Review paper: Critical Issues in Tissue Engineering: Biomaterials, Cell Sources, Angiogenesis, and Drug Delivery Systems

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ABSTRACT: Tissue engineering is a newly emerging biomedical technology, which aids and increases the repair and regeneration of deficient and injured tissues. It employs the principles from the fields of materials science, cell biology, transplantation, and engineering in an effort to treat or replace damaged tissues. Tissue engineering and development of complex tissues or organs, such as heart, muscle, kidney, liver, and lung, are still a distant milestone in twenty-first century. Generally, there are four main challenges in tissue engineering which need optimization. These include biomaterials, cell sources, vascularization of engineered tissues, and design of drug delivery systems. Biomaterials and cell sources should be specific for the engineering of each tissue or organ. On the other hand, angiogenesis is required not only for the treatment of a variety of ischemic conditions, but it is also a critical component of virtually all tissue-engineering strategies. Therefore, controlling the dose, location, and duration of releasing angiogenic factors via polymeric delivery systems, in order to ultimately better mimic the stem cell niche through scaffolds, will dictate the utility of a variety of biomaterials in tissue regeneration. This review focuses on the use of polymeric vehicles that are made of synthetic and/or natural biomaterials as scaffolds for three-dimensional cell cultures and for locally delivering the inductive growth factors in various formats to provide a method of

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Figures 1 and 2 appear in color online: http://jba.sagepub.com

0885-3282/11/04 0383–35 $10.00/0 DOI: 10.1177/0885328211408946

controlled, localized delivery for the desired time frame and for vascularized tissue-engineering therapies.

KEY WORDS: tissue engineering, scaffold, biomaterials, cell sources, angiogenesis, drug delivery systems.

INTRODUCTION

There are serious limitations in using either autologous or allogeneic grafts in traditional transplantation surgeries. These include lack of appropriate donor tissues in autologous transplantation, and risk of disease transmission and extended immune-rejection in allogeneic transplantation. However, tissue engineering offers to circumvent these problems by replacing and restoring various tissues and organs through the delivery of stem cells (SCs) and bioactive molecules onto specific biomaterials in a three dimensional (3D) structure [1].

The cell microenvironment is known to play a remarkable role in determining progenitor cell fate and function. The exact coordination of spatial and temporal cues from the microenvironment is highly essential for SCs to constitute complex functional tissues. Advanced and high throughput assays, such as extracellular matrix (ECM) microarrays and other technologies have unveiled specific cell interactions with ECM components and polymers that can influence SC signaling and response [2–6]. As long as biomaterial research moves forward, new materials and innovations in their manipulation and usage will continue to emerge. By utilizing high throughput arrays to recognize the function of biomaterials, the evaluation of these molecules for ultimate usage can take place in short periods of time [5,7,8]. So, major advances in both the understanding of the local cues required for lineage commitment and the discovery of new biomaterials necessary to support SCs and drug delivery can be advantageous. The promise of cell therapy lies in the repair of damaged tissues and organs *in vivo* and also the production of appropriate tissues *in vitro* for successful transplantation. In the recent years, in order to mimic the SC niche, a variety of biomaterials have been combined with SC cultures, to prepare a suitable microenvironment for their growth and differentiation [2,9–11].

The reconstruction and direct replacement of diseased cells and tissues are becoming a clinical possibility mainly because of correlated advances in the modification of biomaterials and comprehension of SCs behavior [12–14]. Creation of cell-compatible biomaterial microarrays has allowed rapid, microscale testing of biomaterial interactions with cells [2,6]. Several review articles have discussed how specific types of

biomaterials are used as substances to mimic the physico-chemical microenvironments of cells and tissues [14–16]. A number of studies have specifically examined materials for the development of bone [17,18], cartilage [19], skin [20], and blood vessels [21]. Others have reported studies on various pre-differentiated SC populations and their combination together using biomaterials to form hybrid constructs that closely mimic native tissues [22–24].

However, the field of tissue engineering is restricted by the necessity for angiogenesis in large tissues and organs for nutrient, waste, and oxygen transport. Strategies to induce new blood vessel networks will be essential in almost all engineered tissues [25,26]. There have been many reports on the design and improvement of scaffold materials to help local angiogenesis directly *in vivo* and promote gradual penetration of host vessels into the scaffolds [27,28]. Even for *ex vivo* pre-vascularized scaffolds, successful joining of the graft with the host tissues largely relies on vessel and tissue integration. One major theme guiding this approach is delivery of angiogenic factors from implanted scaffolds, which will be discussed in this review. While systemic injection of angiogenic molecules such as vascular endothelial growth factor (VEGF) is counterpart with negative side effects in non-target tissues (hyper-permeable vessels, hypotension, motivation of tumor growth, and unhindered neovascularization), prolonged delivery of angiogenic factors from scaffold materials can be localized to a microenvironment to minimize the negative side effects in non-target regions. In addition, natural processes of secretion and sequestration of angiogenic factors in ECM beds can be imitated in this system by adjusting their release kinetics from the scaffolds [29–31].

In spite of the good progress in bioengineering of tissues composed of thin layers of cells, such as skin, a main dispute for future tissue engineering is the creation of larger organs with more complex structures, such as kidney, heart, and liver. Tissues with a huge mass of cells require a vascular network of capillaries, arteries, and veins for the transport of nutrients and oxygen to each cell. Development of effective methods for angiogenesis of these tissues is critical for obtaining a successful outcome [32]. In this review, we will strive to describe major advances in the field of tissue engineering, including biomaterials, cell sources, engineering of thick tissues or organs, drug delivery systems (DDSs, Figure 1), and novel findings to better mimic the native tissues for preclinical and clinical tissue engineering.

Although classical two dimensional (2D) cell cultures are widely used and have provided many advances in cell biology, but due to the fact that cells (including SCs) reside, proliferate, migrate, and differentiate inside

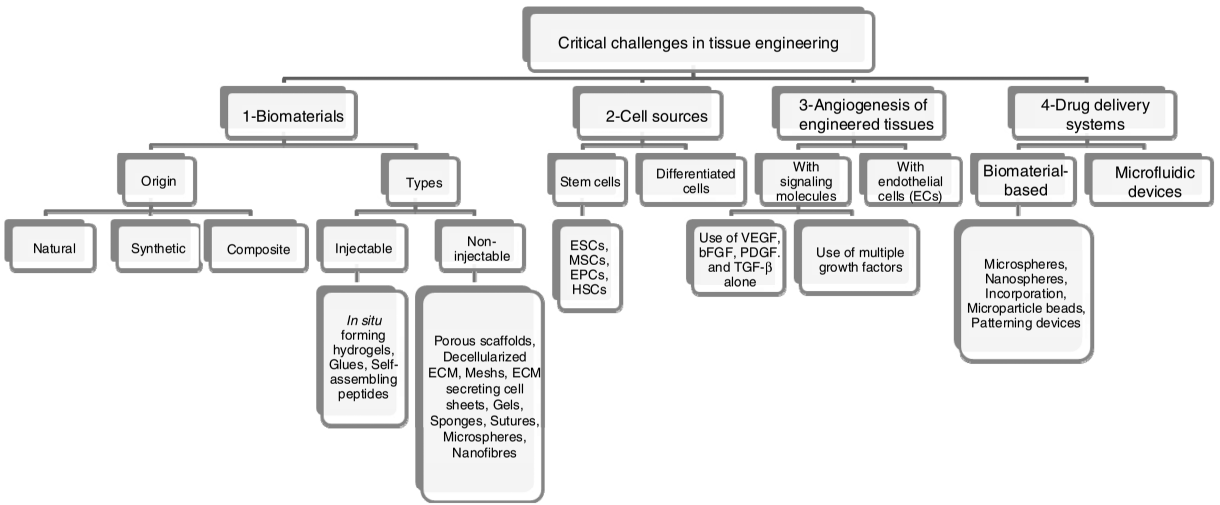

Figure 1. Overview of critical issues in tissue engineering.
Notes: ESC, embryonic stem cell; MSC, mesenchymal stem cell; EPC, endothelial progenitor cell; HSC, hematopoietic stem cell; VEGF, vascular endothelial growth factor; bFGF, basic fibroblast growth factor; PDGF, platelet-derived growth factor; and TGF-β, transforming growth factor-β.

the body within complicated 3D microenvironments, most of the current research in biomaterial-directed SCs manipulation is focused on such 3D environments [11]. This review will primarily focus on the concept of 3D culture, biomaterials and their modifications, and later on we will discuss other important issues in the engineering of thick tissues.

BIOMATERIALS

The term 'biomaterials' has many definitions; one traditional meaning indicates that a biomaterial is a non-living substance used in a medical device, like a joint prosthesis. However, the technology of biomaterials has developed gradually, and the expanded definition includes substances that are designed to control the biological environment of cells and tissues. More than being simply compatible with the host and serving a structural role, biomaterials can now direct cells through microenvironmental cues [33].

Biomaterial-based 3D systems have been the most influential tools in rendering a scaffold to cells, both in culture or inside the body. These 3D structures present an ideal substrate for cell–cell and cell–material communications, and their properties can be modified to induce differentiation of cells into specific lineages [34]. Scaffolds used for tissue engineering perform many functions and their role during tissue formation is dependent on the specific characteristics of the selected biomaterials [35]. It has been proven that 3D scaffolds enhance osteogenic [36], hematopoietic [37], neural [38,39], and chondrogenic [40] differentiation. Thus, in addition to acting as delivery vehicles for biomolecules during tissue development [9], biomaterials promote cell attachment, proliferation, organization, and differentiation [41].

Properties of biocompatible scaffolds, synthetic or natural, can be considered from different aspects including optimal nutrient and waste transport, delivery of bioactive molecules, material degradation rate, cell-recognizable surface chemistries, mechanical unity, and the ability to promote signal transduction pathways. The considerable success of tissue organization and development highly depends on these properties, because they can eventually dictate cell adherence, nutrient/waste transport, cell differentiation, cell viability, and matrix synthesis and organization. Most of the materials in scaffolds can be chemically or physically modified to control all these important parameters, and a variety of synthetic and natural materials have been used for investigating SCs behavior by specifically manipulating these properties. Several articles have reviewed the application of scaffolds in tissue engineering in general [42,43]. In an optimal form, a biomaterial must

degrade without toxicity and with a controlled degradation rate. Contrary to a constantly implanted structural prosthesis, scaffold biomaterials should remain long enough to conduct joining of recruited or applied cells, but not persist so long as to obstruct the final cell–cell physiological coupling necessary for tissue engineering.

An important property of biomaterials is the degradation rate. A rapid degradation can jeopardize the mechanical unity of biomaterials. Therefore, it is desirable to control degradation and stiffness independently. Diverse approaches can regulate biomaterials degradation. The molecular weight and copolymerization ratio can be easily controlled to optimize the degradation rate [44–46]. Kong et al. showed that modifying alginates with various cross-linking strategies could keep stiffness but increase degradation, improving bone formation by bone marrow-derived mesenchymal stem cells [47]. Thus, degradation of polymers can potentially be adjusted for the regenerative strategy as long as transiently supporting mechanical integrity exists.

Another important feature of scaffolds is porosity. The pore size (both length and cross-sectional area), pore numbers, and pore connectivity of scaffolds are key factors in determining their function. Size of pores on a length scale of micrometers to millimeters strongly affects trafficking of cells; extremely large pores could spoil vascularization, since endothelial cells (ECs) are not capable of bridging pores larger than a cell diameter [48]. In contrast, pores smaller than 100 nm will influence diffusion of nutrients, waste, and oxygen. Poor diffusion of factors and nutrients may result in the failure of implant and reduced survival of implanted cells. Porosity needs to be balanced with the integrity of the materials, their mechanical properties and cellular effects [49–51]. Some hydrogels, like those formed by self-assembling peptides, have very small pore sizes that encourage endothelial adhesion and capillary formation, but still permit rapid cell migration because of the hydrogels flexibility [52].

There are natural, synthetic, and composite materials that can be injectable or non-injectable (Figure 1). Injectable polymers are important biomaterials, because of their clinical applicability without surgery, as DDSs for tissue engineering [28,53–56]. Several synthetic and natural biodegradable polymers, including polyesters, poly(amino acids), polysaccharides, and proteins have been studied well [53,57,58]. In addition, chemical and biological modifications of materials can result in better mimicking the SCs niche and create specific microenvironments to control cell responses [11]. Different types of biomaterials and their modifications are discussed, in more details, in the following sections.

Natural biomaterials

Natural biomaterials used for scaffolds include components found in the ECM such as collagen, fibrinogen, hyaluronic acid, glycosaminoglycans (GAGs), and hydroxyapatite (HA), and therefore have the benefit of being bioactive, biocompatible, and having mechanical properties that are common with native tissues [57]. Furthermore, other natural materials including those derived from plants, insects, or animals (e.g., cellulose, chitosan, silk fibroin, etc.) can provide favorable microenvironments for the culture of SCs [59,60]. Drawbacks of using natural materials rather than synthetic materials include restricted control over their physico-chemical properties, inability to moderate their degradation rates, challenges in sterilization and purification techniques, and also pathogen/viral issues when extracting from different sources [61].

There are many natural biomaterials used as 3D scaffolds for tissue engineering. Several natural materials, e.g., chitosan, Matrigel, hyaluronic acid, and fibrin, which have become commercially available, are well characterized, and have reproducible, controlled properties. Chitosan, as an ideal scaffold, has been widely used in the tissue engineering of skin, bone, cartilage, liver, nerve, blood vessels, and heart in the past 25 years [55]. Chitosan has been approved by the US Food and Drug Administration and is used in drug delivery and tissue engineering. Derivatives of chitosan including porous structures, chitosan-based nanofibrous structures, and injectable chitosan hydrogels have different applications in tissue engineering. Chitosan hydrogel responds to a variety of external stimuli such as pH, light, and temperature. The temperature-responsive chitosan in combination with glycerol phosphate (GP) as injectable hydrogel is highly attractive and has great applications, because bioactive factors (such as growth factors, genes, and supportive cells relevant to the repair and regeneration of the tissues) can be easily incorporated into the polymer solution [56]. Then, once exposed to body temperature (37°C), the polymer solution can polymerize rapidly *in situ* within a short time, trapping these factors within the injected area. This ability for *in situ* polymerization makes chitosan-GP a clinically useful scaffold [55,56,62].

Commercially available Matrigel™ is a complex protein mixture including laminin, collagen IV, and heparan sulfate proteoglycans [63]. Laschke et al. demonstrated that the incorporation of Matrigel into poly(lactide-*co*-glycolide) (PLGA) scaffolds can accelerate adequate vascularization of tissue engineering constructs [64]. Other researchers showed that when human endothelial progenitor cells (hEPCs) were

incorporated into Matrigel, and implanted subcutaneously into immunodeficient mice, vascular networks were created *in vivo* [65].

Hyaluronic acid is another attractive natural biomaterial, which is involved in cell signaling and behavior. Although hyaluronic acid is present in tissues as a gel-like substance, it can be chemically modified for effective processing into fibers, membranes, or microspheres. An altered type of hyaluronic acid is commercially available as Hyaff® [66]. Hyaff®-based scaffolds are biodegradable and combine the advantages of being a natural material with allowing the cells to replace the scaffold with their own ECM. Recently, Gerecht et al. [67] reported the use of hyaluronic acid hydrogels for maintaining the pluripotency and undifferentiated state of human embryonic stem cells (hESCs) and showed that addition of soluble growth factors to these hydrogels could successfully trigger lineage specific differentiation of ES cells.

Fibrin is another class of natural materials that can be applied to make 3D scaffold materials [68]. This scaffold, in conjunction with various growth factors, showed remarkable increase in neuron production and neuronal viability [39] and also in clinical cartilage engineering [69]. Fibrin glue is a synthetic substance used to create a fibrin clot. It is made of fibrinogen and thrombin. Thrombin acts as an enzyme that converts fibrinogen into fibrin in 10–60 s, so that fibrin can be used as injectable *in situ* forming gel. Fibrin scaffolds have been used for injection in a preclinical ischemic heart study [70].

Synthetic biomaterials

Poly(glycolic acid) (PGA), poly(lactic acid) (PLA), and the copolymer PLGA have been extensively used as synthetic 3D scaffold biomaterials for assessing cell behavior [71–73]. Necessary criteria such as biocompatibility, processability, and controlled degradation are fulfilled with these polyesters [74]. These biomaterials degrade hydrolytically via mass erosion and the glycolic/lactic acid byproducts are physiologically removed through metabolic pathways. The molecular weight, copolymerization ratio, and polydispersity of the polymers can be easily tunable to control the degradation rate. These properties have made synthetic materials highly attractive for tissue engineering. In addition, standard processing methods (e.g., salt leaching, sintering, porogen melting, and nanofiber electrospinning) have been well established to prepare a wide variety of 3D scaffolds using synthetic biomaterials [75,76].

Synthetic materials provide the versatility of creating 3D microenvironments with adjustable features including mechanical properties,

degradation rates, and porosity. However, in spite of these benefits, they have poor inherent bioactivity (e.g., polyethylene glycol, PEG), acidic byproducts (e.g., PGA, PLA, or PLGA), etc. Therefore, it is critical to modify synthetic materials with biological or chemical compounds to obtain suitable cellular responses. The physical properties of these polymers can also be easily controlled by changing the ratio of lactide:glycolide, molecular weight, and their crystallinity [74,77].

Use of composite scaffolds is another approach to better mimic physiological niche and improve the process of tissue engineering [46,78]. Natural or synthetic hydrogels closely resemble the consistency of soft, native tissues, making them attractive scaffold materials for soft tissue engineering. On the other hand, sometimes composite materials with higher mechanical strength are required to closely mimic the tissue mechanical properties and optimize degradation rate. For example, hydrogel-like materials can be modified to have increased elasticity, making them more suitable for applications in connective tissue engineering. Collagen gels can also be adjusted to have a higher elasticity by adding HA. Calcium HA is the main component of teeth and bones in vertebrates, thereby imitating the composition of bone, which is mainly composed of collagen fibers and phosphate minerals [79].

Zawaneh et al. have reported the design of an injectable synthetic and biodegradable polymeric biomaterial comprising polyethylene glycol and a polycarbonate of dihydroxyacetone (MPEG–PDHA) that is easily extruded through narrow-gage needles, biodegrades into inert products and is well tolerated by soft tissues. This type of polymer holds significant promise for clinical applications in patients going through surgical procedures ranging from cosmetic surgery to cancer resection and tissue engineering [53].

Modification of biomaterials

Physical, chemical, and biological modifications of biomaterials can directly influence SCs behavior by altering scaffold properties, surface interactions, scaffold degradation rate, microenvironmental architecture, and manipulating the signal transduction pathways in SCs. Biomaterials can be designed to precisely control their degradation kinetics, present specific ligand-based signals, and/or control the release of biomolecules in response to the microenvironment [77]. These can influence cell–matrix interactions and may lead to altered gene expression and lineage specificity. Many studies have demonstrated how modified biomaterials and scaffold surface properties introduce specific biological responses in SCs [34,77,80–83]. Therefore, the goal of

biomaterial-directed SC culture is to mimic physical and biochemical properties of the physiological SC niche [77,84].

When anchorage-dependent cells are cultured on various biomaterials, ECM proteins including collagen, fibronectin, hyaluronic acid, GAGs, fibrin, and gelatin are generally used to cover surfaces of various biomaterials to enhance their interactions with cells. Cellular interactions with ECM proteins are very complicated because these proteins contain multiple cell- and growth factor-binding domains. To avoid these problems, short peptides only several amino acids in length, have been derived from ECM proteins as the most primitive subunits needed for normal cell attachment and proliferation. Employment of synthetic peptides in cell cultures and engineered tissues can overcome the need for bulk production and purification of ECM proteins from tissue extracts. Holtorf et al. evaluated titanium fiber mesh scaffolds coated with Arg-Gly-Asp (RGD) sequence, a cell adhesive, integrin-binding peptide found in fibronectin and laminin, and showed that mesenchymal stem cells (MSCs) could attach more strongly to these RGD-coated scaffolds [85]. Cell–matrix interactions were enhanced in RGD functionalized hydrogels, resulting in increased MSCs viability. Nuttelman et al. [86] showed that the viability of the encapsulated hMSCs augmented from 15% to 75% when RGD was added into PEG-diacrylate hydrogels. Also, hESCs cultured on this completely synthetic ECM substitute were shown to be morphologically similar to hESCs cultured on an embryonic fibroblast feeder layer and generated markers typical of undifferentiated ES cells [87]. Many studies have reported that RGD sequence helps in the attachment of various cell types including fibroblasts [88], smooth muscle cells (SMCs) [89], preosteoblasts [90], preadipocytes [91], and MSCs [92]. Specially, ECs have been favorably cultured on RGD-containing polymers including hyaluronic acid hydrogels [93], derivatives of isopropylacrylamides [94], and PEG hydrogels [95].

Tyr-Iso-Gly-Ser-Arg (YIGSR), a laminin-derived short peptide sequence, combined with polyurethanes, could selectively enhance ECs adhesion and proliferation but decreased platelet adhesion [96]. Glass [97] and PEG hydrogels [98] coated with YIGSR also increased EC attachment and migration. Arg-Glu-Asp-Val (REDV) sequence originated from fibronectin, binds to integrins found on ECs, supporting specific adhesion of these cells [99]. Recombinant ECM proteins with REDV sequence were constructed and applied as vascular graft biomaterials to encourage trapping of ECs [100]. These studies have shown that short peptides can replace massive ECM proteins as coating materials and enhance cellular adhesion and functions on biomaterials. Furthermore, these short peptides with adhesive properties could be

micropatterned into specific regions to control spatial arrangement of ECs, and other tissue-specific cell types [95,101,102].

SCs differentiation can be directly mediated by exposure to proper biological or chemical signals in their microenvironment. It is well established that specific growth factors, hormones, and cytokines can increase proliferation and lineage-specific differentiation of SCs. For example, fibroblast growth factor-2 (FGF-2) has been shown to increase self-renewal of MSCs and maintain their multi-lineage differentiation capability [103]. Moreover, bone morphogenetic proteins (BMPs) have been shown to play a significant role in the regeneration of specific cell types including skeletal tissues, especially bone [104].

Growth factors, hormones, and chemicals have classically been directly added into the culture medium. On the other hand, these biomolecules can be directly incorporated within the scaffold structure or encapsulated into the scaffold biomaterials in a variety of ways during the scaffold manufacturing process [105,106]. A highly used method for the delivery of growth/chemotactic factors in tissue engineering is simple physical adsorption of biomolecules on biomaterials or scaffolds surface [35]. The chemotactic factors such as stromal cell-derived factor 1 (SDF1) incorporated in PLGA scaffolds can attract MSCs to the site of implant. Enhanced homing of autologous MSCs improved the tissue responses to biomaterial implants through modifying/bypassing inflammatory responses and jumpstarting SCs participation in healing at the implant interface [71].

Covalent immobilization of soluble signaling proteins on biomaterials enables prolonged signaling by intervening with their endocytosis. By immobilization technique, bioactivity of the proteins can be retained on biomaterials. An important angiogenic factor, VEGF, was covalently incorporated into collagen gels. When this scaffold was implanted on chicken chorioallantoic membrane, the results indicated improved capillary formation and tissue ingrowth [107,108]. It was also shown that genetically modified VEGF, in which N-terminal cysteine was conjugated to fibrin matrix via thiol-directed bifunctional cross-linking reagent, could retain bioactivity, and improved angiogenic performance [109]. Another powerful angiogenic growth factor is basic fibroblast growth factor (bFGF) that has been immobilized into PEG hydrogels with concentration gradient. The resulting materials could direct cell alignment and migration of SMCs [110]. Cell–cell interaction proteins including ephrin-B2 [111] and ephrin-A1 [112], which were incorporated into fibrin matrices and PEG hydrogels, respectively, showed significant roles in the induction of angiogenesis. Therefore, the use of special signaling proteins presents opportunities in the design of angiogenic biomaterials.

CELL SOURCES

Various sources of SCs and differentiated cells are used for tissue engineering as for different target tissues (Figure 1). SCs represent an important building block for regenerative medicine and tissue engineering. These cells are broadly classified into embryonic stem cells (ESCs) and adult SCs. ESCs have a higher regenerative capacity than adult SCs and can be manipulated to differentiate into other cell types [113,114]. Use of hESCs is restricted by ethical problems and the potential to form teratomas [115,116] and the request for autologous grafts has made adult progenitor cells more appropriate for tissue engineering [117]. It has also been shown that hESCs can acquire chromosomal abnormalities [118,119] and therefore are more potent to tumorogenesis. Adult SCs are multipotent cells with high plasticity. They have been isolated from many tissues including bone marrow, blood, brain, liver, muscle, and skin. [120–122] Although adult SCs have a lower plasticity compared with ESCs, they have been demonstrated to differentiate into a variety of cell types and have been used for treatment of various diseases including ischemia, neural degeneration, and diabetes in animal models [123–127].

Here, we do not aim to present an inclusive review to explain the characterization of SCs since several outstanding previous reviews are available [117,128–132]. We further intend to emphasize how SC technology can be made applicable and beneficial in angiogenesis for engineering thick tissues and whole organs.

One emerging issue in the area of SC therapy is homing and engraftment of injected or resident cells to the site of damaged tissue [133]. We investigated the expression of chemokine receptors (such as CXCR4), which are involved in homing, on the surface of human MSCs. [134] For the construction of vascularized tissue, it might be helpful to implant a chemokine (SDF1)-containing scaffold in the site of injury in order to recruit SCs to the site [71,135]. This method may prevent formation of a necrotic core, which results from cell death in the center of scaffolds due to lack of nutrients and oxygen. While the chemokine is released from the scaffold, cells gradually penetrate into the scaffold and full tissue, along with angiogenesis, is formed.

Application of SC technology in engineering of thick tissues

The ECs, which cover the inside of arteries, veins, and capillaries, are one of the major players of angiogenesis process in physiological and

pathological conditions. ECs are involved in thrombo-withstanding effects, regulation of leukocyte interactions, adjustment of blood flow and vessel tone, and selective permeability to various materials. Vasculogenesis is a process that results from the differentiation of endothelial progenitor cells (EPCs) to form new blood vessels. On the other hand, angiogenesis applies to the development of new capillary blood vessels by a process of budding from pre-existing vessels [136,137]. Therefore, whereas vasculogenesis is restricted to embryogenesis, angiogenesis may develop from ECs and EPCs, which take part in the formation of new vessels in normal and pathological situations after birth as well as embryogenesis [137,138].

Since the discovery of EPCs, there has been an interest in their use in tissue engineering [139]. The relative ease of extracting these cells and their capability to be expanded in culture for up to 1000 doublings, while holding their capacity to differentiate, has resulted in their extended use in this field [140]. EPCs can be isolated from bone marrow or peripheral blood. EPCs have the capacity to differentiate into ECs *in vitro* and also integrate into positions of neovascularization *in vivo*. The differentiation of EPCs to ECs was revealed for the first time in vasculogenesis event [139]. Although EPCs exhibit less plasticity and less capacity for self-renewal than their parent SCs, they still have the capacity to differentiate into several cell types. EPCs differentiation, *in vitro*, depends on culture conditions. VEGF and fibronectin can induce the differentiation of EPCs into ECs [141].

Outgrowth endothelial cells (OECs) are EPCs variants that can produce high cell numbers typically required in vascular tissue engineering applications. OECs collected from human peripheral blood can be proliferated more than 20 passages, proposing a very good supply of autologous ECs [142,143]. Blood- or bone marrow-derived EPCs (OEC type) have been examined for the formation of blood vessels [144,145]. The mechanisms by which these different EPC variants give rise to new vessel formation may differ. OECs are classified into early and late outgrowth EPCs isolated from the mononuclear fraction [146]. These show different proliferation capacities, differential secretion of angiogenic cytokines, and have different morphologies [146]. Hence, cells with different functions are present within the mononuclear fraction. Early outgrowth EPCs can enhance neovascularization, mainly by the secretion of angiogenic cytokines (interleukin-8 and VEGF), while on the contrary, late outgrowth EPCs, which have a high proliferative capacity, serve as a source of ECs. Therefore, early outgrowth EPCs may function as sources of angiogenic cytokines with minimal incorporation into the vasculature; [146–148] this paracrine effect has also been described for

MSCs [149,150]. While both early and late outgrowth EPCs are equally efficient in promoting neovascularization [146], the use of two cell types is better than a single type [151]. Interestingly, early outgrowth ones were shown to be cells with spindle shape morphology at first week of culture, while late outgrowth ones were shown to be cells with cobblestone morphology after 2–3 weeks of culture [143,146,151]. In fact, while many independent investigators have shown bone marrow cells to incorporate into the vasculature, there are a few reports showing that bone marrow cells do not incorporate into growing vessels and instead form periadventitial accumulations, where they express angiogenic/arteriogenic cytokines such as VEGF, monocyte chemoattractant protein-1, and FGF [145,152–154]. This may represent various cell populations within the mononuclear fraction, which have different cellular and molecular mechanisms in affecting neovacularization, and can be used in tissue engineering alone or in combination.

Some experiments have suggested that blood- and bone marrow-derived primary EPCs possess extended plasticity. Melero-Martin et al. [65] showed that hEPCs isolated from human umbilical cord blood or from adult peripheral blood, which were combined with Matrigel and implanted subcutaneously into immunodeficient mice, showed vasculogenic activity and created vascular networks *in vivo*. EPCs were also able to differentiate into cardiomyocytes when co-cultured with newborn rat cardiomyocytes [155] and could exhibit a mesenchymal phenotype in response to transforming growth factor β-1 (TGFβ-1) [156]. In addition, expression of TGFβ-1 in SMCs regulates EPCs migration and differentiation [157]. Thus, transdifferentiation of EPCs might be possible via signaling molecules. As a result, one can build biomaterial scaffolds engineered with inductive cues to induce cardiomyocyte formation from EPCs, increasing low-efficiency transdifferentiation event to a clinically relevant level [158].

Many studies have demonstrated that co-culture of vascular cell types (ECs, EPCs, or OECs) with secondary supporting cells is very effective in the production of vascularized tissue constructs [159–164]. Several reports have suggested the use of MSCs for recruitment of EPCs/ECs and promotion of angiogenesis due to their paracrine effects [149,150]. However, MSCs are also able to differentiate into multiple lineages including cardiomyocytes and vascular ECs [149].

Clinical application of tissue engineering is still limited, due to demands for highly specialized cell culture, isolation, and enrichment techniques required for this purpose. More understanding of the proliferation and differentiation processes that occur in transplanted SCs populations within the scaffolds will increase our success in tissue

engineering. An inceptive area of bioengineering investigations is the development of natural or/and synthetic biopolymer matrices as specific environments for EPCs recruitment, growth, and differentiation by providing sites of attachment together with signals that control EPCs migration, survival, and propagation with synchronized differentiation [156]. The combination of phenotypic shifts and further comprehension of progenitor cell behaviors will provide influential tools in advancing thick tissue engineering. It is also important to know that various sources of SCs may have different responses in the same scaffold [117,120,165] and sometimes various scaffolds have diverse effects on the same type of SCs [10,11,166].

Generally, there are three approaches commonly used for vascularization of tissue constructs, as illustrated in Figure 2 [162,167,168]. The second and third approaches are described below.

Induction of angiogenesis by the use of cells and/or angiogenic factors

In spite of considerable attempts in making functional tissues and organs, most applications of tissue engineering have been restricted to

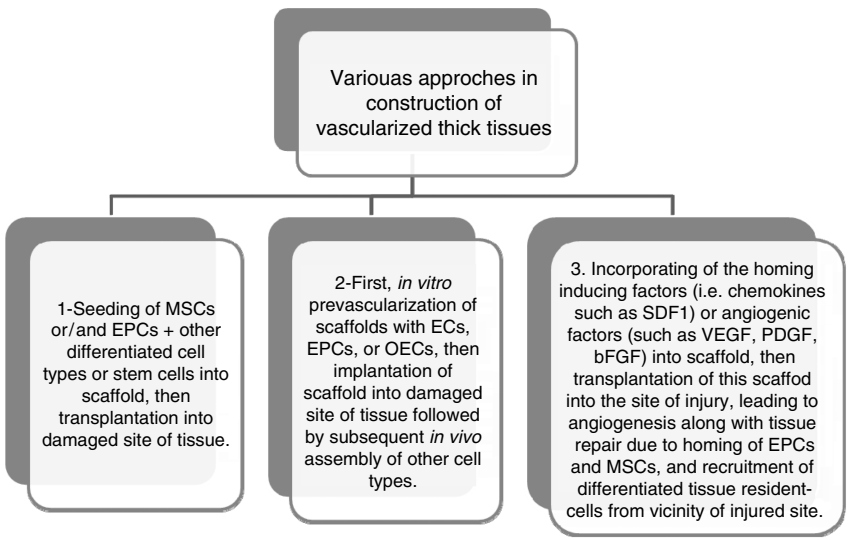

Figure 2. Various strategies that can be used to construct vascularized thick tissues. Notes: EC, endothelial cell; OEC, outgrowth endothelial cell; SDF1, stromal cell-derived factor 1. For the meaning of other abbreviations, see Figure 1.

avascular or thin tissues such as cartilage, skin, or bladder [169,170]. In these tissues, nutrients and oxygen can diffuse into the implants and retain cellular viability. However, as the tissue becomes thicker, cells existing more than a few hundred microns away from nearest capillaries would undergo hypoxia and apoptosis [9]. So, the main obstacle in the creation of more complex tissues is the formation of vascular networks able to deliver nutrients and oxygen through the engineered tissues. Enough neovascularization can be attained by the proper use of angiogenic factors with appropriate cell types in scaffold biomaterials (Figure 1). There have been many efforts to promote and regulate vascularization of engineered tissues and also in pathological situations such as chronic wounds in diabetic ulcers that are resulting from insufficient blood supply, contributing to inflammation and infection at the deficient sites [21], and myocardial ischemia, which is a weakening defect associated with hypoxia and tissue necrosis because of occluded vessels [171]. Therefore, induction of neovascularization in engineered tissues, chronic wounds, and ischemic areas is the main therapeutic goal.

First of all, we should understand angiogenesis process. The process of angiogenesis follows from a complex cascade of events including ECs activation, migration, and proliferation, their arrangement into immature vessels, addition of mural cells (pericytes and SMCs), and matrix deposition as the vessels mature [21,137,172]. The molecular mechanisms regulating each of these stages are being described, and it is obvious that different growth factors act at distinct steps of neovascularization. For instance, bFGF and VEGF, which are heparin binding growth factors, contribute to the initiation of angiogenesis, and induce endothelial cell proliferation and migration. Platelet-derived growth factor (PDGF) is a mitogen and chemotactic agent that recruits pericytes and SMCs. Finally, TGF-β causes ECM deposition for stabilization of new vessels [137,173,174]. However, VEGF and its receptors constitute the key signaling system for angiogenic activity in tissue formation [175]. ECM proteins that participate in neovascularization are laminin, collagen type I, and collagen type IV [167]. So, it is important to properly deliver these signaling molecules locally and temporally to obtain the desired biological outcomes, while avoiding unfavorable side-effects.

Angiogenesis of engineered constructs is encouraged *ex vivo* by biomaterial design, cell seeding, and culture conditions. *In vitro* prevascularized scaffolds would then be transplanted *in vivo* and promoted to unite with the host vascular network. This approach requires various biochemical signals inserted in the scaffolds to imitate normal

microenvironment and would lead to elevated angiogenic potential of the seeded cells. Sometimes, the compatible scaffolds are manipulated with ECM proteins, ECM-derived peptides, and signaling proteins arranged in special micropatterns to direct angiogenesis [95,176,177]. Angiogenic factors are incorporated into scaffold biomaterials and then transplanted *in vivo* for the recruitment of EPCs followed by subsequent assembly of other cell types [178–180]. In another approach, instead of angiogenic factors, one can incorporate chemokines (such as SDF1) into the scaffolds to induce homing of MSCs or/and EPCs from peripheral blood to the site of implant and ultimately augment angiogenesis [71,133,145].

Taken together, in terms of cell sources and angiogenic factors, for successful engineering of vascularized thick tissues, some fundamental guidelines must be considered, which are summarized here.

(1) The scaffold must be compatible with ECs growth, formation of capillaries, and construction of the target tissue [181]. The matrix can be coated with a substance such as collagen, laminin or fibronectin that allows attachment and growth of ECs and tissue-specific cells.
(2) Angiogenic growth factors are required for proliferation of ECs and better formation of blood vessels. Therefore, one can incorporate the source of an angiogenic factor, with slow and sustained-release kinetics, into the bioengineered tissue before implantation, so that enhanced new capillary ingrowth from the host's vascular plexus is achieved after *in vivo* transplantation [25,182]. As an alternative, cells within the engineered tissue can be genetically modified to secrete angiogenic factors [183,184]. Release of these factors and chemokines (such as SDF1) from the implanted site can recruit circulating EPCs or MSCs into the scaffold and induce the angiogenesis of the tissue [71,145,185].
(3) For the augmentation of tissue vascularization, ECs, EPCs, or OECs can be inserted into the bioengineered tissue. These cells can constitute capillaries within the tissues *in vitro* and link to the host's vasculature systems *in vivo* [58]. The combination of these cells with the incorporation of a prolonged reservoir for angiogenic factor secretion from the scaffold would be more effective [186]. It is also possible to use MSCs for induction of angiogenesis in the scaffold through their paracrine effects [150].
(4) The normal angiogenic process in the engineered tissue must be developed so that a functional vascular network will be achieved. Excessive production of angiogenic factors may give rise to

deformed, non-functional vessels. On the other hand, low concentration of these factors may result in non-effective capillary density. Therefore, the creation of new blood vessels should follow the kinetics of normal development in the vasculature [174,187]. A combination of concentrations and various periods of exposure to different angiogenic factors should be tested to find the best conditions for high-efficiency angiogenesis. For this end, it is necessary to design excellent DDSs for engineering of thick tissues, and these systems are discussed in the following section.

DRUG DELIVERY SYSTEMS

Despite the fact that many biomaterials can supply essential mechanical support and attachment sites, they cannot direct changes in cellular phenotype as efficiently as growth factors. Binding of growth factors to various biomaterials appears to be a relatively simple task [188]. For example, biotinylated polymeric biomaterials can be easily coupled to growth factors by streptavidin [189]. This technique has been used to conjugate RGD to PLA–PEG copolymers [190].

Growth factors are released by cells for immediate signaling or are embedded in the ECM and released in a controlled manner. Sequestering of growth factors in the ECM allows their stabilization and provides physical cues for cells through spatial presentation. Controlled release of factors from the ECM is coordinated by extracellular degradation. Generally, these processes contribute to growth factor delivery that is responsive and dynamic, changing in accordance with specific cellular requirements and processes [191].

Although systemic delivery of single proteins is technically simple, the succeeding distribution of them to every place in the body and consequently their rapid degradation result in unfavorable side effects and toxicity, and also an inadequate local concentration for the desired time frame [29]. Polymeric systems can be successfully tricked to deliver small doses of factors at distinct release rates directly to target cells (Figure 1). Polymeric delivery systems made up of various natural and synthetic biomaterials can grant controlled growth factor delivery by diverse mechanisms. Various types of these materials have been applied for regulated release of bFGF and VEGF, including alginate hydrogels, PLG microspheres, and porous PLGA scaffolds [72,192,193]. The release profiles of biomolecules from these carriers can be controlled by diffusion, polymer degradation, the dose of the factors loaded in the system, and the composition of the scaffold. Biodegradable polymer

systems are used to deliver proteins [30,31,194,195] or plasmid DNAs encoding the desired factors [196,197]. However, there is necessity for growth factor delivery systems that are able to deliver multiple factors with distinct release kinetics, which is required for driving normal tissue development [54,198].

Growth factors can be incorporated into the scaffolds by two approaches. The first approach involves adding a lyophilized factor like VEGF to the polymer particles prior to processing the polymer into a porous scaffold, which results in the factor being largely associated with the surface of the polymer. In this method, VEGF is subjected to rapid release (e.g., days to weeks in duration). The second approach involves pre-encapsulating of a factor like PDGF in PLG microspheres. Therefore, fabricating scaffolds from these particles results in a more even distribution of factors throughout the polymer, with release kinetics controlled by degradation rate of the polymer used to construct microspheres. The two approaches may be combined by mixing particulate polymers containing the first factor with microspheres containing a pre-encapsulated second factor to deliver two growth factors with different release rates. The particulate and microsphere PLGAs are then fused to form a homogeneous combined scaffold with an open-pore structure [106].

In a few circumstances, we need to deliver several factors with distinct release kinetics in desired time frames. Therefore, the future of bioactive materials is the design of 'smart biomaterials' that respond to their environment with predetermined responses in which release is initiated by microenvironmental cues. Although the design of smart biomaterials is in its early stages of development, the potential for engineering these biomaterials has been presented by many studies [4,199,200]. Various pathologic conditions can lead to the increase of local temperature or acidity, or to the activation of matrix metalloproteinases (MMPs), and these microenvironmental conditions can be exploited by smart biomaterials [200,201]. MMPs, which cleave specific amino acid sequences, act locally in ECM, and are normally expressed at low levels in restful tissues. Thus, MMP-sensitive linkers could be used to couple factors to biomaterials. Lutolf et al., developed hydrogels with MMP-sensitive linkages between polyethylene glycol chains entrapping BMP2. This strategy allowed rapid bone formation in rats due to proteolytic invasiveness of the gels and subsequent release of BMP2 [202].

Successful repair and regeneration strategies will require quantitative insight of tissue microenvironment and can be engineered via designing biomaterials, which provide quantitative adhesion, growth, or migration signals to direct cellular differentiation pathways including angiogenesis

and vascular maturation. Angiogenesis with biomaterial-based drug- and cell-delivery systems have been reviewed by several papers [25,203,204]. Mimicking biological patterning may be especially useful to control tissue development processes such as neovascularization, where unguided or uncontrolled growth can lead to pathological effects including tumor growth, metastasis, and deformed vessels. Techniques developed for microarray patterning and microcontact printing, micromolding, laser photolithography, and micro-electro-mechanical systems (microfluidic devices) may be useful to form gradients of growth factors within the scaffolds. In addition to biomaterial-based DDSs, these technical micropatterning approaches might be valuable in the generation of complex networks of temporally and spatially controlled growth factor delivery to mimic the micro- and nano-topographies of natural ECM, creating complex tissue architectures in scaffold materials and to regulate angiogenesis [4,7,95,167].

CONCLUSION

Advances in bioactive biomaterials and DDSs allow not only controlled release, but also protection of factors from degradation. Design of these advanced biomaterials will require substantial basic biological insight, since dose, timing, spatial range of growth factors delivery, and also the conditions for environmentally controlled release will be highly specific for each target tissue and disease. Materials can be designed to be multifunctional and smart, in order to provide sequential signals with different release kinetics for individual factors. Thus, new rationally designed biomaterials provide exact control of multiple growth factors release with distinct gradient in response to a specific niche. Identification and manipulation of biomaterials that support appropriate cellular attachment and proliferation and induce optimal angiogenic signaling pathways are critical for engineering of thick tissues.

Organized neovascularization in engineered tissues may allow development of thick tissues with large mass and complexity. To reach this goal, several key issues must be considered; first, since normal tissue regeneration and development follow a specific series of spatially and temporally coordinated signaling events, biocompatible scaffolding biomaterials must be designed in a way to yield tissue constructions in a precisely controlled manner. On the other hand, properties of each scaffold should be specific for full tissue engineering and regeneration including its angiogenesis. For example, the matrix should have a high degree of porosity to allow the penetration of blood vessels into the implant. There are many biomaterials in use today, in clinical settings,

with reasonable biocompatibility; however, performance of these biomaterials in conjunction with incorporated bioactive factors needs to be addressed. Second, the use of better sources of vascular cell progenitors (OEC-type EPCs, for example) in combination with other tissue-specific cell types may alleviate cell sourcing problem that can hinder industrial scale-up of engineered thick tissues. The third issue is the need for better comprehension of the biology behind neovascularization. Understanding the normal route of angiogenesis provides basic data on which we can build and optimize normal growth and development of vessels within scaffolds. The fourth important factor is finding a way to optimize fabrication of scaffold–biomolecule hybrids. For example, organization of biomolecules and cells in biomaterials needs to be optimized to mimic tissue complexity, and micro- and nano-patterning. The fifth issue involves drug loading and delivery methods, release profiles, and gradients, which need to be examined to achieve maximum efficacy of growth factor release, similar to that of normal tissue development. Finally, we need to integrate vascularized tissue constructs with functional cells of interest and also with host's vasculature networks. To create functional heart, muscle, lung, etc., the native cell types or their precursors have to be either included or recruited into the scaffolds along with vascular cell types. The resulting interactions among multiple cell types have to be carefully examined so that functional tissues are regenerated with complete network of blood vessels. Further investigations are required to address these critical issues for improvement and engineering of clinically large tissues, which is an important goal in tissue engineering.

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