiments show a connection between tensile stress and alignment of forming vessels. ECs grown on micro-carrier beads, cultured within a fibrin gel containing smooth muscle cells and subjected to 10% cyclic strain at 0.7 Hz, formed sprouts which aligned in parallel to the strain direction, whereas the unstrained control sprouted radially. The plasticity of this alignment was demonstrated, when the aligned strained vascularized constructs were transferred to static unstrained condition, which led to random alignment of the vessel sprouts [6]. In a similar work, researchers isolated microvessels, seeded them into a collagen gel, and applied 6% cyclic strain at 1 Hz. The forming sprouts aligned in parallel to the strain direction [27]. However, contradictory findings reported by Matsumoto et al. noted that ECs seeded on a dextran micro-carrier surrounded with fibrin gel sprouted outward, at an angle that was perpendicular to the cyclic strain [37]. Similar behavior was recorded when human pluripotent stem cell-derived vascular smooth muscle cells (vSMC) were subjected to uniaxial cyclic strain, which induced their alignment perpendicular to the stretching direction [58]. ECs seeded on collagen on top of a silicone mold and subjected to cyclic uniaxial stretch (20%, 1 Hz), also aligned perpendicular to the stretch direction and formed more vessel sprouts [59]. In a different work, ECs and fibroblasts co-cultured within Gelfoam 3D scaffolds and subjected to uniaxial cyclic stretch (10% and 1 Hz) formed vessels that aligned diagonal (30–600) to the stretching direction (Fig. 2.3a) [45]. However, ECs and fibroblasts seeded into a Gelfoam

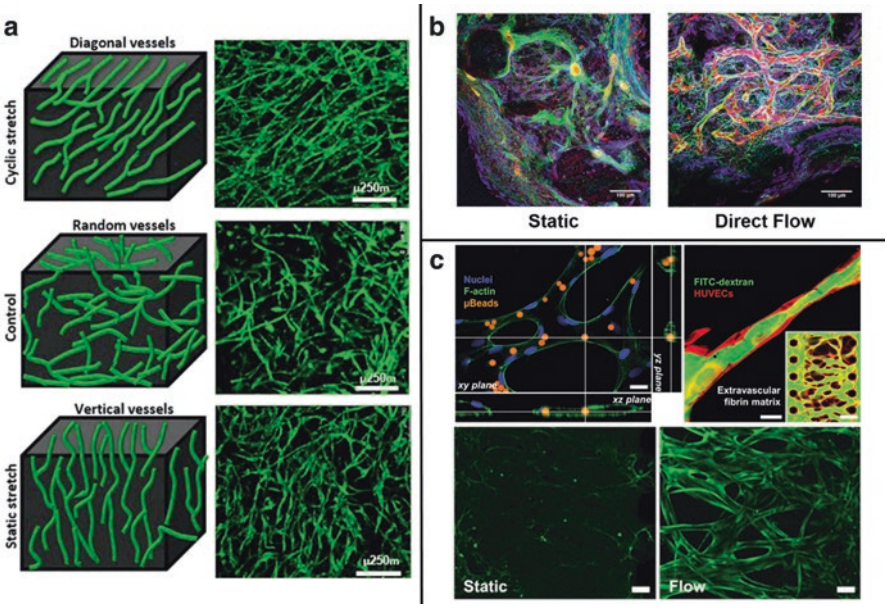

Fig. 2.3 External mechanical regulation of microvasculature by tensile forces and fluid shear stress. (**a**) The orientation of vessel-like structures upon exposure to various mechanical stretching regimens. Free-floating scaffolds (no external force) contained randomly orientated vessels, while cyclic-stretched scaffolds contained diagonal vessels and static-stretched scaffolds displayed vertically aligned vessels. Green, HUVEC-GFP cells (adapted, with permission, from Rosenfeld et al. [45]). Bar 250 μm. (**b**) Scaffold cross sections showing colocalization of collagens I and IV with EC structures. A cross section of a scaffold cultured for 7 days under static conditions and a scaffold cultured for 5 days under static conditions and then for 2 days under direct constant flow conditions (adapted, with permission, from Zohar and Blinder et al. [63]). ECs, red; collagen IV, green; and collagen I, blue. Bar, 100 μm. (**c**) A perfusable vascular network established in a microfluidic device, as demonstrated by perfusing FITC-dextran (top right) and fluorescent microbeads (top left). Endothelial cells responded to 1 h of luminal flow with an increase in nitric oxide (NO) synthesis, as demonstrated with DAF-FM DA fluorescence dye (green) (adapted, with permission, from Kim et al. [23]). Bar 50 μm

scaffold and subjected to static strain aligned parallel to the strain direction [45]. These results agree with another report of ECs and muscle cells embedded within a collagen-Matrigel 3D construct subjected to uniaxial stress [54]. In other work, Chang et al. printed and framed aligned microvessel fragments embedded within a collagen gel which was then subjected to uniaxial stress. While the pre-patterning disappeared during culture in vitro, the vasculature within the constrained constructs aligned in parallel to the stretch direction, and both maintained alignment during culture in vitro and induced invading vessels to align in the same direction post-implantation [7].

The reasons for these recorded alignments are debatable. Ingber hypothesized that local ECM thinning, caused by ECM turnover triggered by ECM modulators, increases ECM compliance, resulting in the production of tractional forces by the surrounding cells leading to local cell distortion. Consequently, increases in trans-

membrane receptor tension results in modification of cellular biochemistry, which eventually impacts cell growth and motility and overall network patterning [20]. Another work showed that tensile forces generated by ECs cause ECM alignment, leading to sprout alignment [26]. In contrast, Ceccarelli et al. claimed that vessel alignment is caused by changes in ECM stiffness (resulting from the applied strain) rather than ECM alignment [6]. Kaunas and Deguchi determined that cell alignment in response to cyclic stretch is guided by remodeling of cell adhesions and actin stress fibers, where myosin II has a major role in maintaining cellular homeostasis under mechanically stimulated environments [22].

Strain was also shown to influence cytokine secretion from stimulated vascularized constructs. Vascular endothelial growth factor (VEGF) and platelet-derived growth factor (PDGF)-ββ secretion levels increased under cyclic strain when compared to the unstimulated control group [45]. This correlates with another study that showed an increase in angiopoietin 2 (Ang-2) and PDGF-ββ secretion from ECs subjected to cyclic strain. Endothelial migration and sprout formation were both increased under cyclic strain conditions [62]. Static strain has been shown to induce vessel formation, without the need for addition of growth factors, and to stimulate muscle cells surrounding the ECs to secrete VEGF [54]. When subjecting tubular constructs composed of fibrin seeded with bovine aortic endothelial cells, to 7 days of 10% cyclic strain, the length and density of the forming sprouts were affected; stimulated constructs displayed wider, less branched sprouts compared to the unstimulated control group [14]. In addition, cyclic strain was shown to induce a short-term increase in gene expression of collagen type I, fibronectin, and elastin within human pluripotent stem cell-derived vSMCs. In the long term, uniaxial strain decreased ECM expression within mature vSMCs, whereas in less mature vSMCs, elastin upregulation was found [58]. Rho-associated kinase (ROCK) is shown to have an important role in vessel alignment and remodeling under cyclic stretch. The addition of ROCK inhibitor to a culture medium resulted in alignment suppression and a decrease in vessel sprouting. In contrast, inhibition of the receptor tyrosine kinase resulted in a decrease in vessel sprout density but had no effect on vessel alignment [59].

Stretch has also been utilized in the field of tissue engineering of large blood vessels. A special bioreactor was developed to enable application of cyclic biaxial stretch on polyglycolic acid (PGA) vessels seeded with SMCs, to mimic the stimulations in native arteries. Biaxial stretch-stimulated constructs formed elastic fibers and aligned collagen fibers, similar to those found in native arteries, with higher suture retention strength. Additionally, biaxial stretch stimulation was shown to increase the strength and compliance of the engineered blood vessels [19].

2.3.2 Fluid Shear Stress

Flow-induced shear stress is correlated with morphological changes in endothelial cells in vitro [17, 18, 39, 51] as well as with changes in gene, protein, and miRNA expression [15, 40]. In microfluidic devices [3, 13, 21, 55] and two-dimensional (2D) monolayer "flow-over" models [2, 56], flow-induced shear stress has been

shown to regulate and enhance angiogenic processes. Several groups have studied the effects of flow-induced shear stress on endothelial cells in vitro, using a parallel plate flow chamber [5, 18, 51]. This experimental setup is simple to model and provides better control over the applied shear stress. However, it does not closely mimic the 3D nature of interstitial flow, as the medium flows over the cells rather than through the scaffold. Moreover, endothelial cell response is limited as the system does not support 3D sprouting and network formation. In an alternative 3D model, consisting of 3D collagen and alginate gel plugs embedded with endothelial cells, direct flow simulation resulted in enhanced vascular morphogenesis and angiogenesis-related gene expression [29, 46]. Furthermore, in our recently published work, we demonstrated the effect of flow-induced shear stress on vascular formation and maturation in an implantable 3D engineered construct. Direct flow conditions resulted in a significant increase (>100%) in vessel network morphogenesis parameters, EC and ECM protein depth distribution, and colocalization incidence of alpha-smooth muscle actin (α-SMA) with endothelial vessel networks. These findings suggest that flow conditions promote 3D neovascularization and vascular network maturation in a 3D engineered tissue (Fig. 2.3b) [63]. In microfluidic-based platforms, luminal flow through a self-assembled microvasculature was applied to generate shear stress stimulation inside microvessels [23, 57]. The microvasculature patency was demonstrated by tracking flowing FITC-dextran and fluorescent microbeads (Fig. 2.3c) [23, 57]. The self-assembled microvessels possessed morphological and biochemical markers characteristic of natural blood vessels and exhibited strong barrier function, long-term stability, cytoskeleton rearrangements, and increased nitric oxide synthesis (Fig. 2.3c) [23].

However, the true nature of the effect of shear stress on vessel formation and behavior is still poorly understood [1]. Many molecular mechanisms and signal transduction pathways are suggested to bear shear-sensing or mechanotransductive roles. These mechanisms involve cell-matrix and cell-cell adhesion and junction molecules, membranous receptors and ion channels, the extra-membranous glycocalyx complex, and cytoskeleton proteins among others [1, 33, 44]. For example, shear stress has been shown to mediate changes in endothelial cell phenotype, by increasing RhoA (a cytoskeletal regulator) activity and promoting the formation of stress fibers [8]. Additionally, a direct connection between flow-induced shear stress and VEGF signaling during angiogenesis has been shown to be mediated by an endothelial cell-specific microRNA, called mir-126, which is regulated by the mechanosensitive zinc finger transcription factor klf2a [11, 40]. This microRNA has a pro-angiogenic function, by naturally repressing VEGF inhibitors ispred1 and pik3r2.

2.4 Conclusions and Future Perspectives

Creation of vascularized 3D engineered tissue requires a multidisciplinary approach. This involves integrating knowledge of vascular biology together with biomaterials engineering and microfluidic design, to recapitulate a microenvironment in which

cells can form mature blood vessels that can maintain tissue viability over time. The basic building blocks for blood vessel engineering are endothelial cells (ECs) and supporting cells. Both cell types can be isolated from different sources or differentiated from different pluripotent stem cells. In most of the discussed studies, ECs and supporting cells were co-cultured on different natural and synthetic biomaterials harnessed to establish a natural 3D environment supportive of vascularization processes. Both matrix stiffness and boundary constraint affect cell-induced contractile forces, which regulates cell-cell communication, vascular network structure, and directionality. External forces, in the form of fluid shear stress and tensile forces, induce vascular network formation and maturation and dictate vascular network structure and directionality. Although the effect of mechanical stimulation on vascularization is clearly demonstrated, the origin of the biological triggers remains in question. The impact of mechanical cues on vascularization can be explained by direct communication, via EC mechanotransduction sensors [1], or by indirect cues related to environmental changes such as neighbor cell responses, matrix deformation, and biochemical changes. A deeper understanding of the mechanisms through which ECs are affected by mechanical stimulation in 3D matrices may be achieved by correlating vascular morphogenesis with the levels of biosensors associated with relevant known biomechanical pathways. In addition, while the vast majority of studies focus on changes in vascular network morphology in response to mechanical cues, technical hurdles limit research on other critical functional parameters key to the success of engineered vessels, such as perfusability and barrier function. Perfusable vascular networks enable generation of both luminal shear stress and circumferential wall stress, both of which play critical roles in vascular mechanobiology [35]. Such physiologic stresses and strains exert vasoprotective actions, mediated by nitric oxide, and provide a homeostatic oxidative balance for further vascular remodeling and maturation [35]. It is very important to clarify that the developmental stage of the vascular network largely impacts endothelial cell responses to mechanical stimulation. For example, during vasculogenesis (when vascular networks are not fully established), ECs can respond to external shear stress by accelerating vascular network arrangement. This pro-angiogenic role of shear stress may be critical in cases of injury, when the tissue is undergoing a wound healing process. Conversely to anti-angiogenic role in vascular network remodeling, maturation and quiescence processes occur under homeostasis conditions regulated by luminal shear stress and circumferential wall stress.

Recently developed experimental approaches, based on evolving 3D microfabrication techniques, may open up new strategies for exploring the impact of biomechanical triggers on vascularization under more physiologically relevant conditions. Moreover, the control and repeatability of patterning vascular and perivascular cells in 3D engineered tissue will allow for a higher degree of regulation over the initial organization of vascular structures and, therefore, will enable to study cell-cell and cell-matrix mechanical interactions in more accurate and controlled systems. Such high levels of accuracy will enhance computational estimations of local mechanical cues, which can be validated by mechanical biosensor-based feedback signals. Conclusively, integration of high-precision fabrication techniques with advanced

computational models and utilization of biomechanical sensors may provide a better understanding of the means through which mechanical forces regulate the different stages of vascularization in 3D engineered tissues.

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