Vascular Engineering Using Human Embryonic Stem Cells

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Engineering vascularized tissue constructs remains a major problem in regenerative medicine. The formation of such a microvasculature—like the vasculogenesis in early embryogenesis that it closely resembles—is guided by biochemical and biophysical cues, such as growth factors, extracellular matrix proteins, hypoxia, and hydrodynamic shear. As they undergo spontaneous and directed vascular differentiation, human embryonic stem cells can be used as a model system to explore central issues in engineering vascularized tissue constructs and, potentially, to elucidate vasculogenic and angiogenic mechanisms involved in such vascular diseases as limb and cardiac ischemia. Because the conventional spontaneous differentiation approach can only isolate small quantities of vascular cells, recent efforts have sought to develop controlled approaches, including the development of three-dimensional scaffolds to reengineer the microenvironments of early embryogenesis. This review focuses on emerging approaches to deriving and directing vasculatures from human embryonic stem cells and efforts to engineer 3D vasculatures from such derivatives. © 2009 American Institute of Chemical Engineers *Biotechnol. Prog.,* 25: 2–9, 2009

Introduction

The emerging field of regenerative medicine arose to address the shortage of organs available for transplantation. The ability to grow stem cells in three-dimensional (3D) scaffolds and to direct their differentiation into specific tissues could reduce the need for organ replacement and accelerate the development of new therapeutics to treat patients. Despite the progress made in developing artificial livers,[1] kidneys,[2] pancreases,[3] skeletal muscles,[4] and blood vessels,[5] the ability to engineer a vascularized tissue construct remains a bottleneck for tissue engineering. Tissues engineered in vitro that do not have their own blood supply are limited in size to the short distance over which oxygen can diffuse before being consumed.[6] Once implanted into a patient, engineered tissue will consume the available oxygen before a supply from the host circulation system is available (through angiogenesis). Therefore, engineering vascularized tissue in vitro and inducing the anastomosation of new vasculature networks in vivo are crucial for the successful implantation, survival, integration, and functionality of the engineered tissue constructs. The formation of microvasculature networks can be explored in spontaneous and directed differentiation of human embryonic stem cells (hESCs). Because of their high proliferative capacity, pluripotency (the ability to differentiate into mesoderm, endoderm, and ectoderm), and low immunogenicity, hESCs are a reliable source for vascular progenitor cells (VPCs), which have been shown to repair ischemic tissues and restore blood flows.[7] In this review, we will discuss methods used to derive and direct vasculatures from hESCs and efforts to engineer vasculatures from such derivatives.

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Factors Involved in Vascular Development and Regeneration

Growth and remodeling of blood vessels are regulated by various cues, including growth factors (GFs), extracellular matrix (ECM) proteins, oxygen tension, and hydrodynamic shear.

GFs

Paracrine signals that control angiogenesis and vasculogenesis include protein ligands, which bind to and modulate the activity of transmembrane tyrosine kinase receptors.[8,9] Among these molecules are vascular endothelial growth factor (VEGF) and its receptor families (Flk and Flt), Ang-1 and Ang-2 and their receptors (Tie 1, Tie 2), basic fibroblast growth factor (bFGF), platelet-derived growth factor (PDGF), and transforming growth factor β (TGFβ). The dominant role of VEGF and its receptors in early vascular development has been demonstrated in gene-targeted animals (i.e. lacking VEGF), which die in early stages of embryogenesis (either homo- or heterozygous animals).[10,11]

ECM proteins

During vascular development and regeneration, endothelial cells (ECs) are immobilized within a 3D structured

environment and are bound to a diverse array of ECM proteins.[12] Through its rich surface topography and soluble factors, this 3D structure provides both biochemical and biophysical cues that affect cellular fate and organization. Those cues are recognized by the transmembrane integrin receptors and their respective cytoskeletal components. Such bidirectional and dynamic interactions are mediated by focal adhesion, resulting in a cascade of downstream signaling and cytoskeletal rearrangement.[13] Several ECM proteins, like collagen, fibronectin, laminin, and tenascin-c, have been identified as providing adhesion signals, GF binding sites, and migration sites that regulate neovascularization.[14–16]

Hypoxia

The regulation of angiogenesis by hypoxia is an important component of homeostatic control mechanisms that link cardio-pulmonary-vascular oxygen supply to metabolic demand in local tissue.[17] During development, proliferation and tube formation by ECs occur within hypoxic regions.[18,19] HIF-1, a heterodimeric transcription factor composed of an HIF-1α subunit and an HIF-1β subunit, has been identified and purified as a nuclear factor induced in hypoxic cells; more recently, the delineation of the molecular mechanisms of angiogenesis has revealed a critical role for HIF-1 in the regulation of GFs and various aspects of angiogenesis.[17] In ESCs, hypoxia results in the upregulation of VEGF mRNA levels and HIF-1d,[19,20] and in the enhancement of angioblast specification.[21]

Hydrodynamic shear

Physical factors associated with blood flow play important roles in the subsequent maturation of blood vessels. In the lumen, ECs are subjected to hydrodynamic shear; while within the vascular media, physiologic stress is manifested as cyclic strain.[22] These biomechanical forces affect the phenotype, proliferation, and differentiation of both ECs and smooth muscle cells (SMCs) within human vasculature. During embryonic development, hydrodynamic shear was found to induce differentiation of ECs, vascular wall cells, and even cardiovascular commitment through epigenetic histone modification.[23–25] Recently, ECs derived from hESCs were shown to respond functionally to changes in fluid shear stress by modulating gene expression and cell morphology.[26]

Human ESCs as an In Vitro Model for Human Vascular Development

By the third week of development, the embryo's oxygen needs can no longer be met by diffusion alone, which induces EC precursors to differentiate, expand, and coalesce to form a network of primitive tubules, a process referred to as vasculogenesis.[27] These microvascular vessels are composed of fragile tubes of ECs encircled by vascular SMCs that protect and control blood flow.[28] Embryonic vascular formation is a sequential process that involves complex regulatory cascades. Spontaneous differentiation of hESCs can occur by means of the formation of embryoid bodies (EBs).[29] We studied the changes of gene expression for 4 weeks along the development of human EBs.[30] Upregulated genes include vasculogenic GFs, such as *VEGFA*, *VEGFC*, *FIGF (VEGFD)*, *ANG1*, *ANG2*, *TGF-3*, and *PDGFB*; as well as the related receptors *FLT1*, *FLT4*, *PDGFRB*, *TGF-R2*, and *TGF-R3*; other markers, such as *CD34*, *VCAM1*, *PECAM1*, *VE-CAD*; and transcription factors *TAL1*, *GATA2*, and *GATA3* (See example of the analysis performed in Figure 1). On then basis of this analysis of the vascular system, we suggested human EBs as an in vitro model for the study of early human development.[30]

Derivation of ECs and SMCs from hESCs

Human ESCs are undifferentiated cells isolated from the inner cell mass (ICM) of the developing blastocyte. To maintain their undifferentiated states in vitro, hESCs are cultured on mouse embryonic fibroblasts (MEFs) as a feeder layer[31] or indirectly as a source of conditioned medium in feeder-free culture conditions,[32] or with a defined medium composition.[33,34] When cultured in suspension, hESCs spontaneously aggregate and form EBs, which contain a population of VPCs with the potential to differentiate into ECs and SMCs.[35]

ECs can be characterized by their cobblestone cell morphology, formation of capillary-like structures when cultured on Matrigel, incorporation of Dil-labeled acetylated low-density lipoprotein (Dil-Ac-LDL), and expression of endothelial markers—such as vascular endothelial cadherin (VE-cad); platelet endothelial cell adhesion molecule-1 (PECAM1), also known as CD31; CD34; and vascular endothelial growth factor receptor 2 (VEGFR 2), also known as Flk1 or KDR. Functional ECs also express von Willebrand factor (vWF), endothelial nitric oxide synthase (eNOS), and E-selectin proteins.[36] In contrast, SMCs are characterized by having a spindle-shaped morphology, being able to contract and relax in response to carbachol and atropine, rarely forming capillary-like structures when cultured in Matrigel, and expressing SM markers such as α-SM actin, SM myosin heavy chain, calponin, caldesmon, and SM22.[37]

ECs were isolated from 13-day-old human EBs using fluorescence-activated cell sorting (FACS) of $CD31^+$ cells. In addition to expressing CD34, Flk1, VE-cad, and taking up Dil-Ac-LDL, the isolated ECs also expressed mature endothelial protein vWF.[38] Wang et al.[39] later showed that $CD31^+Flk1^+CD45^-$ cells isolated from ten-day-old human EBs, constituted a population of primitive precursors which could be induced to mature into ECs. Ferreira et al.[37] reported that VPCs isolated from ten-day-old EBs expressed CD34, suggesting a common origin for endothelial and hematopoietic cells. When cultured in endothelial growth medium (EGM)-2 supplemented with either $VEGF_{165}$ or PDGF-BB, those VPCs differentiated into ECs or SMCs, respectively. Human ESC-derived ECs and SMCs, when implanted into severe combined immunodeficient (SCID) mice, formed functional microvasculatures which integrated with the host vasculatures.[35]

The differentiation of hESCs into ECs has also been achieved using scalable two-dimensional methods that avoid an EB intermediate. We have shown that hESCs can be directed to differentiate into ECs and SMCs by culturing them on collagen IV, with the supplementation of $VEGF_{165}$ or PDGF, respectively.[40] Other researchers have further demonstrated different feeder layers or ECMs—including mouse bone marrow cells (S17, OP9, and MS-5),[41,42] mouse yolk sac ECs (c166),[41] MEFs,[43] and methylcellulose—which induce their differentiation toward early hematopoietic precursors with the potential to differentiate into ECs.[44] Although all ECs believed to differentiate from a common precursor and present many common functional features,

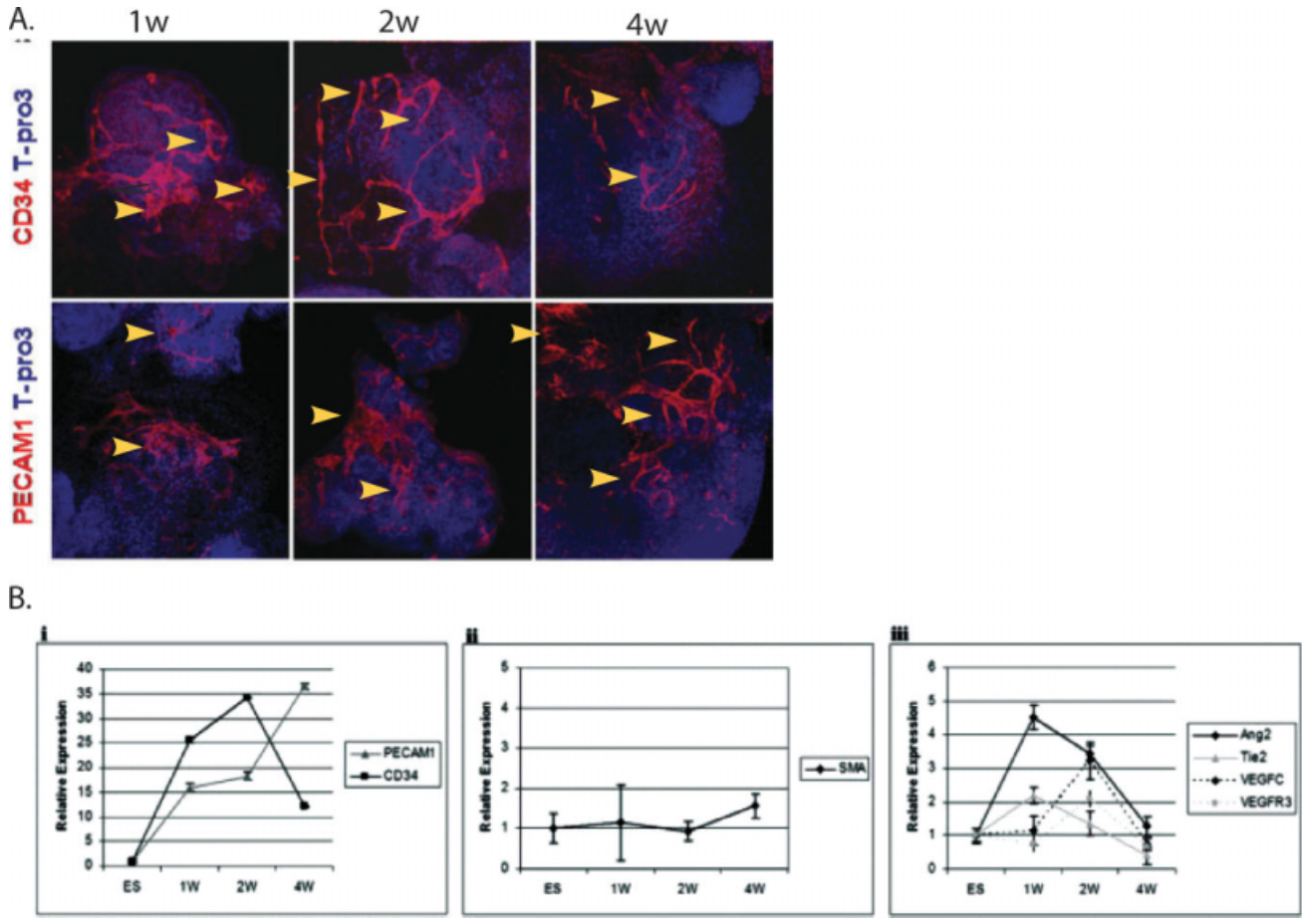

Figure 1. Vasculature structures along human embryoid body (EB) development.

(A) Low-resolution confocal microscopic analysis of vascular structures (indicated by arrow heads) at different time points (1 to 4 weeks) of human EB development reveals progress in arrangement of PECAM1+ (CD31+) cells and peak in arrangement of CD34+ cells after 2 weeks of differentiation. (B) The expression of several vascular genes was verified using real-time RT-PCR for (i) PECAM1, CD34, (ii) SMA, and (iii) ANG2, TIE2, VEGFC, and VEGFR3.[30]

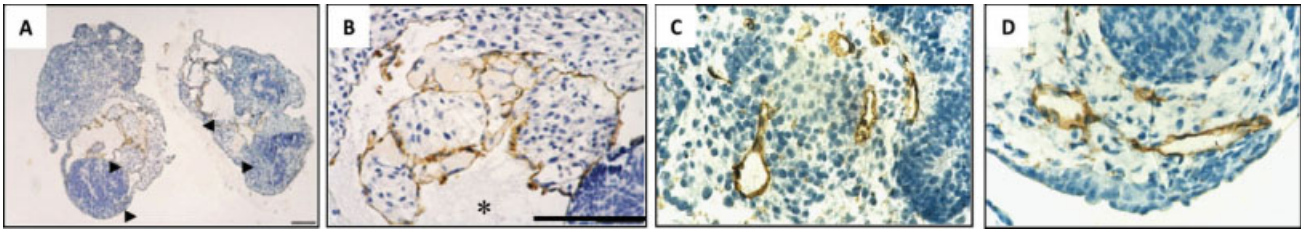

Figure 2. Vasculogenesis in alginate-borne hEBs.

Immunolabeling revealed: (A) CD34+ cells surrounding voids (arrowheads) and (B) higher magnification CD34+ cells demonstrating formation of complex vasculature arrangements along the remaining scaffold (asterisk). Representative images of CD34+ immunolabeled vasculature formed within (C) Petri-dish and (D) bioreactor-borne hEBs.[86]

they display remarkable heterogeneity in different organs.[45] An example for structural heterogeneity are ECs that line the aorta which are larger and thicker than their counterparts in capillaries and veins.[46] Another example are ECs lining capillaries in the brain that have tight junctions,[47,48] while ECs in the kidney are highly discontinuous and fenestrated to allow filtration and transendothelial transport.[49] This heterogeneity also encompasses diversity in cell surface markers, protein, and mRNA expression,[50–53] which are mediated by both microenvironment and epigenetic. Certain site-specific properties of ECs are epigenetically programmed, while others are influenced by spatial and temporal differences in extracellular environment, which include biomechanical cues (i.e. shear stress and cyclical strain) and biochemical cues (i.e. growth factor, cytokines, chemokines, nitric oxide, and oxygen).[54] However, the difficulty to isolate and culture ECs from microcirculation of different organs remains a major barrier to reveal this vast diversity.[52] Challenges remain to differentiate hESCs into a generic type of ECs and incorporate ECs heterogeneity to engineer vasculature networks suitable for a specific organ.

A growing body of evidence suggests the attractive therapeutic potential of ECs derived from hESCs, including neovascularization and the improvement of blood flow in

ischemic disorders. Wang et al.[43] demonstrated that, after transplantation of hESC-derived ECs into SCID mice, the differentiated cells contributed to arborized blood vessels that integrated into the host circulatory system and served as blood conduits for 150 days. VPCs derived from hESCs cultured on an OP9 stromal cell line were shown to differentiate into ECs and SMCs.[7] When transplanted into an SCID mouse model of hind limb ischemia, hESC-derived ECs and SMCs contributed to the formation of new blood vessels and improved blood flow.[7] Cho et al.[55] obtained ECs from differentiated hESCs by mechanical isolation and cell sorting for vWF and demonstrated that these cells improve blood perfusion and limb salvage by facilitating postnatal neovascularization in a mouse model of hind limb ischemia. ECs derived from hESC-blast colonies, when injected into rats with diabetes or into mice with ischemia-reperfusion injury of the retina, were found to localize to the site of injury in the damaged vasculature and appeared to participate in repair.[56] Injection of these cells also reduced the mortality rate after myocardial infarction and restored blood flow in mouse models of hind limb ischemia.[56]

3D Scaffolding

In their native stem cell niche, hESCs are confined in 3D surroundings, where the crucial interactions with neighboring cells and ECM are preserved. This physical constraint can be mimicked in vitro by seeding or encapsulating hESCs in a 3D scaffold, which permits culture at high cell density, enables EB formation, and allows instructive differentiation. In addition, controlled degradation of the scaffolds can be used as a tool for localized, defined GF supplementation.[57] Various biomaterials have been explored to direct hESC differentiation into microvasculatures by providing both biochemical and biophysical cues. Biocompatible materials that have been shown to guide hESC differentiation into microvascular networks can be classified as natural or synthetic biomaterials.

Naturally derived biomaterials

Biomaterials isolated from ECM proteins and plants are particularly appealing to use because of their bioactive, biocompatible, and biodegradable properties.[35,58,59] Naturally derived biomaterials can be cross-linked to form hydrogels with mechanical properties similar to native tissues and with high water content to promote cell viability.[60] Such 3D hydrogels contain recognition signals essential for cell adhesion, proliferation, and differentiation. Hence, hydrogels provide structural support and directed differentiation for hESCs to form microvascular networks in vitro. Upon implantation, hydrogels can be degraded in vivo to facilitate integration of the microvascular construct with the host's vascular system.

Agarose Hydrogels. Agarose, a purified galactan hydrocolloid isolated from marine algae, is a linear polymer composed of alternating D-galactose and 3,6-anhydro-L-galactose units. Because of its large pore size and cell adhesiveness, agarose has been widely used for gel electrophoresis and cell migration assays.[61] Dang et al.[62] encapsulated hESCs in 200 to 300 μm diameter capsules made from low-gelling-temperature agarose in dimethylpolysiloxane (DMPS). After 8 days of differentiation culture, the encapsulation procedure maintained a balance, allowing hESCs to aggregate which is necessary for EB formation while preventing EB agglomeration for efficient cell growth and differentiation. When cultured in low-oxygen conditions, the encapsulated EBs formed a high number of hematopoietic progenitor cells, the building blocks of the vascular system.

Porous Alginate Scaffolds. Like agarose, alginate is a family of polyanionic copolymer extracted from brown sea algae. It is composed of 1,4-linked β-D-mannuronic (M) and α-L-guluronic (G) residues in varying proportions. In the presence of divalent cations (i.e., Ca^{2+}, Ba^{2+}), this viscous solution forms ionically cross-linked hydrogels suitable for cell cultures. The encapsulated cells can also be recovered by adding mild chelating agents to disrupt the ionic bonds of the hydrogels.[63] When undifferentiated hESCs were removed from a feeder layer and seeded in a porous alginate scaffold, EB formation was observed with a relatively high degree of cell proliferation and differentiation.[64] The relatively small pore size (100 μm average diameter) and the hydrophilicity of the scaffold caused the EBs to form mainly within the pores and to distribute evenly over the entire hydrogel. In addition to the chemical cues provided in the medium, this confining environment exerted physical constraints that induced EBs to form complex microvascular networks. Voids and tube-like structures were observed from the histological sections of the early EBs. Immunohistochemical staining also confirmed the presence of CD34, a marker for ECs. Thus, porous alginate hydrogels induce vasculogenesis in the differentiating EBs to a higher degree than observed in conventional static or dynamic culture systems (Figure 2).

Dextran Hydrogels. Dextran, a branched polysaccharide, consists of α-1,6-linked D-glucopyranose residues, synthesized from sucrose by lactic acid bacteria like *Leuconostoc mesenteroides* and *Streptococcus mutans*. Dextran hydrogels have been widely used for drug delivery[66,67] and tissue engineering.[68] They are particularly appealing to use for engineering microvascular networks because of their antithrombotic effect,[69] resistance to protein adsorption,[70] and opportunity to modify with cell adhesive ligands.[71] By cross-linking pullulan, dextran, and fucoidan in a 71:42:5 ratio, Thébaud et al.[72] were able to differentiate endothelial progenitor cells (EPCs) into mature ECs. Recently, Ferreira et al. developed dextran-based bioactive hydrogels for vascular differentiation of hESCs. Dextran-based hydrogels were prepared with or without immobilized regulatory factors: a tethered RGD peptide and microencapsulated $VEGF_{165}$. Undifferentiated hESCs were encapsulated within these hydrogels by photocrosslinking with long-wavelength UV light. Encapsulated hESCs differentiated within the dextran hydrogels while forming EBs. After 10 days in culture, upregulation of vascular markers and well-organized vasculature networks were observed in EBs encapsulated in the dextran hydrogels. Furthermore, when these cells were released from the dextran hydrogels and cultured on a Petri dish in vascular differentiating media (EGM-2 supplemented with VEGF) for 6 additional days, the number of vascular cells increased, suggesting preferable proliferation along a vascular lineage.[35]

Hyaluronic Acid Hydrogels. Hyaluronic acid (HA) is a glycosaminoglycan which consists of a nonsulfated linear polysaccharide of (1-β-4)D-glucuronic acid and (1-β-3)*N*-acetyl-D-glucosamine. As a major component of ECM, HA content is especially high in undifferentiated cells and during early embryogenesis, where HA plays a crucial role in cell proliferation, motility, and metastasis.[73–75] In particular, HA was observed to stimulate EC proliferation, migration, and sprouting.[76] Cellular interactions with HA are mediated by

Table 1. 3D Scaffolds from Naturally-Derived and Synthetic Biomaterials for Vascular Differentiation of hESCs

Biomaterials	Modification/Culture conditions	Pore size diameter (μm)	Young's Modulus (kPa)	Results	Ref.
Agarose	Dissolved in DMPS to create hydrogel microcapsules	200–300	300–400	EB formation with limited agglomeration	62
	Stirred hypoxic	200–300	300–400	High frequency of hematopoietic progenitor cells	62
Alginate	Porous scaffolds	50–100	500–1,136 (dry state)	Efficient and well-vascularized EB formation	64
Dextran (acrylate)	Arc-PEG-RGD or incorporation of VEGF-loaded microparticles	2–35	2–5	Differentiation via EB formation, and enhanced vascular differentiation and maturation	35,58
Hyaluronic acid (methacrylated)	MEF-conditioned media	30–50	2–100	Maintenance of pluripotency	78,86
	Supplementation of VEGF	30–50	2–100	Cell sprouting and elongation	78,86
PLGA/PLLA	50:50 PLGA and PLLA	225–500	65–500	Cell proliferation and 3D vascularization	83
	Triculture system of myoblasts, endothelial cells, and fibroblasts.	225–500	65–500	Vascularized skeletal muscle tissue with improved in vivo vascularization and survival.	84
PGSA	Mixing with glycerol for porosity	20–200	40–60	hESCs proliferated and differentiated. Scaffold promotes *in vivo* tissue ingrowth	85

CD44 and CD168, surface markers which are expressed at high levels in undifferentiated hESCs. CD44 is responsible for cell proliferation and survival pathways,[75] and CD168 is involved in cell locomotion.[77] When encapsulated in HA hydrogels and cultured in MEF-conditioned medium, hESCs maintained their undifferentiated state and self-renewal.[78] Upon release from HA hydrogels, hESCs were found to be viable, undifferentiated, and to have preserved their genetic integrity. HA hydrogels can be used to induce differentiation by altering soluble factors. When the MEF-conditioned medium was replaced by an angiogenic differentiation medium containing VEGF, cell sprouting and elongation was observed after 48 h. Immunohistochemical staining confirmed the presence of vascular α-SM actin and CD34 endothelial marker.[78]

Synthetic biomaterials

The use of synthetic biodegradable polymer scaffolds that mimic their natural counterparts can be more beneficial due to their ease of fabrication and adjustable physical properties.[79] Parameters like material pore size, structural orientation, and degradation rate can be manipulated to favor a specific cell lineage.[80] Therefore, synthetic biomaterials can be made reproducibly with chemistry and cellular responses that are more predictable than naturally derived biomaterials. Synthetic scaffolds not only provide the physical supports for ECs to grow, but can be modified with binding domains to mimic the 3D in vivo interactions to stimulate the formation of microvasculatures.

PLGA/PLLA. Because of its biodegradability and biocompatibility, PLGA/PLLA, a copolymer consisting of glycolic acid, D-lactic acid, and L-lactic acid, has been used widely in drug delivery[81] and tissue engineering.[82] When transplanted in vivo, water catalyzes the degradation of this polymer scaffold into glycolic and lactic acid byproducts which can be removed via metabolic pathways. In addition, the degradation rate can be controlled by adjusting the polymer molecular weight and the copolymerization ratio. Blending 50% PLGA with 50% PLLA generates 3D scaffolds with an average pore size of 225 to 500 μm.[83] PLGA degrades quickly to facilitate cellular ingrowth, while PLLA provides mechanical strength to support the 3D construct. Differentiated human ESCs isolated from eight- to nine-day-old EBs were seeded into either Matrigel-coated or fibronectin-coated PLGA/PLLA scaffolds. In the presence of specific GFs, the 3D cells-scaffold constructs developed into organized primitive tissue structures. The differentiation of hESCs was influenced by the polymer scaffolds and directed by specific GFs. Capillary-like networks were observed throughout the tissue construct on these polymer scaffolds. Immunohistochemical staining for CD34 and CD31 confirmed the presence of ECs. Although PLGA/PLLA is a versatile polymer scaffold for engineering microvascular networks, the seeding of hESCs into scaffolds of this polymer required extracellular-derived matrix (i.e., Matrigel or fibronectin), which may affect cell differentiation.[83] PLGA/PLLA scaffolds were also used to engineer a triculture system where mouse myoblasts were mixed with hESC-derived ECs and embryonic fibroblasts.[84] Immunostaining for vWF and SM actin indicated that embryonic fibroblasts, after 2 weeks of culture, differentiated into SMCs which were colocalized around ECs in vessel-like structures. When implanted into SCID mice, the vascularized skeletal muscle constructs showed improved implant vascularization and survival in vivo.[84]

PGSA. This porous bioelastomer is another promising biodegradable polymer for vascular differentiation of hESCs. Glycerol added to poly(glycerolcosebacate) acrylate (PGSA) forms a porous, flexible bioleastomer hydrogel which is suitable for 3D cell culture. When encapsulated in this hydrogel, undifferentiated hESCs adhered to the scaffold wall and formed protrusions and apparent interconnections between each other within 24 h. After 7 days, undifferentiated hESCs proliferated and differentiated in the PGSA scaffold, forming 3D tissuelike structures.[85] Subcutaneous transplantations showed that porous scaffolds have biocompatibility profiles similar to nonporous PGSA, in which minimal inflammatory zones surrounding the scaffolds decrease along the seven-week experiment; however, porous PGSA promotes tissue ingrowth and integration with the host vasculature, unlike nonporous PGSA.[85] PGSA is a promising flexible scaffold that can further be explored for vascular differentiation (Table 1).

Conclusion and Future Perspectives

In recent years, advances have been made in the vascular differentiation of hESCs, positioning them as a reliable source for vascular therapeutics and for engineering

vascularized tissue constructs. Various methods have been developed and have demonstrated the efficient isolation and, in some cases, expansion of VPCs and their derivatives, ECs and SMCs, from hESCs. The development of biomaterials capable of presenting specific chosen environmental cues offers a unique opportunity to generate instructive 3D environments for vascular assembly. Both naturally-derived and synthetic biomaterials have unique properties that are suitable for various applications. For instance, 2% methyl arcylated HA hydrogel is very soft, which makes it suitable for in vivo transplantation into soft tissues, but cell-material interactions including hydrogel degradation by the cells, present a challenge for long term in vitro cultures. On the other hand, alginate scaffold is effective for EB formation because of its large pore sizes and good mechanical properties. Given the complex microenvironments involved in vasculogenesis during early embryogenesis, challenges remain: to better monitor, control, and quantify the kinetics of vascular differentiation, maturation, and tissue organization within the 3D scaffolds.

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