# Scaffold biomaterials for nano-pathophysiology

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**Abstract**

This review is intended to provide an overview of tissue engineering strategies using scaffold biomaterials to develop a vascularized tissue engineered construct for nano-pathophysiology. Two primary topics are discussed. The first is the biological or synthetic microenvironments that regulate cell behaviors in pathological conditions and tissue regeneration. Second is the use of scaffold biomaterials with angiogenic factors and/or cells to realize vascularized tissue engineered constructs for nano-pathophysiology. These topics are significantly overlapped in terms of three-dimensional (3-D) geometry of cells and blood vessels. Therefore, this review focuses on neovascularization of 3-D scaffold biomaterials induced by angiogenic factors and/or cells. The novel strategy of this approach in nano-pathophysiology is to utilize the vascularized tissue engineered construct as a tissue model to predict the distribution and subsequent therapeutic efficacy of a drug delivery system with different physicochemical and biological properties.

**Keywords:** scaffold biomaterials, angiogenic factor delivery, angiogenic cell delivery, vascularized tissue engineered constructs

## 1. Introduction

In the body, cells and tissue are organized into three-dimensional (3-D) architecture. To engineer such functional tissues, scaffold biomaterials have to be fabricated through different methodologies that facilitate cell distribution and guide growth into a 3-D space. Traditional static two-dimensional (2-D) cell culture systems allow us to understand many complex biological processes in a homogeneous population of cells. However, in the native physiological system, cells exist in a complex microenvironment consisting of various biological cues, such as signaling molecules, extracellular matrix (ECM) molecules, biomechanical forces, and cell–cell interactions [1]. Although the simplicity of the 2-D culture systems can elucidate individual cell phenomena, the 2-D culture systems reduces the biological importance and complexity of dynamic tissue architectures that affect the physiological behavior of cells *in vivo*. For instance, unlike in 2-D culture, mammary epithelial cells embedded in a 3-D environment stop growing, form acinar structures, and establish a *de novo* basement membrane [2-4]. For this reason, 3-D tissue constructs better reflect native biophysical and biochemical environments, and hence have become a focus of recent investigation [5].

In addition to the geometric effect, the microenvironment plays a pivotal role in regulating the spatial distribution of nutrients, oxygen, and soluble signaling molecules, such as growth factors, hormones, and cytokines. These chemical gradients are essential for the regulation of fundamental cell processes, such as cell migration [6], angiogenesis [7, 8], and tissue patterning in development [9-12]. This spatial distribution of diffusible factors is complex and is affected by the surrounding ECM, the organization of vasculature, and distribution of neighboring cells. Furthermore, the sustained presence of a 3-D concentration gradient is required to elicit chemotactic events such as cell migration and polarization. However, under conventional 2-D culture conditions, cell-secreted or exogenously added soluble factors diffuse ubiquitously throughout the medium, thereby forming a rapid equilibrium devoid of these gradients.

Similar to the signaling molecule, the diffusion of soluble therapeutic drugs, which is one of the important determinants in the therapeutic efficacy of the drug, is regulated by microenvironmental properties including oxygen concentration, vascular structure, local pH, and inner pressure in pathological tissues [13]. For instance, Kano et

al. demonstrated that the efficacy of anticancer drug-incorporated nanoparticles with a diameter of several tens of nanometer was largely determined by the structure of vascular walls [14]. In order to act effectively to the cancer cells, these nanoparticles must pass through the vascular wall towards the interstitial tissue [14-17]. By depleting alpha smooth muscle actin (αSMA) positive cells from the tumor vasculature with an inhibitor of transforming growth factor (TGF)-β receptor type I, the resistance to the anticancer drug in a model of pancreatic cancer is diminished, and thus alteration of the tumor microenvironment enhances the therapeutic efficacy of the anticancer drug even in the intractable tumor. This environmental effect in the cancer treatment is not predictable by the conventional drug screening based on the regular 2-D culture, where cancer cells derived from the intractable tumor are always sufficiently sensitive to conventional anticancer drugs.

Normal blood vessels and tumor-induced blood vessels differ greatly in morphology and function. Normal blood vessels recruit pericytes and vascular smooth muscle cells to the endothelial cells (ECs) to stabilize the vessels. Depending on the type of tumor, tumor-induced blood vessels can be either less stable, disorganized, and leaky, or conversely, less leaky with increased pericyte-coverage and collagen deposition [16, 17]. The leakiness of the tumor vasculature leads to the increased accumulation of nanoparticles depending on their size, a phenomenon which has been called the “enhanced permeability and retention (EPR) effect” that could enhance the efficacy of the treatment with a less adverse effect [18, 19]. On the contrary, it is likely that tumor vasculature that has increased pericyte-coverage and collagen deposition will impair the permeation of the nanoparticle. Taken together, the complexity of the tumor vasculature requires the consideration of multiple confounding factors in determining drug efficacy, some of which may be better evaluated with interdisciplinary approaches between engineering and basic clinical medicine.

Quantitative understanding of how the number of the pericyte layer and thickness of the collagen deposition affect the penetration, accumulation, and efficacy of nanoparticles plays a pivotal role in developing nanotherapeutics for intractable tumors. However, the lack of the reproducibility and standardization of conventional animal models, such as tumor zenograft models, is a major drawback to tackle the issue. One interdisciplinary approach to accurately mimic a nano-pathophysiological microenvironment in tumors is to create vascularized tissue engineered constructs with

particular vascular structures seen in pathological tissues by utilizing scaffold biomaterials and following controlled positioning of different cells therein. There are two strategies to create vascularized tissue engineered constructs (**Figure 1**). The first is "top down approach" based on the ECs and their ability to form vascular networks *in vivo*. The second strategy, "bottom-up approach", deals with the vessels themselves *in vitro*. In these approaches, scaffold biomaterials function not only as a 3-D template for cell attachment, cell positioning, ECM presentation, and subsequent tissue formation, but also as a spatio-temporally controlled delivery system of angiogenic factors and/or cells. We will now explore these scaffold biomaterials and their use as controlled delivery systems of angiogenic factors and/or cells, and review how angiogenic growth factors and/or cells play an integral role in introducing vascular networks into tissue engineered constructs. We also discuss how this knowledge might inspire the development of novel vascularized tissue engineered constructs for nano-pathophysiology in the future.

## 2. Scaffold biomaterials for tissue fabrication

Scaffold biomaterials have been extensively investigated as a 3-D template for cell attachment and subsequent tissue formation. Unlike conventional 2-D cultures, which involve growing cells in a non-physiological 2-D environment, positioning of cells within 3-D scaffolds may provide more natural microenvironments that enable cells to promote physiological functions *in vitro* [5, 20-23]. On the contrary, it has been demonstrated that fibroblasts and surrounding cells are regulated by pathological ECM proteins in fibrosis and tumor [24-28]. In addition to the interactions between the cells and the ECM, the mechanical properties of the substrate (i.e., stromal tissues) also regulate multiple cellular processes, and thus have numerous effects on tissue development and disease processes [29-31]. Indeed, mimicking such natural microenvironments seems to be a promising strategy to design scaffold biomaterials. However, there still remain some substantial challenges to create natural microenvironment-mimicking scaffold biomaterials. One possible way to tackle this issue is to encourage cells to create their own ECM microenvironments in scaffold biomaterials. Thus, design of scaffold biomaterials facilitating cell secretion of pathological ECM proteins therein or an appropriate combination of biological/biophysical cues, such as ECM structures, stiffness, and mechanical stimuli,

may modify cell activities in tissue engineered constructs with the pathological microenvironment.

### 2.1. Biological/biophysical cues

Several biological/biophysical cues, such as ECM structures, stiffness, and mechanical stimuli, may modify cell activities in scaffold biomaterials. By utilizing such cues, people could expect cell secretion of ECM proteins therein and following tissue formation similar to pathological tissues. Among them, the ECM that forms the structural framework of tissues is one of the major contributors to the development of a unique microenvironment [32]. Fibroblasts are one of the abundant cell types that secrete ECM in connective tissues [33]. Under pathological conditions, alteration in cytokine secretion patterns activates fibroblasts, such as myofibroblasts and cancer associated fibroblasts (CAF), which secrete a different composition of ECM proteins in stromal tissues when compared to their healthy counterparts [26-28] (**Table 1**). In tumor tissues, several ECM proteins, such as tenasin-C (TNC), fibronectin (FN), and SPARC (secreted protein, acidic and rich in cysteine), are considerably up-regulated, and some of them exists as alternatively spliced isoforms that promote tumor growth and invasion [34-47]. For instance, fibroblast expression of caveolin-1 *in vitro* and *in vivo* favors an tumor-specific organized 3-D stromal architecture of fibrin matrices that promotes spindle morphology, facilitates tumor cell invasion, and increases p190-dependent metastatic potency [48]. Moreover, DeQuach et al. developed naturally derived ECM coatings for cell culture that provide important tissue-specific cues unlike traditional cell culture coatings, thereby enabling the maturation of committed C2C12 skeletal myoblast progenitors and human embryonic stem cells (hESCs) differentiated into cardiomyocytes [49]. Taken together, it is possible that coating of pathological ECM proteins may provide better microenvironments for disease cells to create tissue engineered constructs with the pathological microenvironment.

In addition to the interactions between the cells and the ECM, the mechanical properties of the substrate (i.e., stromal tissues) also regulate multiple cellular processes, and thus have numerous effects on tissue development and disease processes [29-31]. Stiffness of a substrate, measured by the Young's modulus, plays an important role in the adherence of the anchorage-dependent cells [1, 50]. Soft tissue has a low Young's modulus (0.5-2 kPa). Normal mammary tissues are quite soft (0.15 kPa), while this low

stiffness shows a 10-20 fold increase in an advanced invasive mammary tumor microenvironment. This increase in stiffness greatly influences cell behavior and facilitates tumor progression [51]. Furthermore, increased collagen crosslinking has been shown to lead to enhanced acquisition of malignant features [43]. Elevated expression of lysil-oxidase, an enzyme necessary for natural collagen type I crosslinking has been linked to increased fibrillar collagen deposition and linearization. Levental et al. demonstrated that ECM stiffness increases from normal to tumor. Strikingly, the stiffness of the tumor adjacent stroma was shown to be elevated in conjunction with the above-mentioned increases in levels of the crosslinker and linearization of collagen. Mammary epithelial cells grown within 3-D environments at physiological Young modulus (E values of 160–170 Pa) form small growth-arrested colonies with polarized $\beta_4$-integrin and apical-lateral cortical actin, which are all features found in normal mammary epithelium [52]. By contrast, a small increase in stiffness (400 Pa) promoted the formation of double-sized colonies while further increases in matrix stiffness, closer to those exhibit by tumor-associated ECM, stimulated the formation of greater colonies with atypical (tumorigenic) acini structures and altered integrin and actin polarization [52]. Increased matrix stiffness generates the tension necessary to cluster $\alpha_5\beta_1$-integrin in cell–matrix adhesions, thus facilitating cell migration and invasion. Furthermore, stiffness experienced by cells during adhesion to a substrate modulates intracellular mechanisms from gene expression to cell movement through integrin clustering and phosphorylation of integrin-regulated effectors, such as non-receptor tyrosine kinase like focal adhesion kinase (FAK) and $p^{130}$Crk-assosicated substrate [43]. It has been demonstrated that results from cell culture experiments carried out on a 2-D rigid substrate such as tissue culture plastic may not be comparable to those obtained under *in vivo* conditions [53]. For instance, the stiffness of the ECM can regulate epithelial cell growth, differentiation, and migration, and reduction of ECM stiffness can suppress the malignant behavior of mammary epithelial cells [51]. Thus, the typical 2-D culture not only lacks the unique chemical and physical properties that modulate cell-cell interactions, but also influence the morphology of the individual cell unit.

Mechancial stimuli are also very important in tissue engineering. Different types of mechanical stresses are experienced by various tissues. *In vitro* systems have been developed to model the effect of hydrostatic pressure or fluid shear stress on ECs,

and cyclic strain or compressive stress on chondrocytes. For instance, Dewey et al. demonstrated that confluent monolayers of ECs undergo a time-dependent change in cell shape from polygonal to ellipsoidal and become uniformly oriented under a laminar shear stress of 5-10 dynes/cm$^2$ [54]. Obi et al. reported that fluid shear stress induces differentiation of circulating endothelial progenitor cells [55]. Using mouse embryonic stem cells differentiated *in vitro*, Adamo et al. showed that fluid shear stress increases the expression of Runx1 in CD41(+)c-Kit(+) haematopoietic progenitor cells, concomitantly augmenting their haematopoietic colony-forming potential, that increases with an increase in shear stress *in vitro* [56]. To stimulate chondrocytes, Mizuno et al demonstrated that cyclic hydrostatic fluid pressure that mimics the biophysical stimuli in the joints enhances matrix synthesis and accumulation by bovine chondrocytes in 3-D collagen scaffold biomaterials *in vitro* [57]. When a force is experienced by cells, the cells sense the microenvironmental cues and transmit the mechanical signal to intracellular biochemical signals via signal transduction. Several cellular mechanosensors, such as integrins and ion channels have been implicated [58]. For instance, integrins allow for a direct mechanical connection between the cell cytoskeleton and ECM, transmitting forces from the outside to the inside of the cell [59]. Such stimuli influence the cytoskeleton assembly directly, thereby translating the mechanical signal into changes in biochemical signaling pathways [60].

**2.2 Design of polymeric scaffold biomaterials**

To encourage cells to create their own ECM microenvironments, scaffold biomaterials should meet several requirements in terms of structural properties, bulk properties, and biological properties [61]. A major structural issue is to create interconnected pore structures that allow cells to migrate homogeneously into scaffold biomaterials. Moreover, a large surface area and space in the scaffold biomaterial enables cells to attach, grow, and produce their own ECM microenvironments. An optimal pore size ranging between 100 and 500 μm is necessary for cell migration and vascularization [62, 63]. Several technologies and methodologies have been developed to create highly porous scaffold biomaterials. Among them freeze-drying and porogen leaching [64] have been widely used to generate porous structures. Another sophisticated technologies include rapid prototyping and electrospinning that can create a defined structure of micropatterns and nanofibers, respectively [65-68]. However, at

present, it is practically difficult to fabricate a stable 3-D micropattern or a thick porous nanofiber in scaffold biomaterials.

The mechanical property and biodegradability greatly affect the bulk property of scaffold biomaterials and subsequent tissue formation. Scaffold biomaterials should have excellent mechanical properties and remain for a certain period of time to keep a space for cells therein. Otherwise, the remaining material may impair the tissue formation. Several biodegradable polymers have been extensively utilized as biomaterials for scaffold fabrication [69]. Among them polyglycolide (PGA) and its copolymers, such as lactide–glycolide copolymer (PLGA), have been widely employed in many studies on tissue engineering. However, they degrade very quickly when used as a scaffold biomaterial. In contrast, poly(L-lactide) (PLLA) degrades extremely slowly (3–6 years) [61]. These synthetic polymers degrade through non-enzymatic hydrolysis, whereas natural polymers, including collagen, gelatin, and hyaluronic acid, undergo enzymatic hydrolysis. In general, most of natural polymers are hydrophilic and yield scaffold biomaterials with low mechanical strength in comparison with PGA, PLGA, and PLLA. Alginates do not contain any hydrolysable bonds, but are often used as a resorbable biomaterial when decomposed to a form with a smaller molecular weight enough to be excreted from the body [70, 71]. Water-soluble polymers are mostly rendered water-insoluble through covalent crosslinking. For instance, poly (ethylene glycol) (PEG) diacrylate has been widely used by crosslinking with thiol-group-containing enzyme sensitive peptides [20, 72, 73]. The thiol group was coupled with an acrylate group through the Michael reaction under a mild condition, which allows us to incorporate cells in the resulting PEG hydrogels. Several peptides with functional moieties, such as a protease cleavable peptide and a ECM binding peptide, have been employed to prepare cell-encapsulating PEG-based hydrogels with bioactivities [20].

Since the bulk property of the living tissue is extremely complicated to be mimicked using conventional biomaterials, biomolecules with high bioactivities, such as signaling molecules and ECM, have been utilized to improve biological properties of scaffold biomaterials and facilitate tissue formation [61]. There are two methods to combine biomolecules with scaffold biomaterials. One is the controlled release of biomolecules from scaffold biomaterials. The other is the surface modification of scaffold biomaterials with biomolecules, such as graft polymerization, micropatterning,

and protein coating or immobilization, that allows us to change the surface property including wettability, electric charge, morphology or roughness, and bioactivity [74]. Upon seeding onto the scaffold biomaterial with an appropriate surface property, cells could produce their own microenvironments and eventually form new tissues [75]. In the living system, cells receive external cues from the microenvironment through the interactions of cell-ECM, cell-cell, and cell-soluble factor, which are mediated by cell adhesion molecules, cell signaling molecules, and growth factors, respectively. However, since the biomolecules act upon receptors presented on cells in a highly sophisticated manner, people cannot always expect the bioactivities of the biomolecules outside the living system. Considering the biomolecules mainly made up of proteins, the immobilization of the proteins on biomaterial surfaces has been investigated as artificial microenvironments to achieve desired cellular functions for tissue regeneration [76]. Several covalent and non-covalent bonding methods, such as tethering with PEG [77-79], complexing with natural polymers [80-83], and fusion protein engineering of growth factor and different tagged molecules [84-90], have been investigated as a means to mimic cellular microenvironments through protein immobilization without losing the bioactivities for the induction of tissue regeneration.

**3. Enhanced neovascularization in scaffold biomaterials**

Another big issue of scaffold biomaterials is the neovascularization that is essential to supply nutrients and oxygen to the cells for tissue regeneration or to elucidate the distribution of nanoparticles in tissue engineering constructs for nano-pathophysiological researches. Neovascularization can be obtained by three approaches using angiogenic genes, angiogenic factors, and progenitor or stem cells [91-95]. Several attempts have been made to fabricate vascularized tissue engineered constructs *in vitro* [91, 92]. By contrast, *in vivo* neovascularization will be expected if the tissue engineered construct can provide adequate stimuli to the surrounding tissues. Neovascularization is the formation of vasculature in an avascular tissue. The formation of vasculature is based on two underlying mechanisms: angiogenesis and vasculogenesis. The basic idea of angiogenesis is the formations of new blood vessels from pre-existing ones by sprouting of capillaries. Vasculogenesis denotes the *de novo* assemble of ECs to capillaries *in situ* and regulates the first primitive vessel formation in early embryonic development. In this process, endothelial progenitor cells (EPCs)

migrate to avascular areas and differentiate to mature ECs in response to local cues, such as angiogenic factors and ECM, resulting in creating first primitive vessel networks [96]. Once the primitive vessel network formed, new blood capillaries emerge from the pre-existing vessels and generate more complex capillary networks, which are governed by angiogenic factors secreted from ECs and stromal cells, and/or cancer cells. To introduce vascular networks into tissue engineered constructs, similar processes are required (**Figure 1**).

### 3.1. Angiogenic factors to enhance neovascularization

Angiogenic factors, such as vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF), have been utilized to promote neovascularization [97]. The VEGF family has been most widely investigated as one of the crucial modulators involved in several steps of neovascularization [98]. In some clinical trials, significant neovascularization and increased blood perfusion were observed [99, 100]. FGF functions not only as an angiogenic factor but also as a regulatory factor to maintain the integrity of vasculatures [101]. Among the FGF family, FGF-1 and FGF-2 have been extensively studied and utilized in therapeutic angiogenesis. FGF-1 gene therapy has been conducted using FGF-1-expressing plasmid DNA and showed the safety of angiogenic gene therapy in Phase I/II trials for patients with critical limb ischemia [102], while Phase II trial failed to demonstrate its benefit. Besides VEGF and FGF, hepatocyte growth factor (HGF) and platelet-derived growth factor (PDGF)-BB have also been studied. It has been shown that HGF stimulates ECs proliferation via VEGF production, and promotes secretion of proteases to be involved in ECM degradation and cell migration [103]. PDGF-BB promotes FGF-2 release and FGF receptor activation in vascular smooth muscle cells [104], and plays an important role in maturation of vasculature [105]. Another cytokines or chemokines, such as granulocyte colony-stimulating factor (G-CSF), stroma cell-derived factor (SDF)-1, and substance P, are also known to contribute to neovascularization by mobilizing progenitor cells or neutrophil granulocytes from bone marrow and subsequently recruiting the cells to the site of angiogenesis [93].

In the *in vivo* system, most of angiogenic factors exist in an inactive form by binding to ECM components, such as proteoglycan, collagen, and fibrin [106], and with the onset of angiogenesis, their active form will be released from ECM through

enzymatic activations. Therefore, regardless of the factor type, it is necessary to develop a delivery system that can realize stable concentration of the angiogenic factor suitable for inducing neovascularization *in vivo*. Although prolonged expression of an angiogenic gene could expect a higher local concentration of the resulting protein enough to induce angiogenesis, protein administration may be more predictable in terms of initial dose and pharmacokinetics of the angiogenic factor than the genetic approach. Several controlled release system comprises hydrogels, microspheres, porous scaffolds, and nanofibers, and both natural and synthetic polymers have been explored to enhance biological activities of angiogenic factors (**Table 2**) [92, 95]. Among natural polymers, fibrin, collagen, gelatin, alginate, and hyaluronic acid are widely investigated. Angiogenic factors have been incorporated into fibrin matrices together with heparin, leading to better control of the release rate of heparin-binding growth factors, such as FGF-2, than fibrin matrices alone [107]. Hubbell et al. created a fibrin-binding VEGF fusion protein via recombinant protein engineering that enables the introduction of a specific binding domain into growth factors at a desired location not to lose their bioactivities [108]. The fibrin-binding VEGF fusion protein allows VEGF to be released in a controlled manner via proteolysis for the matrix remodeling after ECs infiltration.

Marui et al. reported that the dual release of a lower dose of FGF-2 and HGF from the collagen microspheres could achieve equivalent blood perfusion recovery and more mature vasculature in ischemic limbs than a higher dose of FGF-2 or HGF alone [109]. To improve the affinity of growth factors to collagen [110], heparin was covalently introduced into collagen-based scaffold biomaterials [111]. Similar to the fibrin-binding VEGF fusion protein, a collagen-binding VEGF fusion protein demonstrated better retention in collagen-based scaffold biomaterials compared with VEGF alone, and showed the promoted angiogenesis in the ischemic heart [112, 113].

Tabata et al. demonstrated the effectiveness of FGF-2 protein released from gelatin hydrogels in various animal models either non-diabetic or diabetic for acute myocardial infarction [114, 115], prevascularization for cardiomyocyte transplantation to the ischemic heart [116], limb ischemia [117], and bone regeneration [118]. Furthermore, Marui et al. have shown the safety and feasibility of the controlled release system in patients with critical limb ischemia, who had no option of medical or surgical treatment [119].

We utilized an alginate hydrogel patch to deliver SDF-1 and demonstrated the

accelerating healing and reducing scarring in an animal model that closely mimics human wound healing using Yorkshire pigs with acute surgical wounds (**Figure 2**) [120]. Alginate microspheres can also be employed for controlled release of entrapped proteins. Protein loss during microsphere fabrication has been a major drawback for application as a controlled release system [92]. On the other hand, a hyaluronic acid hydrogel conjugated with cell adhesion peptides and matrix metalloproteinase-cleavable peptides to entrap angiogenic factors and endothelial colony-forming cells. By using the hyaluronic acid hydrogel with angiogenic activities, Hanjaya-Putra et al. showed that a functional vascular network forms *in situ* and integrates with the host vasculature [121].

Synthetic polymers, such as PGA, PLA, and their copolymer [122-129], and PEG derivatives [130-132], have been investigated as controlled release systems for angiogenic growth factors. To avoid the bioactivity loss caused by exposure to organic solvents in fabrication processes, many researches have employed heparin for controlled release systems of angiogenic growth factors from PLGA [126]. Combination of an angiogenic factor-encapsulating PLGA microspheres with another angiogenic factor-incorporating porous PLGA scaffolds achieves sequential angiogenic factor delivery, such as VEGF and Angiopoietin-I [127], VEGF and PDGF-BB [128], or FGF-2 and PDGF-BB [129], to promote more mature and stable vessel formation than simultaneous delivery of the angiogenic factors.

Hubbell et al. fabricated PEG hydrogels by crosslinking with cleavable linkers, such as matrix metalloproteinase-cleavable peptides, and demonstrated the cell-demanded release of VEGF and the subsequent cell ingrowth into the PEG hydrogel [130]. West et al. introduced a VEGF-mimetic peptide, a cell adhesion peptide of Arg-Gly-Asp, and a collagenase-cleavable peptide into PEG diacrylate hydrogels using a succinimidyl ester linker and showed the promoted angiogenesis into the hydrogel [131]. Salimath et al. reported the dual delivery of HGF and VEGF from a protease-degradable PEG hydrogel could improve cardiac function in rat with acute myocardial infarction caused by ischemia and reperfusion [132]. Werner et al. demonstrated that a local concentration gradient of SDF-1 generated by PEG hydrogels containing a sulfated-glycosaminoglycan could enhance the recruitment of circulating EPCs and subsequent angiogenesis *in vivo* [133].

### 3.2. Angiogenic cells to enhance neovascularization

Cell source is also an important topic for enhanced neovascularization. Primary sources are ECs that include human dermal microvascular ECs (HDMECs), human umbilical vein ECs (HUVECs), bovine aortic ECs (BAECs), and bovine capillary ECs (BCEs). Furthermore, Jarrell et al. have used microvessel endothelium isolated from a liposuction-derived fatty tissue through a Percoll density gradient [134]. However, the complicated isolation protocol and low proliferation rates for autologous ECs may become a major drawback to their application. Therefore, EPCs from various adult sources, such as umbilical cord blood, peripheral blood, bone marrow-derived mononuclear cells, and fat tissue, have been proposed as an alternative cell source for neovascularization.

Asahara and Isner identified EPCs in 1997 and described their role in vasculogenesis for hind limb ischemia [135]. EPCs can differentiate into mature ECs as verified by endothelial markers, such as von Willebrand factor (vWF), platelet endothelial cell adhesion molecule-1 (PECAM-1/CD31), and vascular endothelial-cadherin (VE-Cad) [136]. EPCs have the potential to migrate and incorporate into ischemic tissue, and blood flow recovery and capillary density were significantly improved after EPC transplantation in animals [137]. As a result, EPCs reduced the rate of limb loss and increased blood flow in diabetic mice with impaired circulation [138]. They also improved neovascularization and myocardial function in mouse cardiac infarction models [139]. These findings suggest that exogenously administered EPCs augment neovascularization based on the cross talk between EPCs and other cell types mediated by angiogenic factors or cell-cell interactions. Systemically administering granulocyte macrophage-colony stimulating factor (GM-CSF) increased the circulating EPC population and augmented neovascularization of ischemic tissues [140]. VEGF increased mobilization of EPCs in peripheral blood of mice. Other mobilizing factors include angiopoietin-1 (Ang-1), placental growth factor (PlGF), and erythropoietin. Mobilizing factor release plays a crucial role in recruiting EPCs to sites of neovascularization. Another important recruitment factor is SDF-1. SDF-1 binds to the chemokine receptor CXCR-4, which is highly expressed on EPCs in addition to bone marrow stroma. Once sites of neovascularization are reached, EPCs may recruit additional EPCs by releasing growth factors, such as VEGF, HGF, G-CSF, and GM-CSF.

EPCs have been demonstrated to differentiate towards ECs in culture both

with and without VEGF [141-143]. Because of their angiogenic potential, EPCs have been utilized not only for therapeutic intervention in ischemic diseases but also for endothelialization of implants, such as vascular grafts and stents [144, 145]. Moreover, engraftment of EPCs is an attractive possibility for enhanced neovascularization in scaffold biomaterials. EPCs accelerated the vascularization of heparin-immobilized PCL scaffold biomaterials in the presence of VEGF [146]. It has been shown that the co-culture of EPCs with different cell types, such as smooth muscle cells, fibroblasts, adipocytes, or osteoblasts, plays a crucial role in supporting angiogenesis [147-150]. The presence of EPCs enhanced dermal vascularization with capillary-like structures homogeneously in scaffold biomaterials [151]. The integrative use of bioreactor culture systems and co-cultured osteogenic cells and EPCs promotes maturation of vascularized bone tissue engineered constructs *in vitro* [152].

In contrast to normal tissues, the vasculature of solid tumors has a limited diffusion, resulting in the tissue hypoxia with a low oxygen concentration. This tissue hypoxia triggers a cascade of hypoxia-inducible factor-1 (HIF-1) and VEGF-mediated signaling events that initiate tumor neovascularization. Hida et al. demonstrated that ECs in solid tumors are cytogenetically abnormal and aneuploid with multiple chromosomes and multiple centrosomes [153, 154]. Unlike normal ECs, which remain diploid in long-term culture, the aneuploidy of tumor ECs increases in culture suggesting that these cells are inherently unstable. Moreover, tumor ECs show resistance to paclitaxel compared with normal ECs [155]. Taken together, it is likely that heterogeneity of ECs with different phenotypes needs to be concerned in mimicking a pathological endothelium in vascularized tissue engineered constructs. However, a source of viable and stable ECs *in vitro* is a major challenging. One possible way to tackle this issue is to use embryonic stem cells (ESCs) or induced pluripotent cells (iPSCs). We have recently demonstrated the ability of hESCs to self-renew and to generate ECs [156, 157]. In addition to their self-renewal capacity, hESCs have the potential for large-scale differentiation into any adult cell types *in vitro*, and are thus an attractive alternative source of ECs. The emergence of ECs from differentiating hESCs could be monitored by an EC-specific genetic reporter, whereby the VE-Cad promoter drives expression of green fluorescent protein (hVPr-GFP). This novel approach for vascular monitoring in combination with the ability to grow cells on 3-D scaffold biomaterials will allow us to examine the contribution of the hVPr-GFP positive ECs to

the neovascularization process in tumors.

## 4. Vascularized tissue engineering constructs for nano-pathophysiology

Substantial efforts have already been made to understand angiogenic processes taking place in ischemic tissues and tumors. On the basis of the angiogenic process, different approaches have been attempted to introduce vascular networks in tissue engineered constructs (**Figure 1.**). Two strategies have been proposed to create vascularized tissue engineered constructs. The first is "Top down approach" based on the ECs and their ability to form vascular networks *in vivo*. The second strategy of "Bottom-up approach" deals with the vessels themselves *in vitro*.

### 4.1. Top-down approaches (*in vivo*)

Enhanced neovascularization *in vivo* aims to create vascular networks for following cell transplantation or to connect host vasculatures with prefabricated *in vitro* vascular networks in tissue engineered constructs. The newly formed vascular network could be expected to supply nutrients and oxygen for cells enough to survive and function *in vivo*. To build a functional vascular network for tissue engineered constructs, two approaches have been developed. One is to utilize controlled release systems of angiogenic factors for neovascularization. Arteriovenous (AV) loops are the other neovascularization approach to generate vascularized tissue engineered constructs *in vivo*. Angiogenic factors could enhance neovascularization based on their bioactivities, that stimulate ECs or EPCs to migrate towards the factor gradient and subsequently promote cell assembly, vessel formation, and maturation. The controlled release systems as described above have been used with or without scaffold biomaterials. To induce neovascularization in scaffold biomaterials, the physical parameters are critical. Specifically, pore size has been evaluated in a mouse ectopic implantation model using poly(vinyl alcohol) porous scaffold biomaterials combined with a controlled release system of FGF-2. A pore size above 250 μm was necessary for vascular ingrowth into the scaffold biomaterial [63]. This area of research remains critical with unexplored issues of microenvironment contribution to vascularization of tissue engineered constructs. Neovascularization *in vivo* prior to cell transplantation enables cells to survive and function even in ischemic sites [116]. For instance, Sakakibara et al. reported that neovascularization induced by the controlled release of FGF-2 enables

transplanted cardiomyocytes to improve the left ventricular function in rats with myocardial infarction [158]. Furthermore, using the same controlled release system, Gu et al. demonstrated that combination of gelatin microspheres incorporating FGF-2 and collagen scaffolds allows the xenogeneic islets-containing bioartificial pancreas devise to induce neovascularization even at the subcutaneous site of streptozotocin-induced diabetic rats, resulting in improved function of islet transplantation [158]. One disadvantage of this neovascularization approach is the lack of reproducibility and standardization in terms of the structure of the resulting vascular network.

By contrast, AV loops enables us to fabricate vascularized tissue engineered constructs with capillaries of artery and vein in a controlled fashion. The resulting AV roop can be utilized to connect with host vessels by suturing under a surgical microscope [159-164]. To obtain such a functional vascular network, a scaffold or chamber is placed within an AV loop in a site of rich vascularization *in vivo*, mainly muscle. After the formation of an artery-capillary-vein network by the surrounding tissue, the construct can be transferred to the reconstructive defect [165]. Mian et al reported that an isolated chamber is capable to generate new vascularized tissue even in the absence of added ECM [163]. Polykandriotis et al. performed the AV loops for neovascularization of bovine cancellous bone matrix to obtain vascularized hard-tissue constructs [166]. The application of the AV loop provides a promising tool for fabrication of vascularized tissue engineered constructs.

### 4.2. Bottom-up approaches (*in vitro*)

Unlike the “Top-down approaches”, the 3-D positioning of cells together with angiogenic cells *in vitro* could recreate physiological or pathological vasculatures in a controlled fashion in terms of structures and cell types. This reproducibility and standardization in fabricating vascularized tissue engineered constructs *in vitro* is a major advantage of “Bottom-up approaches”. The controlled assembly of different vascular cells, such as ECs and pericytes, could realize vascularized tissue engineered constructs with particular vascular structures seen in pathological tissues, and quantitatively recapitulate the *in vivo* dynamic behavior of nanoparticles in *in vitro* conditions. Unlike conventional animal models, the vascularized tissue engineered construct connected with host vasculatures will function as an alternative experimental platform with a higher reproducibility and quantitativity even in the *in vivo* conditions.

However, building a natural connection of the engineered vascular network with the existing host vasculature, a process called anastomose, is a major issue to be challenged. Although the difficulty in anastomosis requires numerous investigations both through *in vitro* and *in vivo* approaches, there still remain some substantial challenges to develop technologies for rebuilding vascular networks in tissue engineered constructs [91, 92].

Recently two different strategies have been proposed to rebuild vascular networks in tissue engineered constructs *in vitro*. The first strategy is to fabricate synthetically vascularized scaffold biomaterials. Decellularization of tissues and organs is the second strategy termed biologically vascularized scaffold biomaterials, that maintain intact 3-D geometry, vasculature, and ECM structure. Effective neovascularization in tissue engineered constructs is inherently linked to intelligent design and sophisticated fabrication for scaffold biomaterials. Smart 3-D design and high resolution manufacturing technologies are used to define cell alignment and angiogenesis. Several technologies, such as sacrificial templates [167, 168], microfluidics [169-171], organ printing [172-174], and 3-D scaffold biomaterials [175, 176], have been developed.

Chen et al. developed a technique that can print rigid 3-D filament networks of carbohydrate glass, and used them as a cytocompatible sacrificial template in tissue engineered constructs containing living cells, such as primary rat hepatocytes [167]. Yoshida et al. used silica tubes as a sacrificial template to reconstruct a bilayered vessel structure consisting of a monolayer of ECs and surrounding smooth muscle cells *in vitro* [168]. Using the sacrificial templates allows independent control of network geometry, endothelialization, and extravascular tissue, and is compatible with a wide variety of cell types, and synthetic and natural scaffold biomaterials.

On the basis of a microfluidic technique, Zheng et al. fabricated living microvascular networks in 3-D tissue engineered constructs and demonstrated their biofunctionality *in vitro* [169]. The endothelialized microfluidic vessels within a native collagen matrix recapitulate endothelial characters in terms of the morphology, mass transfer processes, long-term stability of the endothelium, angiogenic activities, and nonthrombotic nature. Another approach is to combine vascularized native tissues with a microfluidic perfusable bioreactor [170]. In this approach, triple-layer cardiac cell sheets produced from co-culture with ECs were placed on resected tissue with a connectable artery and vein as a vascular bed and were maintained in a media perfused

bioreactor. As a result, ECs connect to capillaries in the vascular bed and form tubular lumens, creating *in vitro* cardiac tissues with vascular networks to be transplanted with blood vessel anastomoses. Beside the resected tissue, Shimizu et al. also used a perfusion bioreactor having collagen-based microchannels [171]. When triple-layer cardiac cell sheets are incubated within this bioreactor, ECs in the cell sheets migrate to vascularize in the collagen gel, and finally connect with the microchannels.

Bioprinter technique could use computer-aided design (CAD) data sets to realize freeform 3-D printing [172-174]. Tissue engineered constructs, built by those layer-by-layer manufacturing processes, can match the *in vivo* characteristics of porosity, mechanical strength and vascularization, with the correct spatial positioning and morphology. Xu et al. fabricated functional 3-D tissue engineered contractile cardiac hybrids by arranging alternate layers of biocompatible alginate hydrogels and mammalian cardiac cells according to pre-designed 3-D patterns [174].

Fischbach et al. engineered 3-D human tumor models using carcinoma cells in polymeric scaffold biomaterials that recreated microenvironmental characteristics representative of tumors *in vivo* [175]. Interestingly, the angiogenic characteristics of tumor cells were dramatically altered upon 3-D culture within this system, and corresponded much more closely to tumors formed *in vivo*. We have recently succeeded in generating stable cultures of vascularized cells in a honeycomb alginate scaffold biomaterials self-organized into capillary-like structures with an average channel diameter of 300 μm (**Figure 3**) [176]. The porous 3-D alginate depots containing cells, in a serum-free condition, were further exposed to laminar flow to recapitulate the vasculature *in vivo*. The scaffold biomaterial remained intact with the cells remaining adhered to it and aligned in the direction of flow, demonstrating its suitability for establishing durable angiogenic modules that may ultimately enhance organ revascularization or model tumor neoangiogenesis.

Despite the recent progress in the development of the synthetic vascularized scaffold biomaterials, mimicking and rebuilding natural vasculatures *in vitro* is still challenging. Therefore, the reuse of biological vascular structures by decellularizing tissues and organs is an alternative approach to tackle this problem. This decellularized tissue and organ are designated as “biologically vascularized scaffold biomaterials” that maintain intact 3-D geometry, vasculature, and ECM structure. Several organs, such as heart [177], lung [178], liver [179], kidney [180], and small intestinal submucosa [181],

have been proved to hold promising performances after decellularization and subsequent repopulation with functional cells including ECs. For example, the decellularization and the repopulation with neonatal cardiac or aortic ECs of rat hearts as well as the culture under simulated physiological conditions led to the formation of contractile myocardium [177]. Indeed, this biologically vascularized scaffold biomaterial provides some advantages including physiological or pathological vascular perfusion, biocompatible ECM structures, and relevant geometries for cell positioning. However, a major limitation of this approach is the lack of the standardization and reproducibility in 3-D structures due to the animal-derived material. Therefore, combination of a defined 3-D geometry based on natural tissue and organ structures and decellularized ECM components will open up a new generation of synthetic vascularized scaffold biomaterials for tissue regeneration as well as nano-pathophysiology.

## 5. Conclusions

Scaffold biomaterials are used in a variety of tissue engineering and drug delivery purposes to promote angiogenesis, and hence influence the regeneration of tissues and organs in the body. Cellular interactions with their environment are of central importance to many biological processes and are important in the context of tissue engineering and regenerative medicine. Several approaches such as sacrificial templates, microfluidics, organ printing, and 3-D scaffold biomaterials, will enable one to grow, or engineer, long-lasting tissues and organs using 3-D depots, which may serve to guide new tissue formation therapeutically in the body or *in vitro* as relevant models to study disease nano-pathophysiology. Insights gained from 3-D platforms may advance our understanding of cancer and contribute to the development of antiangiogenic therapies.

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**Figure legends**

**Figure 1.** Schematic drawing of two strategies to create vascularized tissue engineered constructs. The first is "top down approach" based on the ECs and their ability to form vascular networks *in vivo*. The second strategy, "bottom-up approach", deals with the vessels themselves *in vitro*.

**Figure 2.** SDF-1 treatment accelerates healing of acute surgical wounds. (A) Percent cumulative release of SDF-1 *in vivo*. (B) Percent wound healing over time shown for SDF-1 protein (○) treated wounds and nontreated sham (△) or saline (▲) in a porcine model of acute dermal wound closure.

**Figure 3.** A representative image of alginate scaffolds and GFP-labeled HUVECs in the scaffolds obtained by scanning electron microscope and confocal microscope. (A–C). Alginate scaffolds with aligned-pore structure. Average pore diameter is 352 mm and porosity is 93.7%. (A) Horizontal images and (B) vertical images of scaffolds. Magnification of each image is 10x. In addition, Z-stack images were taken after 1 day of cell seeding at a magnification of 10x and reconstructed through a projection mode using LSM510 software.

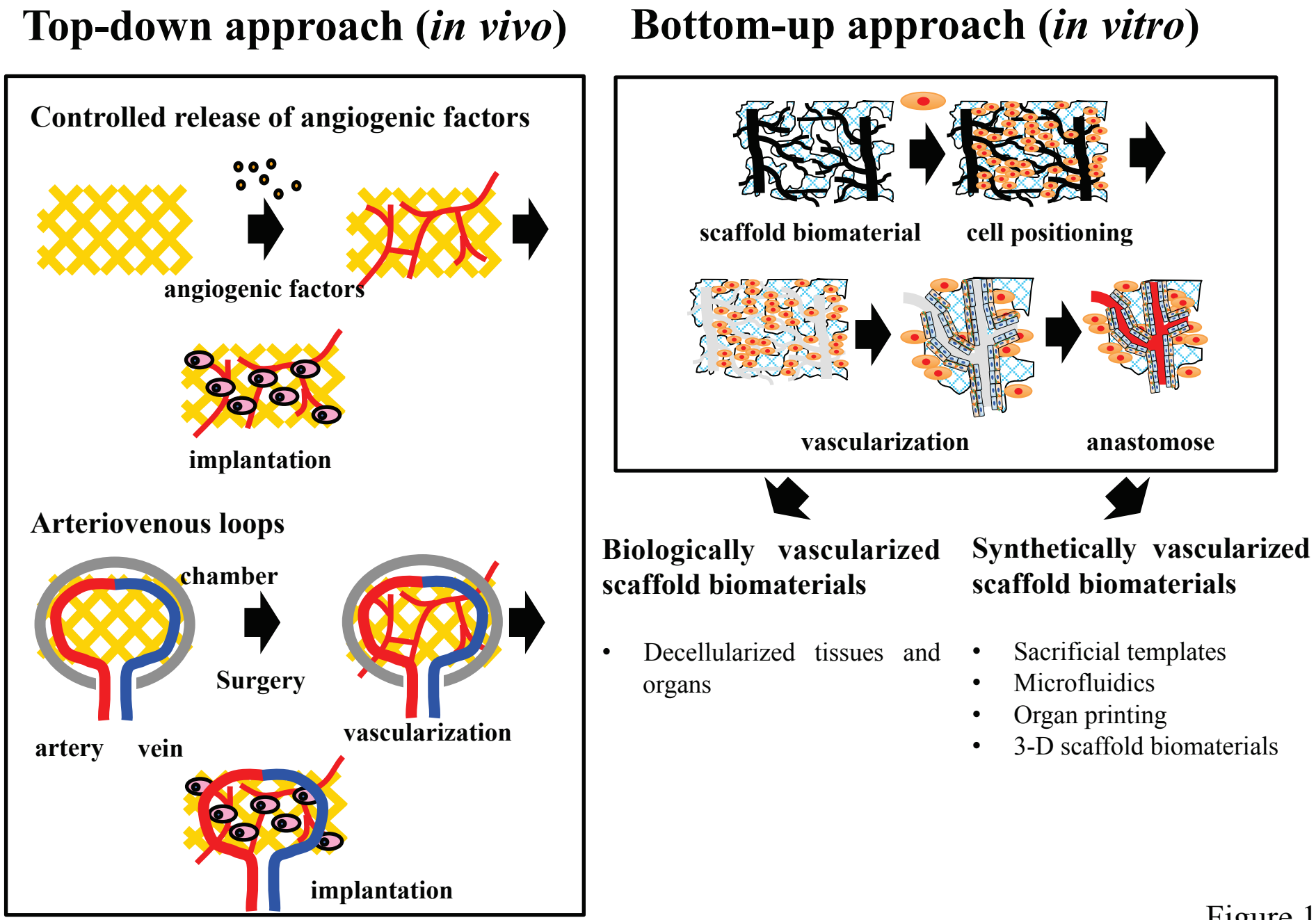
Top-down approach (*in vivo*)
Bottom-up approach (*in vitro*)
Controlled release of angiogenic factors
angiogenic factors
implantation
Arteriovenous loops
chamber
Surgery
artery
vein
vascularization
implantation
scaffold biomaterial
cell positioning
vascularization
anastomose
Biologically vascularized scaffold biomaterials
• Decellularized tissues and organs
Synthetically vascularized scaffold biomaterials
• Sacrificial templates
• Microfluidics
• Organ printing
• 3-D scaffold biomaterials

Figure 1

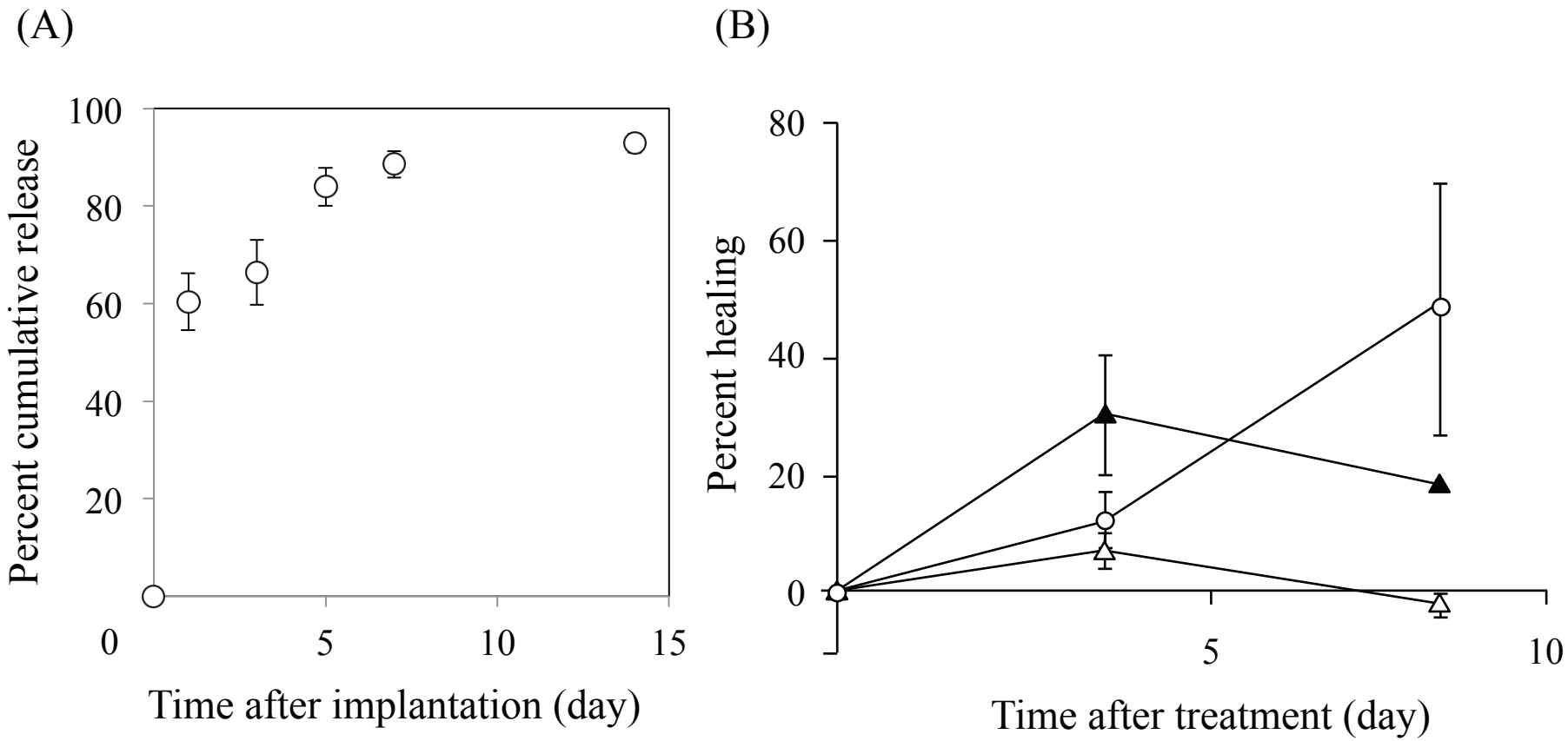
(A)
Percent cumulative release
100
80
60
40
20
0
0
5
10
15
Time after implantation (day)
(B)
Percent healing
80
60
40
20
0
5
10
Time after treatment (day)

Figure 2

(A) (B) (C)

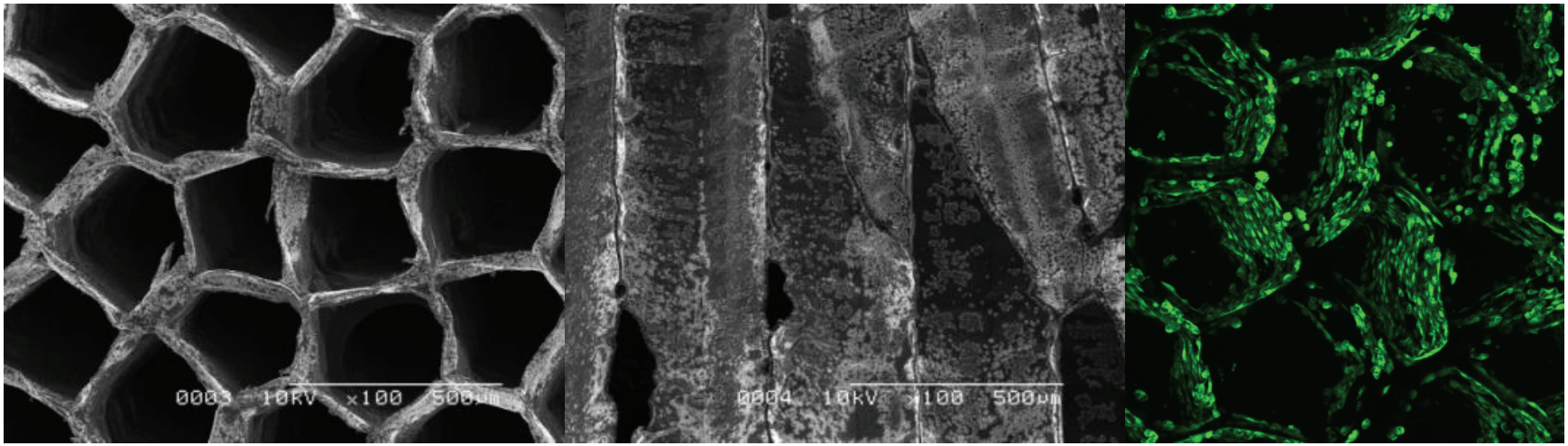


Figure 3

Table 1. ECM proteins with a tumor-specific modification

| ECM proteins | Tumor-specific modifications | Distribution or function | References |
|---|---|---|---|
| tenascin-C | full-length unspliced isoforms | pancreatic and prostate cancer | [37, 42] |
| | isoform containing domain C | gliomas | [35] |
| | isoform containing domain A and D | breast and ovarian carcinoma | [34, 38] |
| fibronectin | FN-extradomain (ED)A | conversion fibroblasts to myofibroblasts | [45] |
| | FN-EDB | seen in neovascular structures in many different tumor types | [36, 40] |
| collagen and elastin | crosslinked with lysyl oxidase (LOX) | elevated ECM stiffness | [43] |

Table 2. Controlled release systems for angiogenic factors

| Origin | Materials | Forms |
|---|---|---|
| Natural polymer | fibrin | hydrogel, sponge |
| | collagen | hydrogel, microsphere, sponge |
| | gelatin | hydrogel, microsphere, sponge |
| | alginate | hydrogel, microsphere, sponge |
| | hyaluronic acid | hydrogel |
| | chitosan | microsphere |
| Synthetic polymer | lactide–glycolide copolymer (PLGA) | microsphere, sponge |
| | polycaprolactone (PCL) | sponge, nanofiber |
| | poly(ethylene glycol) | hydrogel |