Integrated Vascular Engineering: Vascularization of Reconstructed Tissue

16

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Abstract

In this chapter, we describe culture methods to construct microvascular networks as well as approaches to integrating capillary networks with 3D epithelial tissue-engineered constructs. First, culture models of microvascular networks such as *in vitro* angiogenesis and vasculogenesis models are introduced. Using these culture models, the roles of endothelial cells (ECs), such as endothelial tip, stalk, and phalanx cells, are demonstrated. Additionally, regulatory factors, including both biochemical and biophysical factors, are discussed in the context of 3D capillary formation, including the process of vascular development, growth, and maturation. Next, we focus on the use of microfluidics technologies for investigating capillary morphogenesis. Examples of 3D capillary formation assays with growth factor gradients and different extracellular matrix materials are described. Cocultures of ECs and the other cell types in microfluidic devices are also introduced to show the potential of microfluidic vascular formation models. The vascularization of constructed tissues is discussed from the viewpoints of horizontal and vertical approaches for combining capillary structures and epithelial tissues *in vitro*. Finally, the concept of integrated vascular engineering and future perspectives are discussed.

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16.1   In Vitro Culture Models of Microvascular Networks

16.1.1 General Introduction for In Vitro Capillary Formation

Blood vessels are tubular structures through which blood passes to provide oxygen and nutrients to individual cells within tissues and organs. Because oxygen and nutrients are the most important fundamental factors for vital activity, blood vessels are essential tissues in the body. The luminal surface of blood vessels is covered by endothelial cells (ECs). Thus, we need to understand how ECs form blood vessels for the development of vascular tissue engineering. In particular, in the context of tissue engineering for three-dimensional (3D) tissues and organs, construction of microvascular networks, rather than large blood vessels, has a high priority.

There are two processes of microvessel formation *in vivo*, referred to as "angiogenesis" and "vasculogenesis" (Risau 1997). Angiogenesis is the formation of new capillaries branching from preexisting blood vessels. To investigate this angiogenic process, an *in vitro* 3D angiogenesis model has been developed. In this model, hydrogels, such as collagen gel and fibrin gel, are formed in a culture dish, and ECs are seeded on the surface of the 3D hydrogel scaffold. ECs grow on the gel surface and finally form a confluent monolayer. When the cells are cultured with no growth factors, they maintain a confluent monolayer, which is similar to the ECs covering the luminal surface of blood vessels *in vivo* (step 1, Fig. 16.1a). However, when ECs are cultured and supplemented with angiogenic growth factors, such as vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF), some ECs penetrate into the underlying gel to form vascular sprouts, which is the beginning of vascular formation (step 2, Fig. 16.1a). These vascular sprouts gradually extend and branch into the gel and finally develop into 3D capillary-like structures (step 3, Fig. 16.1a).

The other process of microvessel formation, vasculogenesis, is the process of vascular formation in the absence of preexisting blood vessels, which occurs in the developmental process and the process of adult vascular growth (Risau and Flamme 1995). First, cells from the mesoderm form aggregates that are called blood islands. The peripheral cells of the blood islands are angioblasts, which are precursors of ECs and have not yet formed a lumen. Then, angioblasts differentiate into ECs while fusion of blood islands results in the formation of a primary capillary plexus, which, subsequently, generates primitive blood vessels.

This vasculogenesis process can also be reproduced in an in *vitro* 3D vasculogenesis model. In this model, ECs are mixed in hydrogel prepolymer solution. The prepolymer solution is then poured into a culture dish and incubated for gelation, resulting in a hydrogel with embedded individual ECs (step 1, Fig. 16.1b). ECs embedded in the hydrogel, such as a collagen gel or fibrin gel, extend in 3D and connect with each other, while some cells form vacuoles within their cytoplasm (step
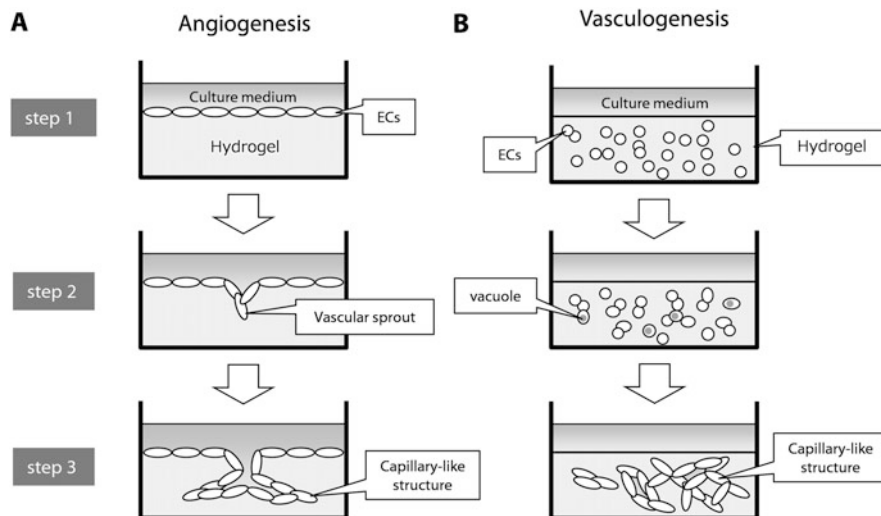
Fig. 16.1 *The process of microvascular network formation in angiogenesis and vasculogenesis culture models.* (**a**) In the angiogenesis model, ECs form a confluent monolayer covering the surface of the hydrogel. Some cells within the monolayer form vascular sprouts, which finally develop into three-dimensionally extending capillary-like networks. (**b**) In the vasculogenesis model, individual ECs are embedded in a hydrogel. These cells extend within the hydrogel and connect with each other. Some cells start to form vacuoles within their cytoplasm. These cells finally connect and develop into capillary-like structures with partial lumens

2, Fig. 16.1b). These vacuoles, formed in individual cells, subsequently fuse with each other, resulting in capillary-like structures with partial lumens (step 3, Fig. 16.1b).

Control of microvascular network formation is important not only in physiological but also in pathological conditions. For example, the formation of microvascular networks is required to promote 3D tissue formation in the field of tissue engineering and regenerative medicine. In contrast, it is also important to inhibit the formation of microvascular networks in terms of cancer therapy because *inhibition* of vascular formation is an important strategy to treat cancer. Thus, *in vitro* culture models of both angiogenesis and vasculogenesis are used to investigate how to control the formation of microvascular networks.

16.1.2 In Vitro Angiogenesis and Vasculogenesis Models

In vitro culture models of angiogenesis and vasculogenesis play important roles in investigating the mechanisms of vascular generation, growth, stabilization, and pathophysiology because cells in these models can be cultured under controlled conditions, which are independent from the complex cellular microenvironments *in vivo*.

Numerous *in vitro* angiogenesis and vasculogenesis models have been used to reveal molecular mechanisms and cell-to-cell interactions for constructing

microvascular networks by ECs in 3D extracellular matrix (ECM) environments, focusing on biochemical factors (Davis et al. 2002; Newman et al. 2011). Another aspect to controlling vascular formation is that of biophysical factors, such as flow-induced mechanical signals and ECM stiffness. Ueda et al. (2004) investigated the effects of shear flow in a 3D angiogenesis model. They applied a laminar shear stress of 0.3 Pa to the surface of a confluent monolayer of ECs seeded on the surface of a collagen gel. Microvessel formation within the gel was promoted by shear flow application. The length of the capillary networks was significantly longer than that in static conditions. Microvessel formation was promoted in the gel whereas shear flow was applied to the cells on the surface of the gel, suggesting that the behavior of the cells *on* the gel is important to understand capillary morphogenesis of the cells *in* the gel.

A 3D vasculogenesis model can also be used to investigate biophysical factors, such as ECM stiffness, on capillary morphogenesis. For example, Sieminski et al. (2004) cultured ECs in floating or constrained collagen gels with different collagen concentrations to control matrix stiffness. EC morphogenesis differed depending on the matrix stiffness. Additionally, their results suggested that the relative magnitudes of cellular force generation due to the different matrix stiffness regulated capillary morphogenesis.

16.1.3 Endothelial Tip, Stalk, and Phalanx Cells

In the process of angiogenesis, ECs form vascular sprouts and gradually develop into branching networks with continuous lumens. Although vascular structures are formed by a single cell type, the EC, three different EC phenotypes are involved in the process of angiogenesis: endothelial tip, stalk, and phalanx cells (Hellström et al. 2007; Tammela et al. 2008; Carmeliet et al. 2009; Mazzone et al. 2009). Thus, we need to understand the characteristics of each EC phenotype and consider them in controlling the construction of microvascular networks *in vitro*.

Endothelial tip cells are the cells that locate at the tip of branching vascular networks. These cells extend filopodia to sense the surrounding microenvironment, such as growth factor gradients and ECM signaling molecules and stiffness, leading to the extension of vascular networks through tip cell migration. Thus, tip cells play an important role in determining the direction in which the capillary extends, while the cells rarely proliferate. In the context of vascular tissue engineering, the control of endothelial tip cells is important in guiding the direction of capillaries.

Endothelial stalk cells are the cells that follow the leading tip cells. Stalk cells proliferate to fill the elongating stalk in capillary networks. Although endothelial tip cells have no lumen, stalk cells create a lumen in the process of vascular formation. The assignment of ECs into the tip and stalk cells is regulated by endothelial cell-cell communication via Notch receptors and their transmembrane ligands, the delta-like ligand 4 ('Dll4') (Carmeliet et al. 2009). When ECs are activated by cytokines, such as VEGF, Dll4 expression is induced in the cells. The cells that strongly express Dll4 become endothelial tip cells. Dll4 expressed in tip cells activates

Notch in neighboring cells, which induce stalk cells. This Dll4/Notch signaling from tip to stalk cells downregulates expression of VEGFR2 in stalk cells.

Endothelial phalanx cells are the third phenotype, which are located far from the tip cells. These ECs form a monolayer with a cobblestone appearance, which also resembles a phalanx, a Greek military formation. Phalanx cells form continuous and tightly packed monolayers as a quiescent endothelium (Mazzone et al. 2009).

16.1.4 Regulatory Factors in Vascular Formation

Advances in angiogenesis and vasculogenesis studies have revealed that both biochemical and biophysical factors regulate vascular formation. *In vivo*, ECs are exposed directly to blood flow, which supplies biophysical factors directly and indirectly. Blood flow delivers soluble factors, such as cytokines and growth factors, produced in different tissues. ECs are not only exposed to blood flow but also exposed to mural cells and ECM, such as basement membrane matrices, which also give rise to biophysical and biochemical factors.

The effects of biophysical factors, such as shear flow, interstitial flow, and ECM stiffness, have been investigated using *in vitro* models. For example, shear stress has been recognized as a promoter of angiogenesis (Ueda et al. 2004; Kang et al. 2008; Kaunas et al. 2011). In addition to shear flow, interstitial flow also plays important roles in capillary morphogenesis (Ng et al. 2004; Helm et al. 2005; Semino et al. 2006; Hernández Vera et al. 2009). ECM stiffness is also important in the regulation of angiogenesis. Yamamura et al. (2007) revealed that ECs cultured in rigid and flexible gels formed 3D networks via different processes; cells formed dense, thin networks in a flexible gel, while thicker and deeper networks were formed in a rigid gel. Sieminski et al. (2004) also reported that the relative magnitudes of cellular force generation and ECM stiffness modulated capillary morphogenesis *in vitro*.

Biochemical factors, such as growth factors, cytokines, chemokines, and their concentration gradients, have also been investigated in the context of vascular formation using *in vitro* angiogenesis and vasculogenesis models. VEGF and bFGF are major growth factors promoting vascular formation (Montesano et al. 1986). Phorbol myristate acetate (PMA), a tumor promoter that increases the production of collagenase and plasminogen activator, is also a promoter of angiogenesis. Montesano and Orci (1985) revealed that bovine microvascular ECs grown on collagen gels invaded the underlying collagen gel and formed extensive capillary networks when they were treated with PMA, whereas control ECs were confined to the surface of the gels.

Not only proangiogenic factors but also antiangiogenic factors have been identified, such as endostatin, tissue inhibitor of matrix metalloproteinase 3 (TIMP-3; Anand-Apte et al. 1997), and leukemia inhibitory factor (LIF; Pepper et al. 1995). Some of these factors are summarized in Table 16.1. Transforming growth factor β (TGFβ) has been reported to have biphasic effects on vascular formation; low concentrations (0.1-1 ng/mL) promoted angiogenesis while higher concentrations (5-10 ng/mL) inhibited capillary lumen formation (Pepper

Table 16.1 Pro- and antiangiogenic factors

Proangiogenic factors	Antiangiogenic factors
PMA (Montesano and Orci 1985)	Thrombospondin (Good et al. 1990)
bFGF (Montesano et al. 1986)	Platelet factor-4 (Maione et al. 1990)
Sodium orthovanadate (Montesano et al. 1988)	Angiostatin (O'Reilly et al. 1994)
VEGF (Bikfalvi et al. 1991; Pepper et al. 1992)	LIF (Pepper et al. 1995)
Fibroblast-derived factors (Montesano et al. 1993)	Endostatin (O'Reilly et al. 1997)
Membrane-type-matrix metalloproteinases (MT-MMPs; Lafleur et al. 2002)	Chondromodulin (Hiraki et al. 1997)
Sphingosine-1-phosphate (Bayless and Davis 2003)	TIMP-3 (Anand-Apte et al. 1997)

et al. 1993). In addition to individual factors, their combined effects are also important. For example, Vernon and Sage (1999) demonstrated that VEGF and bFGF have synergistic proangiogenic effects using an *in vitro* culture model. Newman et al. (2011) also demonstrated the importance of growth factor combinations. They showed that a combination of angiopoietin-1 (Ang-1), angiogenin, hepatocyte growth factor (HGF), TGFα, and tumor necrosis factor (TNF) could substitute for the effects of fibroblast-derived factors on sprout formation whereas no protein alone could function in this way. They also identified fibroblast-derived factors required for lumen formation, which were collagen I, procollagen C endopeptidase enhancer 1, "secreted protein acidic and rich in cysteine" (SPARC), the TGFβ-induced protein "ig-h3," and insulin growth factor-binding protein 7 (Newman et al. 2011). The combined effects of multiple biochemical and biophysical factors need further clarification in future studies.

16.2 Microfluidic Devices for the Culture of Microvascular Networks

16.2.1 Endothelial Monolayers in Microfluidic Devices

This section provides an introduction to the use of microfluidic devices to study microvessel formation, which deals with the culture of ECs or monolayers in microfluidic channels. After EC loading, the cells adhere to the channel surface and form a confluent endothelial monolayer. This mimics the intima of the endothelium, the blood contact surface formed by a single layer of ECs. Mechanotransduction is an important issue for such a monolayer; signal transduction is regulated by the shear stress of blood flow.

Many studies since the 1990s have reported remodeling and alignment of ECs in monolayers due to shear stress gradients (Tardy et al. 1997). Another role for shear stress, in changing endothelial barrier function and permeability, has also been

reported (Seebach et al. 2000; Colgan et al. 2007). This process involves cytoskeletal remodeling and molecular expression within ECs. Precise regulation based on fluidic shear stress types – laminar, turbulent, or pulsatile – has been an important challenge, attracting many interesting microfluidic approaches.

16.2.1.1 Endothelial Monolayer on Microfluidic Channel Surface

A realistic *in vivo*-like microenvironment of the endothelial monolayer would include shear stress, physiological pressure, and stretch. A microfluidic device has been used to culture endothelial monolayers on a stretchable and thin planar membrane (Estrada et al. 2011) (Fig. 16.2a). The membrane was made on the bottom of a microfluidic channel, forming a concave shape under pressure, representing a segment of a blood vessel wall. Precision regarding the physical stimuli can be achieved by a computer-controlled microcirculatory support system integrated with microfluidic devices (Song et al. 2005). It has microfluidic valve and pump units, and the advantages achieved by the integration show a good correlation between levels of shear stress that should be applied to the ECs and pump capacity. The tiny volumes of microfluidic channels have made it challenging to connect "conventional" pumps and microscale channels in parallel. Fluctuation, even due to tube compliance between a pump and a microfluidic device, can make the precise regulation of microfluidic shear stress inefficient. Other types of microfluidic units have been reported enabling pulsatile or oscillatory regulation of shear stress to mimic the *in vivo* situation (Shao et al. 2009) (Fig. 16.2b). Another study included sensor integration in microfluidic channels to precisely measure flow rate, pressure, and shear stress around an endothelial monolayer (Liu et al. 2013). The studies showed an important advantage of the microfluidic strategy: the potential for parallelization and increased throughput over conventional assays. It is also clear that the endothelial monolayer can discriminate pulsatile environments from a static flow condition. However, as yet, the response and mechanism(s) involved cannot be investigated fully, even using microfluidic
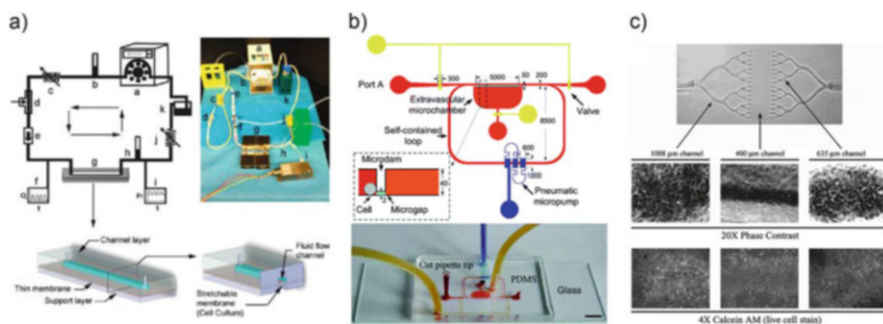

Fig. 16.2 *Formation of an endothelial monolayer and tube-like endothelium in the microfluidic channel*. (**a**) EC culture model integrated with flow loop. (**b**) Integrated microfluidic chip exposed to a pulsatile and oscillatory shear stress. (**c**) Reconstituted functional endothelialized microvascular networks with circular cross sections

approaches. This is because of the complexity of pulsatile flow patterns and also the scale of microfluidic devices.

16.2.1.2 Endothelial Monolayer as Part of In Vitro Blood Vessels

The mechanotransduction studies with shear stress mentioned above revealed interesting features of ECs and/or endothelial monolayers as the interface of a blood vessel and the blood flow within. ECs are reorganized and remodeled actively by shear stress and circulating cytokines, and these effects can be explored precisely with microfluidic regulation. Other factors in recapitulating the functionality of blood vessels include the permeability of the endothelial monolayer, the tube-like morphological characteristics, and interactions between ECs and other neighboring cells. These factors characterize many functions of blood vessels as an important transport system of blood throughout the body (Srigunapalan et al. 2011). When an endothelial monolayer forms in microfluidic channels, the cells cover every surface inside the microfluidic channels, in general, and form a conformal tube-like endothelium. Microfabrication techniques can draw two-dimensional (2D) pipelines and complex networks, with square, rectangular, or hemi- or fully circular cross sections (Borenstein et al. 2010) (Fig. 16.2c). Cell-cell interactions and tube-like morphological characteristics can also be examined with various antibodies and signaling molecules to test the effects of drugs and to study the interaction between different human vascular cell types (àvan der Meer et al. 2013).

However, these models lack the possibility of exploring one of the most important features of blood vessels: the barrier function for oxygen, nutrients, molecules, and/or cells. The barrier is semiselective and can control the passage of many materials into or out of blood. However, molecules cannot pass through an endothelial monolayer cultured on a channel surface. Layers *beyond* the endothelium are needed to support molecular transport through the monolayer. In blood vessels *in vivo*, layers with smooth muscle cells (SMCs), fibroblasts, and ECM are located outside the endothelial monolayer. They support the monolayer, providing rigidity for the vascular tube structure.

Hydrogels mimicking ECM have been used as suitable supports for endothelial monolayers in microfluidic channels (Wong et al. 2012; Zheng C et al. 2012; Zheng Y et al. 2012; Bischel et al. 2013; Baker et al. 2013). With ECM, the endothelial monolayers finally form endothelial tubes mimicking endothelium in blood vessels. Type I collagen hydrogel, fibrin, and Matrigel have been used widely in many applications to form tubes attached to ECM. They are perfusable by medium containing nutrients, oxygen, proteins, chemical reagents, drugs, and cells. Microfabrication techniques can also make planar structures behave as complex 2D networks, with tube-like cross sections of ECM attached to the endothelial monolayer (Zheng C et al. 2012; Zheng Y et al. 2012; Bischel et al. 2013) (Fig. 16.3). Not only permeability but also other important factors regarding the physics of blood vessels, thrombosis and angiogenesis, can be analyzed *in vitro*. Compared with "traditional" *in vitro* assays with ECs, this is physically relevant to *in vivo* vascular networks, with liquid flow inside and ECM materials outside (àvan der Meer et al. 2013). A gradient of diffusive molecules
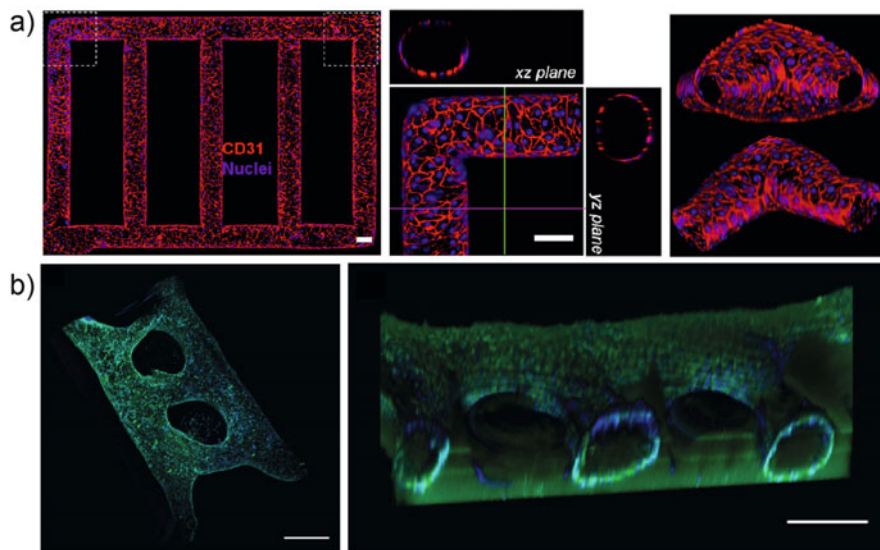
Fig. 16.3 *Microvessel formation in a microfluidic system incorporating ECM hydrogel.* Microfluidic-based reconstitution of 3D lumens with circular cross-sectional geometries within ECM hydrogel

three-dimensionally within ECM can be applied to the formed 3D endothelial tubes, to mimic the guidance provided by the molecules in many processes within development, angiogenesis, and cancer (Baker et al. 2013). The applied molecules, distributed spatiotemporally within 3D ECM, and their diffusion patterns, are affected by the tubes. At the same time, the diffusion patterns also affect the tubes, guiding locations and driving morphogenetic events, such as angiogenesis.

16.2.2 3D Capillary Formation Assays Using Microfluidic Devices

16.2.2.1 3D Angiogenesis Assay

The microfluidic assays described can be applied to the basic morphogenetic analysis of blood vessels; however, they lack any ability to induce fully functional structures due to the high stiffness of the ECM used to support the fluidic channel structure (Borenstein et al. 2010) and limited ECM space around the endothelial tubes (ávan der Meer et al. 2013). Hydrogel-incorporating microfluidic devices have been developed to investigate the morphogenetic nature of blood vessels, angiogenesis (Chung et al. 2009b; Shin et al. 2011, 2012; Jeong et al. 2011a, b; Song and Munn 2011), and vasculogenesis (Kim et al. 2013; Whisler et al. 2014; Chen et al. 2013). Angiogenesis denotes the formation of new blood vessels from preexisting vessels, and vasculogenesis is the process that occurs when there are no preexisting vessels. Angiogenesis is a normal and vital process in growth and development. It is also a fundamental step in the transition of tumors (Penn

2008). Many mechanical and chemical factors regulating angiogenesis have been revealed, including VEGF and angiopoietins. For diseases due to poor or abnormal vascularization, such as cardiovascular diseases and tumors, angiogenesis has already emerged as an effective therapeutic target.

When ECs are seeded in a hydrogel incorporating microfluidic channels, the seeded cells attach to the hydrogel and form an endothelial monolayer. Type I collagen, Matrigel, and fibrin are used widely to mimic the ECM surrounding blood vessels. Chung et al. (2009b) developed an optimized protocol to induce sprouting angiogenesis into type I collagen hydrogel and confirmed its 3D tube-like structure by confocal microscopy (Fig. 16.4a). They found that a VEGF gradient induced sprouting angiogenesis formation but also that additional signals are probably necessary to determine which segments sprout, which dilate, and which remain quiescent. Combined treatment with angiogenic factors and VEGF can induce a complex and integrated network of blood vessels, angiopoietin (Shin et al. 2011),
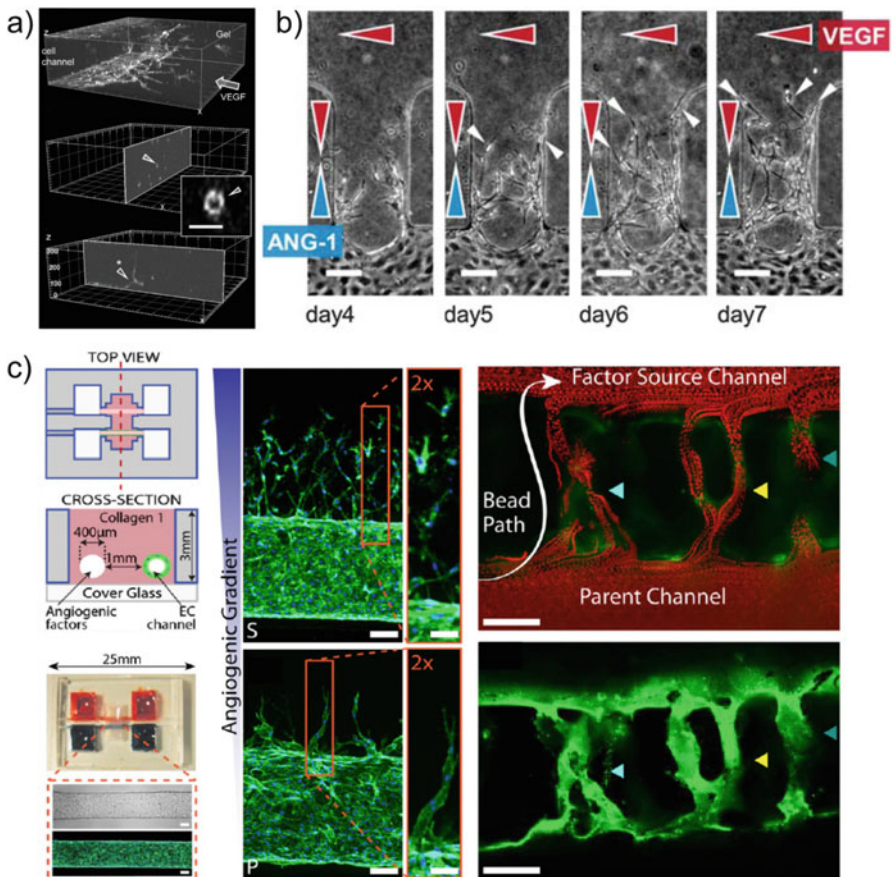

Fig. 16.4 *3D sprouting angiogenesis in a microfluidic system.* (**a**) (**b**) (**c**) Formation of 3D capillary sprouting into ECM hydrogel under gradients of various angiogenic factors

and sphingosine (Nguyen et al. 2013) (Fig. 16.4b, c). Microfluidic schemes enable *in situ* monitoring of collective and complex angiogenic procedures with enhanced views. Many interesting phenomena involved in 3D angiogenesis have been reported, including 3D cooperative migration of tip cells (with filopodia-like protrusions) and stalk cells (with apical-basal polarity), lumen formation, and the connection and division of capillary branches with perfusion inside the lumens. Anti- or proangiogenic drugs can be evaluated (Nguyen et al. 2013; Lee et al. 2014), as can angiogenic inhibitors and matrix metalloproteinase inhibitors.

Another study on the combined effects of growth factors and fluid shear stress also revealed the important nature of capillary angiogenesis (Shin et al. 2011). Endothelial monolayers are always under fluidic shear stress and interstitial (leaking) flow, and these effects, combined with various growth factors, are now under investigation. It also makes the assay more quantitative and robust if these issues are considered. Studies on local and global gradient formation, 3D diffusion, convection, and interactions with cells (barrier function and materials consumption) can provide a basis for understanding the true distribution of the many factors involved and how they interact with blood vessels (Jeong et al. 2011b; Song and Munn 2011; Helm et al. 2005). Local gradients of the factors regulate angiogenic structures but are also interrupted by endothelial monolayers. When the monolayer prohibits the molecule(s) from passing through, a rapid change in concentration occurs across the monolayer. When the cells in the monolayer take up the molecule (s), a local gradient forms around the cells. Studies should also consider the synergistic interaction of molecule distributions under convection by blood flow and interstitial flow and interactions with cells and endothelial monolayers. It is still a very challenging issue to understand the true mechanism of angiogenesis.

Coculturing with stromal fibroblast, pericytes, or SMCs (Kim et al. 2013; Mack et al. 2009) has also been investigated to confirm these cells' roles in adaptive remodeling of capillary structures. Mack et al. (2009) found that KLF2-expressing ECs cocultured with SMCs showed significantly reduced SMC migration. The cells form vascular smooth muscle, the majority of the wall of blood vessels outside the endothelial monolayer, to support the lumen structure and stabilize ECs. Coculturing enables a deep investigation of complex interactions between blood vessels and neighboring cells in development and many diseases, which cannot be achieved readily with conventional cell culture assays.

16.2.2.2 3D Vasculogenesis Assay

The hydrogel-incorporating vasculogenesis assays developed have a similar structure to angiogenesis assays. However, vasculogenesis assays usually involve culturing ECs embedded in ECM hydrogel. Before the development of hydrogel-incorporating microfluidic devices, the differentiation of angiogenesis and vasculogenesis procedures in conventional *in vitro* assays was almost impossible, while current vasculogenesis assays can form very complex, highly connected, functional, and perfusable 3D microvascular networks (Kim et al. 2013) (Fig. 16.5a). The assays show strong barrier function and long-term stability of the networks. Precise control of growth factors and coculturing with stromal
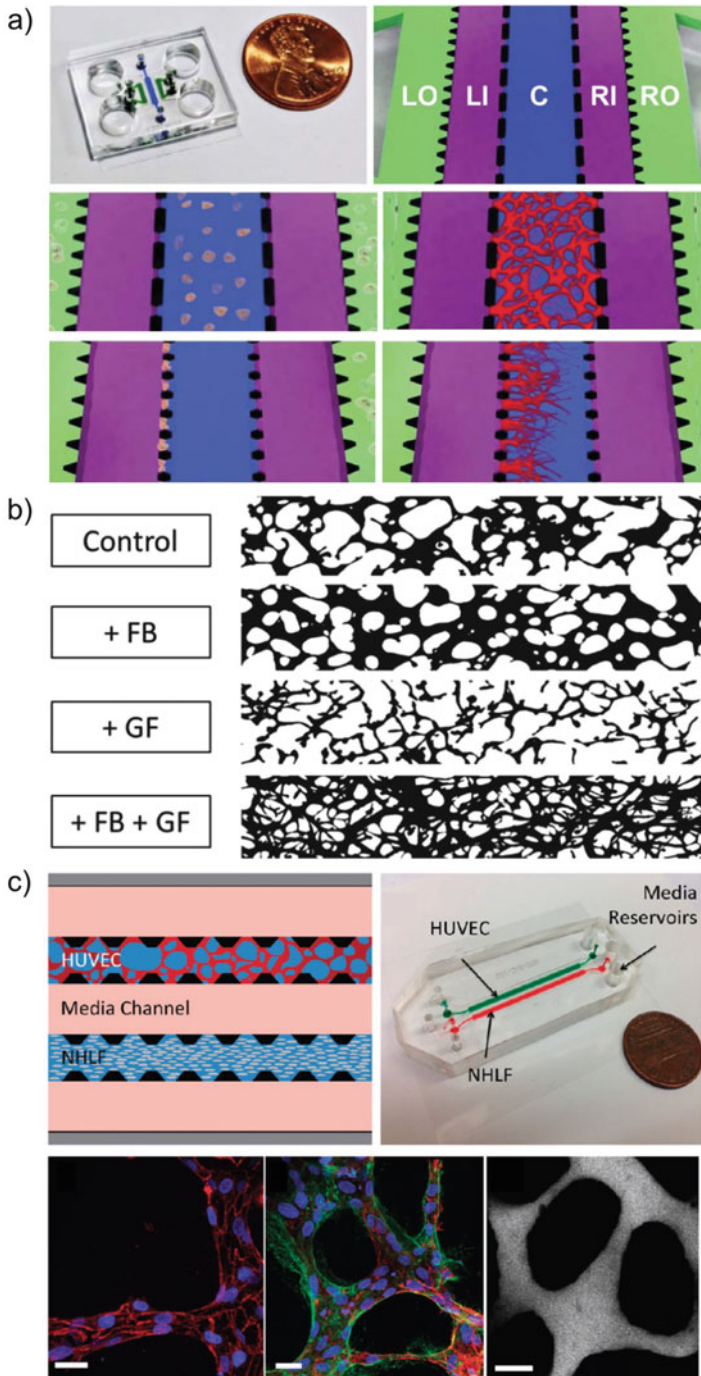
Fig. 16.5 *Vasculogenesis in a microfluidic system.* (**a**) (**b**) (**c**) 3D formation of microvascular network within ECM hydrogel in coculture with stromal cells such as fibroblasts

fibroblasts, SMCs, or pericytes help to form stable but complex vascular structures (Whisler et al. 2014) (Fig. 16.5b). These assays are also used for tumor transendothelial studies, intra- and extravasation (Chen et al. 2013) (Fig. 16.5c). This will be discussed in detail later.

16.2.3 Vascular Formation Under a Gradient of Growth Factors in Microfluidic Devices

The generation and precise control of biochemical factor distributions is one of the most useful features of microfluidic devices. Concentration gradients formed around cells induce 2D chemotactic responses of ECs. ECs exposed to growth factor gradients displayed an increased number of filopodia and an asymmetric distribution of filopodia, with enhanced numbers of protrusions present along the leading edge (Shamloo et al. 2008). The absolute concentration of VEGF enhanced the total number of filopodia, while the steepness of the VEGF gradient induced filopodia localization, cell polarization, and subsequent directed migration.

A study on chemotactic responses in three-dimensionally aligned endothelial monolayer-induced angiogenic responses of ECs, in ECMs with varying densities and stiffness, has been reported. The mechanical properties of ECMs could regulate angiogenic sprout and lumen formation when combined with gradients of vascular growth factors (Shamloo and Heilshorn 2010). Microfluidic chips enable the precise regulation of gradients and *in situ* investigations and monitoring of complex 3D cellular morphogenesis. They also enable quantitative studies on chemical release systems to deliver growth factors, therapeutic agents, and drugs. Kim et al. (2012) evaluated the proangiogenic potential of factors secreted from encapsulated fibroblast cells on an endothelial monolayer. An extensive network of functional lumen structures was made *in vitro*, perfused with blood cells.

The true distribution of a chemical gradient around an endothelial monolayer is not easy to define because many factors are involved synergistically, as described in Section 16.2.2 (Jeong et al. 2011b; Song and Munn 2011; Helm et al. 2005). Pioneering works to precisely estimate actual distributions of chemical gradients by computational modeling have been reported, with experimental verification by monitoring 3D sprouting angiogenesis from an endothelial monolayer (Jeong et al. 2011b). Moreover, molecular diffusion perturbed by the endothelium helps to regulate the degree of gradient around the endothelial monolayer. The orchestration of multiple growth factors (e.g., VEGF and angiopoietins) has also been simulated, with experimental confirmation by 3D cooperative migration of tip and stalk cells and the lumen structures formed (Shin et al. 2011).

16.2.4 ECM Materials for Microfluidic Angiogenic Studies

Various ECM materials, including type I collagen, fibrin, hyaluronic acids (HA), Matrigel, fibronectin, and gelatin, have been used in angiogenic studies as a base for

Table 16.2 ECM materials for angiogenic studies

ECMs	Applications	References
Type I collagen	Basic ECM for angiogenic sprouting, angiogenesis, blood vessel formation, thrombosis study, formation of brain blood barrier model, and cancer metastasis study	Zheng C et al. (2012), Zheng Y et al. (2012), Nguyen et al. (2013), Sudo et al. (2009), and Chrobak et al. (2006)
Fibrin	Basic ECM for angiogenic sprouting, angiogenesis, vasculogenesis, and cancer metastasis study	Chen et al. (2013), Yeon et al. (2012), and Carrion et al. (2010)
HA-collagen	Basic ECM for cell migration	Jeong et al. (2011a)
Matrigel	Reducing cell movement in angiogenesis, blood vessel formation, and cancer metastasis study	Chaw et al. (2007), Dai et al. (2011), and Bischel et al. (2013)
Fibronectin	Enhancing cell movement in angiogenesis, cell migration, and cell adhesion	Shamloo et al. (2011) and Young et al. (2007)
Gelatin	Sacrificial element for blood vessel formation	Golden and Tien (2007) and Chung et al. (2010)

ECs (Table 16.2). Matrigel has been widely accepted as an ECM in 2D angiogenic studies, because ECs form tube-like "2.5-dimensional" structures on Matrigel (Soriano et al. 2004; Kieda et al. 2006). However, the structure formed is far from that of the *in vivo* vasculature. It grows on the ECM (Matrigel), with biased polarity, of the top to the medium and bottom to the ECM, which is the opposite of the *in vivo* vasculature, which has ECM outside containing blood flow inside.

Pioneering microfluidic works incorporating ECM have enabled the study of angiogenic responses in ECM. The vasculature in the ECM was made by combined effects of collective and cooperative migration of ECs, with active remodeling of the ECMs by soluble proteases and matrix metalloproteinases (MMPs). Type I collagen and fibrin are the major ECMs used for microfluidic angiogenesis and vasculogenesis studies. In some studies, the ECMs have been modified with other ECM components, such as laminin and fibronectin, to mimic the ECM composition of specific target organs. For example, the ECM of adult brain tissue has a unique composition. It has lecticans, proteoglycans with a lectin domain and a hyaluronic acid-binding domain. Hyaluronic acid and tenascin family adhesive/antiadhesive proteins are also abundant (Ruoslahti 1996). Brain also has high concentrations of matrix proteins common in other tissues, such as laminin and collagen, but only near the brain vasculature. The unique composition of brain tissue has trophic effects on specific brain cells and in diseases. ECM-incorporating microfluidic studies enable the exploration of the unique effects of cellular microenvironment on specific cells, precisely mimicking *in vivo* tissue physiology (Shin et al. 2014).

16.3 Coculture of ECs and the Other Cell Types in Microfluidic Devices

16.3.1 Using ECs as a Vascular Microenvironment for Other Cell Types: Mesenchymal Cells

16.3.1.1 Proangiogenic Effects of Fibroblasts

Fibroblasts are a type of mesenchymal cell; they play important roles in the synthesis and maintenance of ECM. Since it was first reported that factors produced by mouse embryo 3 T3 fibroblasts stimulated the proliferation of cultured ECs (Birdwell et al. 1977), fibroblasts have been cocultured with ECs to promote angiogenesis in 3D culture models. Montesano et al. (1993) investigated the effect of various fibroblasts, such as Swiss 3 T3, BALB 3 T3, NIH 3 T3 cells, and epithelial cells, such as MDCK and intestine 407 cells, on the angiogenic responses of bovine microvascular ECs. Their results revealed that only Swiss 3 T3 fibroblasts, among the various fibroblasts and epithelial cells tested, were able to induce an angiogenic response when cocultured with the ECs in their 3D angiogenesis model. This proangiogenic effect of fibroblast-derived factors was also used in recent *in vitro* angiogenesis models created in microfluidic devices (Yeon et al. 2012; Kim et al. 2012, 2013; Moya et al. 2013). Fibroblasts are known to produce various proangiogenic soluble factors, including VEGF, bFGF, IL-8, TGFα, TGFβ, HGF, angiogenin, Ang-1, TNF, and PDGF (Mansbridge et al. 1999; Hartlapp et al. 2001; Hurley et al. 2010; Newman et al. 2011). In addition to the growth factors secreted by fibroblasts, fibroblast-derived matrix proteins also play important roles in angiogenic responses (Berthod et al. 2006). Newman et al. (2011) identified collagen I, procollagen C endopeptidase enhancer 1, SPARC, ig-h3, and insulin growth factor-binding protein 7, which were expressed in fibroblasts and were essential for EC lumen formation.

16.3.1.2 Capillary Stabilization by Pericytes or SMCs

ECs have been cocultured with fibroblasts because of their ability to secrete various proangiogenic factors. In the context of vascular tissue engineering, not only the formation but also the stabilization of capillary networks is important to construct long-lasting capillary networks *in vitro*. Other types of mesenchymal cells, such as SMCs and pericytes, may be expected to interact with ECs because they localize around blood vessels *in vivo*. Pericytes wrap themselves around small vessels, such as capillaries <10 μm in diameter, as well as microvessels, 10-100 μm in diameter, while SMCs form many layers around larger blood vessels (Wanjare et al. 2013). These perivascular cells have different effects on angiogenesis compared with fibroblasts. SMCs and pericytes inhibit EC proliferation and angiogenic responses in cocultures as well as *in vivo* (Orlidge and D'Amore 1987; Wanjare et al. 2013; Crocker et al. 1970; Shepro and Morel 1993; Díaz-Flores et al. 2009). Activated TGFβ was the key factor for the inhibitory effect of pericytes on EC growth in EC-pericyte coculture (Antonelli-Orlidge et al. 1989). Saunders et al. (2006) found that EC-derived TIMP-2 and pericytes-derived TIMP-3 coregulated capillary tube

stabilization. Pericytes-derived TIMP-3 inhibited vascular sprout formation by ECs in an *in vitro* angiogenesis model, while TIMP-3 contributed to capillary tube stabilization in an *in vitro* vasculogenesis model. Numerous studies suggest that Ang-1/Tie2 signaling between pericytes and ECs is required for vessel stabilization (Armulik et al. 2005).

16.3.1.3 Use of Stem Cell Source for EC-Pericyte Coculture

Mesenchymal stem cells (MSCs) have also been used for coculture with ECs. Because it is difficult to establish primary culture of pericytes, MSCs have been used to derive pericytes. Au et al. (2008) demonstrated that MSCs derived from human bone marrow differentiated into perivascular cells, which stained positively for α-smooth muscle actin (α-SMA), a pericyte marker, *in vivo* when coimplanted with ECs. Although capillary structures quickly regressed when only human umbilical vein endothelial cells (HUVECs) were implanted, pericytes differentiated from MSCs stabilized capillary structures when coimplanted with ECs, resulting in the maintenance of functional capillary structures for >130 days *in vivo*. Furthermore, these capillary structures constricted in response to stimulation by endothelin-1, an endogenous vasoconstrictive peptide.

Differentiation of MSCs into pericytes has also been confirmed in recent studies using *in vitro* culture models. Carrion et al. (2010) performed HUVEC-MSC coculture in an *in vitro* vasculogenesis model created in a microfluidic device and demonstrated that MSCs localized around the periphery of the vessel structure formed by HUVECs. These MSCs became positive for α-SMA and eventually wrapped themselves around the forming vessels. Yamamoto et al. (2013) reported that HUVEC-MSC direct contact was required for the differentiation of MSCs into pericytes in 2D culture. In this study, HUVEC-MSC coculture was also performed in a microfluidic device. Results showed that MSCs attenuated vascular sprout formation of HUVECs in the early stage of 3D angiogenesis as well as the extension of microvascular networks in the later stage, suggesting that MSCs have stabilization effects on the formation of microvascular networks. Jeon et al. (2014) also reported that HUVEC-MSC direct contact was required for the differentiation of MSCs into pericytes in 3D culture. In this study, HUVEC-MSC coculture was performed in an *in vitro* vasculogenesis model created in a microfluidic device. MSC-derived α-SMA-positive cells colocalized with HUVECs and wrapped around the newly formed microvascular networks, which resulted in a smaller vessel diameter versus HUVECs alone. According to the recent advances in *in vitro* culture systems mentioned here, there is increasing evidence that HUVEC-MSC coculture is promising for the development of functional, physiological, and long-lasting microvascular networks *in vitro*.

16.3.2 Using ECs to Create a Vascular Microenvironment for Other Cell Types: Neural Stem Cells

Cellular microenvironments include signaling molecules, small molecules, neighboring cells, ECMs, small organs, and mechanical stimuli. These provide structural and trophic support for cells, regulating their life cycle. The vascular microenvironment, one of the cellular microenvironments formed by blood vessels, supplies neighboring cells with nutrients, oxygen, and other soluble factors and actively regulates their life cycle. For example, neural stem cells (NSCs) reside in a brain vascular microenvironment. Brain vessels play an important role in maintaining an appropriate balance between NSC self-renewal and differentiation (Shin et al. 2014). NSCs in the subventricular zone maintain contact with the brain vasculature, maintaining its multipotency by self-renewal. Brain tissue damage by, for example, ischemic stroke, neurodegenerative diseases, and brain tumors, promotes homing and integration of NSCs. The NSCs integrated into the damaged brain tissue choose the proper differentiation lineage into neurons, astrocytes, or oligodendrocytes. In the process, the vascular microenvironment plays an essential role, and a study of the microenvironments may facilitate basic research on the development of potential therapies for degenerative disorders.

Shin et al. (2014) developed a microfluidic assay to reconstitute NSC-vascular microenvironments with a 3D brain vasculature and ECMs. The reconstituted vascular microenvironment regulated self-renewal, proliferation, and colony formation of NSCs and suppressed neuronal generation. It also promoted NSC differentiation into astrocytes and oligodendrocytes. Cell culture on a microfluidic scale was shown to emphasize cell-cell communications, by enrichment of secreted factors in the tiny microscale environment. It dramatically enhanced the proximity effect on NSCs from the brain vasculature, pushing NSCs to differentiate into astrocytes to form a brain-specific vascular structure, the blood-brain barrier (Shin et al. 2014).

Not only NSCs but also the other stem cells require an assay for assessing vascular microenvironments. Many *in vivo* models from mouse and Drosophila have helped to explore the role of stem cell microenvironments on stem cell maintenance and activity. However, details of morphogenetic processes of stem cells under specific microenvironments cannot be systematically monitored *in vivo*, raising the need for an *in vitro* assay regulating the microenvironmental factors. Another challenge involves demonstrating the combined effects of multiple microenvironmental factors. For example, low oxygen tension induces angiogenesis by increased expression of various angiogenic factors. The expressed angiogenic factors not only affect ECs but also stem cells and other stromal cells. Newly formed vasculature also affects the previously low oxygen tension and other stromal cells. These cell-cell and cell-microenvironment interactions are complex, affecting each other in a very complicated manner. Reconstitution of each microenvironmental factor and their combinations markedly increases the chances of developing new knowledge.

16.3.3 Using Other Cell Types to Interact with ECs: Immune and Cancer Cells

Cancer occurs by genetic damage to normal cells, leading to uncontrolled proliferation, resulting in a primary heterogenic tumor. The tumor induces angiogenesis and recruits new blood vessels to enhance its nutrient and oxygen supply. Some cancer cells acquire the ability to invade neighboring ECMs and/or tissues, and finally intravasate into lymphatic or blood vessels. They become circulating tumor cells and extravasate to secondary tumor sites. These extravasated tumor cells may die or become dormant due to the different microenvironment of the secondary site from that of the primary site, but sometimes proliferate and form micrometastases, resulting in secondary tumor site(s). Proliferation in the secondary tumor site also requires angiogenic responses from neighboring blood vessels. Angiogenesis is thus an essential process for a cancer to acquire malignancy by metastasis; it supplies the tumor site with nutrient and oxygen, and also works as a major route for tumor cells to escape from the original site.

Cancer angiogenesis is thus a promising drug target; suppressing it could starve tumor cells and minimize the chances of metastasis. The permeability and 3D structures of blood vessels are related to the metastasis procedure; they also work as a route for drug delivery, directly targeting tumor cells. The whole process of a cancer acquiring malignancy by metastasis has been addressed and defined as described above, but there is still a need for appropriate *in vitro* assays to investigate the individual steps involved in metastasis.

16.3.3.1 Remote Interaction Between Tumor Cells and ECs: Angiogenic Response

A microfluidic platform was developed to evaluate and quantify capillary growth from an endothelial monolayer, when placed in coculture with physiologically relevant cell types, including cancer cells and SMCs (Chung et al. 2009a) (Fig. 16.6a). Three-dimensional capillary structures were grown into type I collagen ECM, under a precisely controlled coculture microenvironment. Breast cancer cells were shown to induce capillary formation, while SMCs suppressed it. A similar interaction between endothelial cells and tumor cells was also realized on the 2D surface of a microfluidic array (Zheng C et al. 2012; Zheng Y et al. 2012). The device offered 16 parallel arrays to study interactions between cells. It successfully provided an accurate spatiotemporal microenvironment in cocultures of multiple types of cells (Zheng C et al. 2012; Zheng Y et al. 2012) (Fig. 16.6b).

16.3.3.2 Remote Interaction Between Tumor Cells and ECs: Antiangiogenic Response

It is now widely recognized that cancer cells induce angiogenesis (Folkman 1971). However, antiangiogenic responses were also observed in *in vitro* coculture models. Kalchman et al. (2013) reported the coculture of HUVECs and HepG2, a hepatocellular carcinoma cell line. HUVECs and HepG2 cells were cultured in separate microfluidic channels. When HUVECs were cultured alone, they migrated
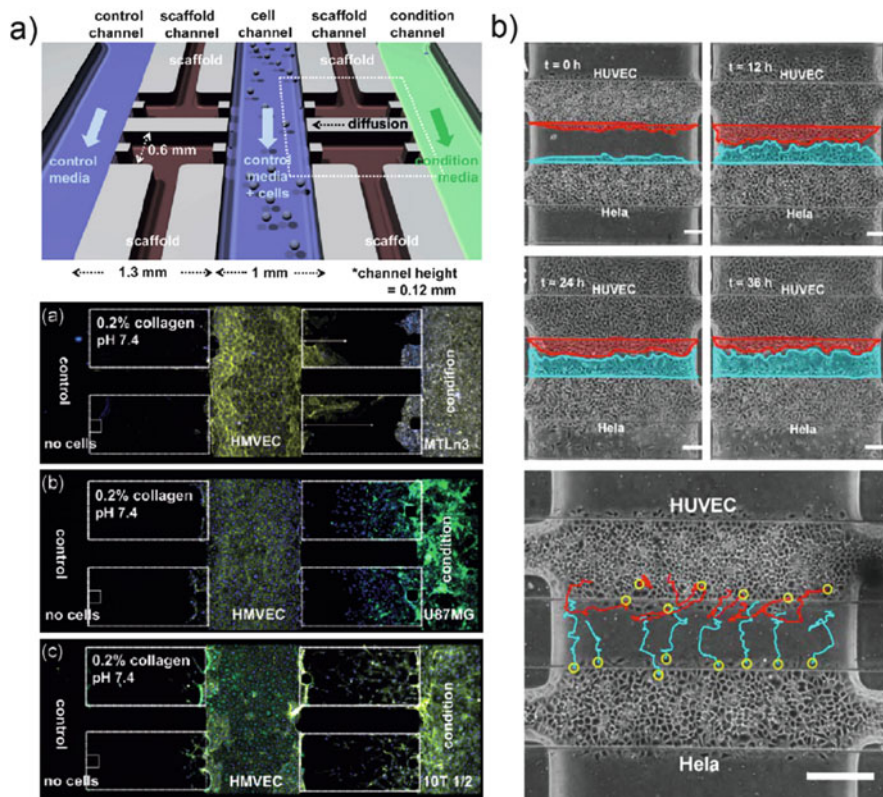
Fig. 16.6 *Interactions between endothelial cells and cancer cells in a microfluidic system.* (**a**) Cancer angiogenesis within a microfluidic system incorporating ECM hydrogel. (**b**) A coculture assay for the quantitative study of the interaction between endothelial cells and cancer cells

into collagen gel and formed microvascular networks. However, when HUVECs were cultured with HepG2, the formation of vascular sprouts was completely inhibited and HUVECs formed monolayers (Fig. 16.7). Instead, HUVECs induced the formation of protrusions by HepG2, indicating that there are interactions between HUVEC and HepG2, although no angiogenic response was observed. These results suggest that tumor-induced angiogenesis can be confirmed in culture models, depending on the cell lines used. Further investigations are needed to clarify interactions between cancer cells and ECs in terms of angiogenesis.

16.3.3.3 Direct Interaction Between Tumor Cells and ECs: Intravasation and Extravasation

Many microfluidic devices have been used to investigate intravasation, entry into the bloodstream, or extravasation, movement out of the blood stream, of tumor cells. A microfluidic device reconstituting the 3D tumor-vascular interface,
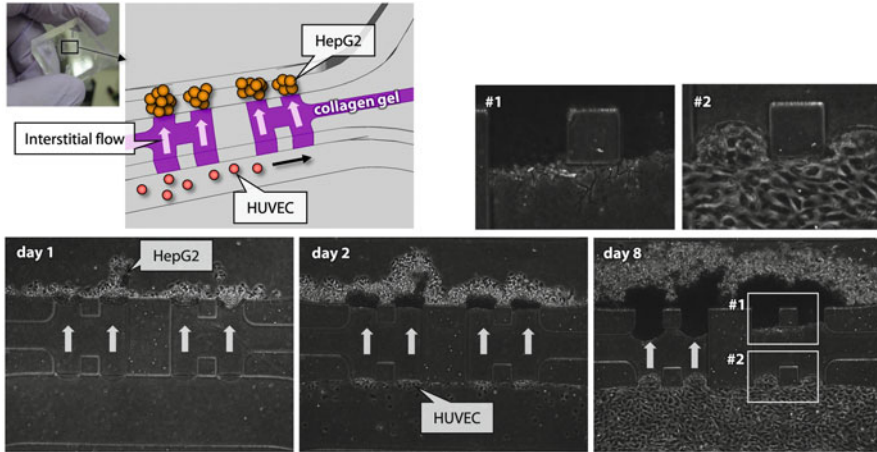
Fig. 16.7 *HUVEC-HepG2 coculture in a microfluidic device.* HepG2 cells were cultured in interstitial flow conditions. HUVECs were added to the other side on day 2. Phase-contrast images #1 and 2 correspond to box # 1 and 2 in the image of day 8

allowing real-time monitoring of cell-cell interactions and precise quantification of endothelial barrier function, such as endothelial permeability has been developed (Zervantonakis et al. 2012) (Fig. 16.8a). The endothelial monolayer was shown to pose a barrier to tumor cell intravasation that could be regulated by factors present in the tumor microenvironment. Another microfluidic device provided an endothelial monolayer culture in a microfluidic device to study the intravascular adhesion of circulating breast cancer cells on an endothelial monolayer at potential sites of metastasis (Song et al. 2009). Spatially restricted stimulation of an endothelial monolayer with the chemokine CXCL12 from the basal side mimicked the organ-specific localization and polarization of chemokines and other signaling molecules, and was shown to affect cancer cell adhesion on the endothelial monolayer.

Another study dealt with the effects of CXCL12 on tumor cell aggregates near the endothelial monolayer (Zhang et al. 2012) (Fig. 16.8b). CXCL12 stimulation damaged the integrity of the endothelial monolayer and attracted cancer cells transmigrating through the monolayer. Thus, the evaluation of antimetastasis agents by inhibiting transendothelial invasion or the adhesion of cancer cells on the endothelial monolayer can be performed with such microfluidic devices. A similar device was developed to mimic tumor cell extravasation, transmigrating across an endothelial monolayer and migrating into a hydrogel, to model the extracellular space (Jeon et al. 2013). Precise measurement of endothelial permeability and *in situ* monitoring of cancer cell transmigration can help in understanding permeability changes during tumor cell intra/extravasation events.

A tumor extravasation study in a microfluidic device was enhanced dramatically by reconstituting complex 3D vascular networks (Chen et al. 2013) (Fig. 16.8c).
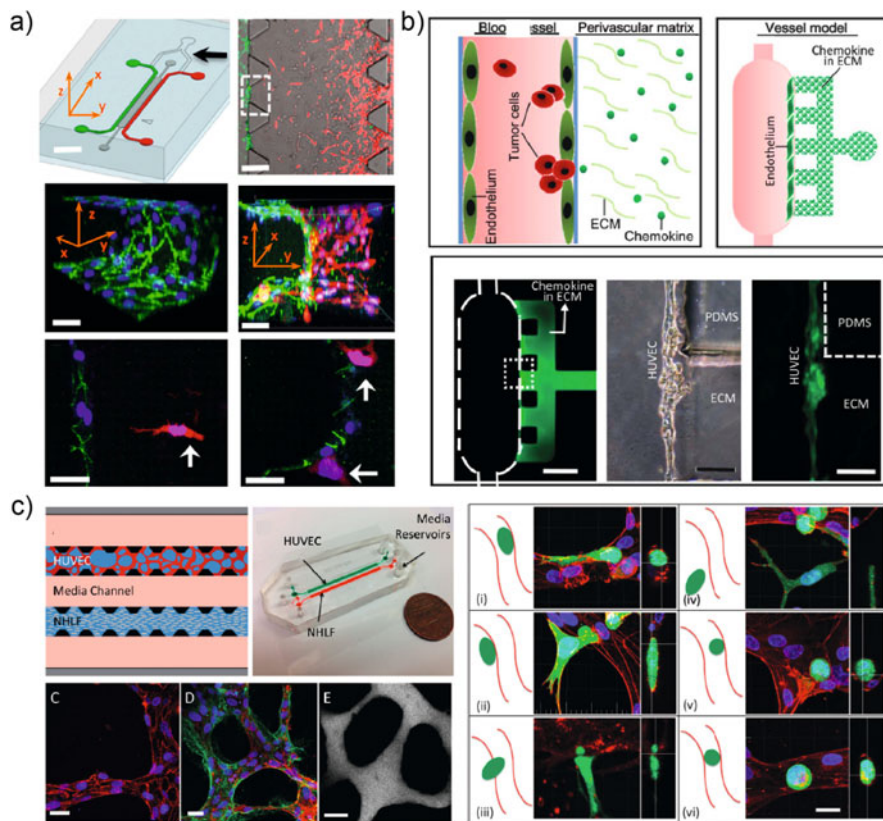
Fig. 16.8 *Intravasation and extravasation in an in vitro model*. (**a**) Microfluidic tumor-vascular interface model for intravasation. (**b**) Blood vessel formation for the study of transendothelial migration of cancer cells. (**c**) Microfluidic microvascular network for the study of tumor cell extravasation

The network was made by vasculogenesis in a fibrin ECM, having tight endothelial junctions with low vessel permeability. Extravasation efficiency was increased by inflammatory cytokine stimulation and trapping cancer cells in complex junction structures. Dynamic interactions of tumor cells and ECs were monitored: protrusion formation, extrusion of the cell body through a very small opening in the endothelial barrier, and nuclei transmigration. These methods can be applied not only to tumor cell metastasis studies but also to the spatiotemporal analysis of the inflammatory response to characterize transendothelial migration behaviors of neutrophils under the influence of various inflammatory stimuli (Han et al. 2012).

16.4   Vascularization of Reconstructed Tissues

16.4.1 General Introduction to the Vascularization of Tissue-Engineered Constructs

Tissue engineering is an engineering technique to construct tissues and organs from individual cells (Langer and Vacanti 1993). Since the concept was first reported, many tissue engineering approaches have been studied with a view to producing various tissues and organs *in vitro* and *in vivo* (Khademhosseini et al. 2009; Griffith and Naughton 2002). The development of tissue engineering has allowed the construction of 2D tissues, such as skin substitutes and cornea, and 3D avascular tissues, such as cartilage. Although these engineered tissues have been assessed in clinical trials, construction of 3D vital organs, such as liver, heart, and pancreas, remains a key challenge in this field.

In contrast to 2D tissues, 3D organs include abundant microvascular networks, because cells in 3D tissues require oxygen and nutrients to be provided from blood flow. Because the diffusion distance of oxygen in a living tissue is limited to 100-200 μm (Carmeliet and Jain 2000), microvessels in 3D tissues have to be located within the interval of this critical distance. Thus, complex 3D tissue structures, including microvascular networks, make it difficult to construct 3D vital organs.

To construct 3D vital organs, formation of microvascular networks is important, as is constructing 3D epithelial tissues composed of parenchymal cells. In previous studies, the formation processes of the microvascular networks and the 3D epithelial tissues have been investigated individually. However, in recent studies, not only constructing the microvessels but also incorporating the microvessels within engineered 3D tissues, referred to as vascularization, has become increasingly important. Thus, this vascularization of tissue-engineered constructs is a key challenge in achieving 3D thick tissues *in vitro* (Rouwkema et al. 2008; Lovett et al. 2009; Auger et al. 2013).

16.4.2 Coculture of ECs and Hepatocytes in a Microfluidic Device: A Horizontal Approach

To achieve the vascularization of tissue-engineered constructs, it is essential to investigate the heterotypic interaction between microvascular ECs that form the microvascular networks and host organ cells (*e.g.*, hepatocytes) used to construct the 3D tissues. Liver is one of the most highly vascularized organs and is composed mainly of functional epithelial cells, the hepatocytes. Because hepatocytes assemble into organized 3D tissues *in vivo*, various 3D hepatocyte culture models have been developed (Sudo et al. 2005; Sudo 2014; Hwa et al. 2007; Toh et al. 2007). Culturing hepatocytes in a 3D configuration is useful to maintain differentiated hepatic functions *in vitro*. However, the current techniques fail to incorporate microvascular networks within the 3D hepatocyte tissues. Vascular tissue

engineering has developed separately from liver tissue engineering. The formation of microvascular networks has been investigated with various *in vitro* 3D angiogenesis models, and the molecular mechanism of capillary formation has been studied (Davis et al. 2002; Vailhé et al. 2001). However, little is known concerning the interaction between the resulting capillary structures and other epithelial cell types, such as hepatocytes. Thus, there is a need to establish a coculture system where microvascular ECs and the other parenchymal cell types can interact in a controlled microenvironment with physiological realistic topology and cell organization (*e.g.*, capillary networks formed by ECs and 3D structures, composed of hepatocytes). Because 3D tissue formation and vascularization can be achieved in 3D tissues *in vivo*, there is an increasing demand for *in vitro* models that capture the physiological complexity such as controllable biochemical (*e.g.*, local chemotactic gradient and ECM properties) and biophysical factors (*e.g.*, interstitial fluid flow, shear flow, and mechanical properties of scaffold materials) (Griffith and Swartz 2006; Yamada and Cukierman 2007). Although *in vitro* models that incorporate these features are difficult to create, recent advances in microfluidic technologies (Kim et al. 2007; Khademhosseini et al. 2006) have allowed the development of such an *in vitro* culture model.

A microfluidic platform for the vascularization of 3D tissue-engineered constructs was reported, integrating knowledge from microfluidics, *in vitro* angiogenesis models, and liver tissue engineering (Sudo et al. 2009). The microfluidic device was made of polydimethylsiloxane (PDMS) and a cover glass. There were two microfluidic channels and an intervening collagen gel (Fig. 16.9a). To investigate the interaction between hepatocytes and ECs, hepatocytes were seeded into a microfluidic channel, while ECs were seeded into the channel of the other side. This microfluidic design allowed locating hepatocytes and ECs in close proximity, separated by collagen gel, the width of which was 750 μm (Fig. 16.9b).

First, hepatocytes were seeded in one of the two microfluidic channels and cultured in interstitial flow conditions (day 0, Fig. 16.10). This interstitial flow application during day 0-1 in the beginning of the culture induced 3D tissue formation by the hepatocytes. Hepatocytes maintained a 3D tissue structure even
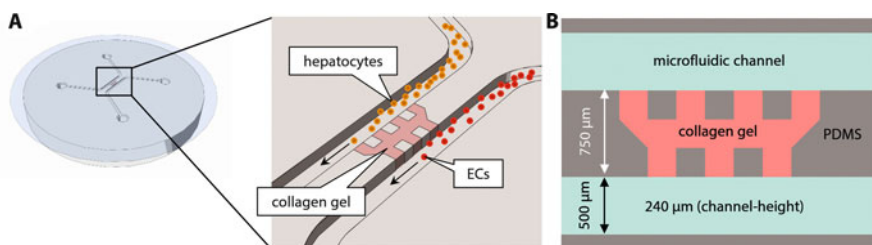

Fig. 16.9 *Microfluidic device for coculture of hepatocytes and microvascular ECs.* (**a**) A schematic image of a microfluidic device made of PDMS and a cover glass. There are two microfluidic channels and an intervening collagen gel. Hepatocytes were seeded in the microfluidic channel while ECs were seeded on the other side for a coculture. (**b**) Enlarged image of collagen gel scaffold in a microfluidic device
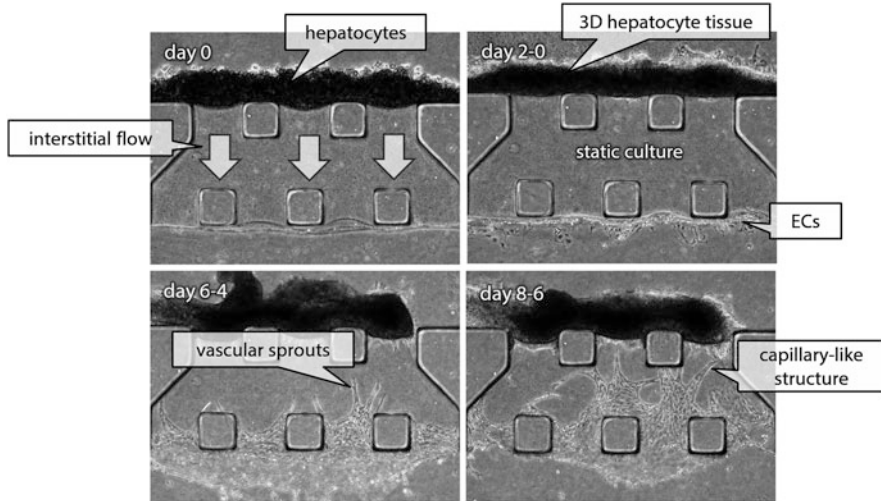
Fig. 16.10 *Phase-contrast images from cocultured hepatocytes and microvascular ECs in a microfluidic device.* Day 0: hepatocytes were seeded into a microfluidic channel and cultured in interstitial flow conditions for 1 day. Day 2–0: Hepatocytes form 3D tissues. ECs were added to the other side of the microfluidic channel, and coculture was performed in static conditions to enhance interactions between hepatocytes and ECs. Day 6–4: ECs formed vascular sprouts in the collagen gel. Day 8–6: Vascular sprouts extended toward the 3D hepatocyte tissues and developed into capillary-like structures

when cultured in static conditions going forward from day 1. ECs were then added to the other side to start the coculture (day 2–0, Fig. 16.10). Although rat ECs failed to form capillary-like structures in the absence of hepatocytes, they formed vascular sprouts extending in the collagen gel only in the case of coculture (day 6–4, Fig. 16.10). Vascular sprouts subsequently extended toward 3D hepatocyte tissues and developed into capillary-like structures (day 8–6, Fig. 16.10). Phase-contrast images of the capillary-like structures formed in the coculture suggested luminal spaces (day 10–8, Fig. 16.11), which was investigated further by confocal microscopy. Cells were fixed and stained for actin to visualize 3D structures. Confocal images revealed that rat ECs formed capillary-like structures with continuous lumens (day 12–10, Fig. 16.11). In summary, hepatocytes formed 3D tissue structures while ECs formed 3D capillary-like structures, penetrating into the collagen gel adjacent to the 3D hepatocyte tissue. Both cell types were initially cultured in separate microfluidic channels but could communicate directly with each other via the gel scaffold located between the channels. The microfluidic platform allowed monitoring of cell behavior and heterotypic interactions. This experimental approach showed the potential for constructing vascularized organs *in vitro*. Further investigations are needed to achieve vascularized liver tissues.
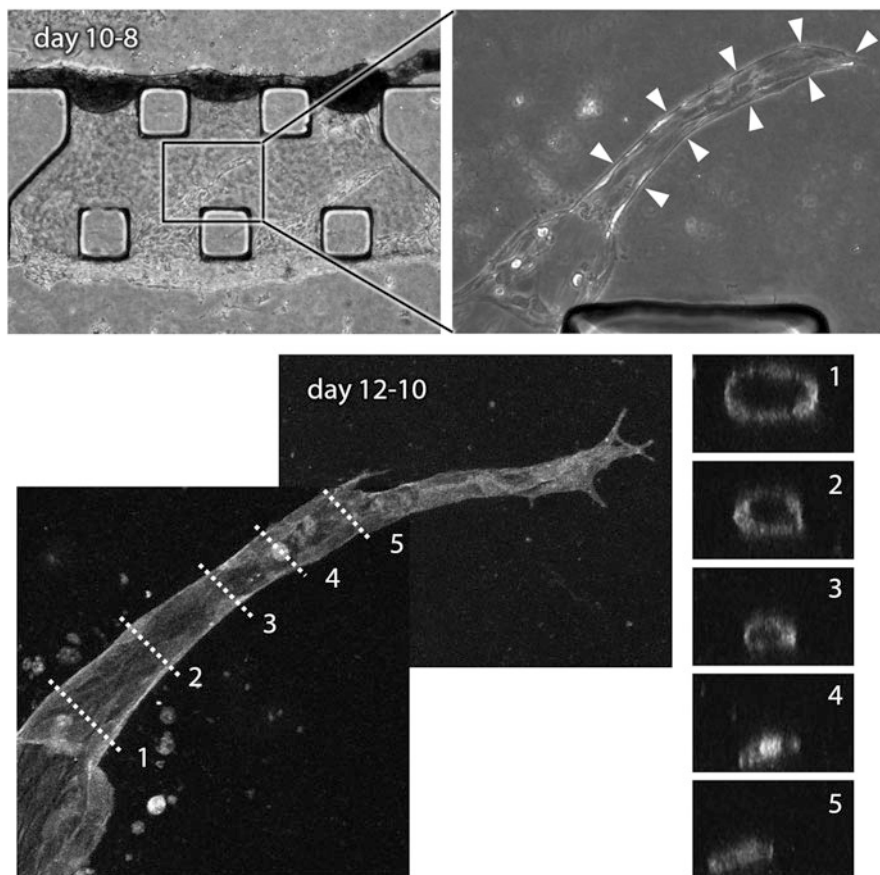
Fig. 16.11 *Capillary-like structures formed in hepatocyte-EC coculture.* Phase-contrast images taken on day 10–8 showed that ECs formed capillary-like structures extending toward the 3D hepatocyte tissues (arrowheads). Day 12–10: Cells were fixed on day 12–10 and stained for actin. A projection image was reconstructed from *z*-stack confocal images. Images #1-5 are cross section images corresponding to the dotted lines #1-5

16.4.3 Coculture of ECs and Hepatocytes Using a Microporous Membrane: A Vertical Approach

Another approach for constructing vascularized tissues is a method using microporous membranes. The microfluidic approach mentioned in Section 16.4.2 is a method to combine liver and vascular tissues horizontally. However, liver and vascular tissues can also be combined vertically, by stacking microporous membranes.

Heterotypic interactions between hepatocytes and ECs were investigated in a coculture model using microporous membranes. First, small hepatocytes (SHs), hepatic progenitor cells, were cultured on microporous membranes. SHs grew on
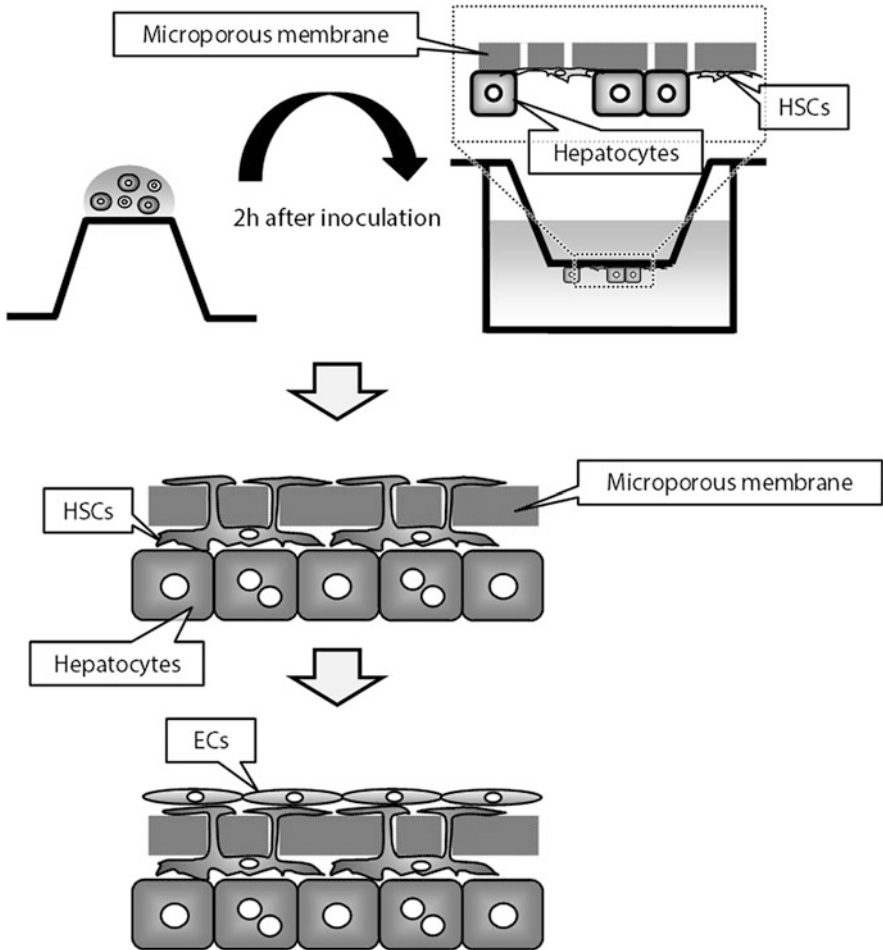
Fig. 16.12 *A triculture model using a microporous membrane*. First, SHs and HSCs were seeded on the bottom surface of a microporous membrane. The membrane was turned over in the holding well after 2 h. The SHs proliferated and formed colonies within 2 weeks in culture, while HSCs located between the SH colonies and the membrane. ECs were seeded onto the top surface of the membrane on day 14, resulting in the HSC-mediated layered architecture of SHs and ECs

the membranes, and the membranes were stacked onto other membranes to construct 3D stacked structures (Sudo et al. 2005). This 3D culture model was extended to coculture with ECs and hepatic stellate cells (HSCs), resulting in a 3D triculture model, established using microporous membranes, to create the functional unit of proximal layers of hepatocytes, HSCs, and ECs (Kasuya et al. 2011) (Fig. 16.12). HSC behavior was controlled by the membrane pore size, which was key for achieving proximal cell layers. With a specific pore size, the HSCs intercalated between layers of hepatocytes and ECs. When only cytoplasmic processes of

quiescent HSCs were adjacent to ECs, while the HSC bodies remained on the side of the hepatocytes, the ECs changed morphologically and were capable of long-term survival. HSCs mediated communication between hepatocytes and ECs in terms of EC morphogenesis. This triculture model allowed investigating the roles of HSCs as both facilitators and integrators of cell-cell communication between hepatocytes and ECs and is useful for investigating heterotypic cellular communication *in vitro*. However, in terms of vascularization of the hepatocyte tissues, ECs in the triculture model formed monolayers rather than capillaries. Thus, next, this triculture model was modified to have ECs forming capillary-like structures.

To modify the hepatocyte-HSC-EC triculture model, the effects of direct HSC-EC contacts on EC capillary morphogenesis were investigated using the triculture model where HSC behavior was controlled spatially to achieve HSC-mediated proximal layers of hepatocytes and ECs. EC capillary morphogenesis was induced by overlaying Matrigel on an EC layer (Fig. 16.13). Direct HSC-EC contacts inhibited EC capillary morphogenesis, suggesting that the HSC-EC contacts may be an important factor in capillary formation (Kasuya et al. 2012a). Next, the hypothesis that, in addition to spatial control, temporal control of HSC behavior is also important in achieving capillary morphogenesis in the triculture was tested. ECs responded to the induction of capillary morphogenesis before the formation of direct HSC-EC contacts, while the ECs remained to form monolayers when capillary morphogenesis was induced after the HSC-EC contacts were established (Kasuya et al. 2012a) (Fig. 16.13). When capillary morphogenesis was achieved in the triculture, HSCs tended to preferably localize near the preformed capillary-like structures, resulting in reconstruction of liver sinusoid-like structures. In these structures, maturation of the hepatocytes was induced. These findings indicated that control, both spatially and temporally, of HSC behavior is a key engineering strategy for the vascularization of engineered liver tissue *in vitro*.

In liver sinusoids, HSCs line the outer surface of microvessels to form a functional unit with endothelia and hepatocytes. To reconstruct a functional liver tissue *in vitro*, recapturing the HSC-lined sinusoidal structures is essential. We previously demonstrated capillary formation in triculture where a Matrigel angiogenesis assay and SH-HSC coculture were combined using polymeric microporous membranes. However, the large thickness and low porosity of the membranes limited the heterotypic cell-cell interactions that are essential in forming HSC-lined structures. In addition to the achievement of capillary formation in the triculture, we previously established a stack-up culture method for reconstructing 3D hepatic tissues using thin and highly porous poly (D,L-lactide-co-glycolide acids) (PLGA) membranes (Kasuya et al. 2012b). We thus focused on the effective use of the PLGA microporous membranes in a hepatocyte-HSC-EC triculture to reconstruct the HSC-lined liver sinusoidal structures *in vitro*. First, the formation of EC capillary-like structures was induced on Matrigel-coated microporous PLGA membranes (Fig. 16.14). Then, the membranes were stacked on hepatic organoids
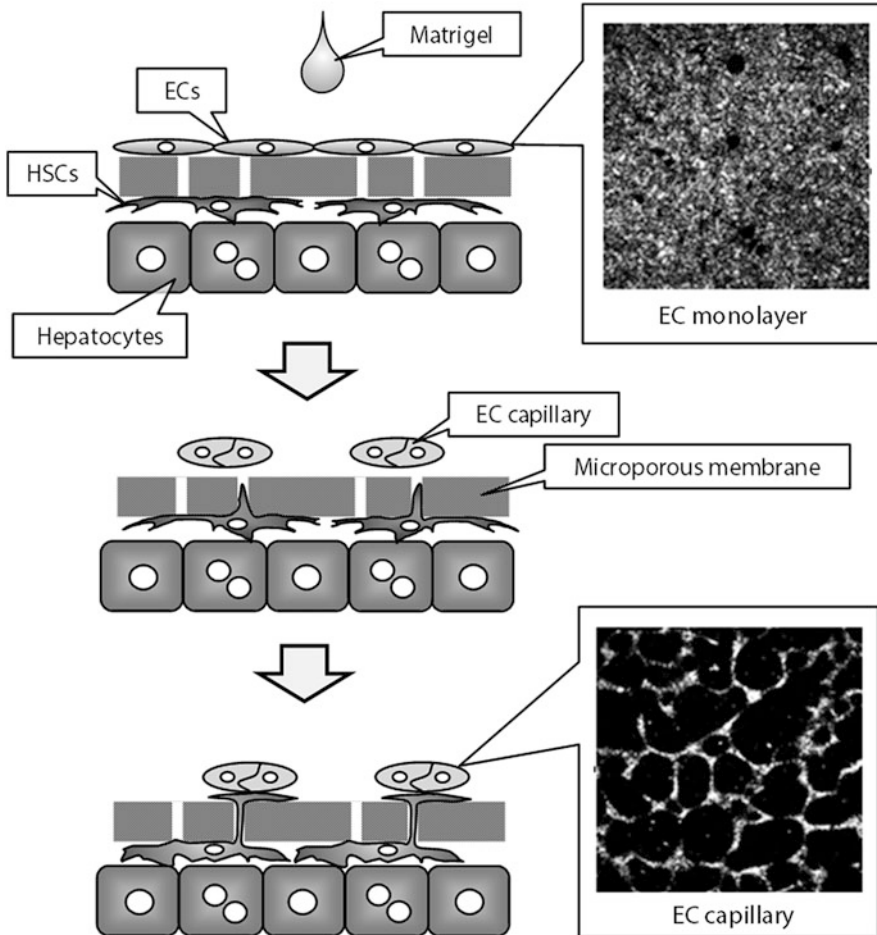
Fig. 16.13 *A triculture model with modification of capillary morphogenesis.* First, a SH-HSC-EC triculture model was constructed, in which ECs formed a confluent monolayer. Then, capillary morphogenesis was induced by overlaying Matrigel on the confluent EC monolayer. HSCs were recruited by the capillary-like structures, resulting in a triculture model where HSCs mediated an SH layer and capillary-like structures

composed of SHs and HSCs. When membranes with optimized configurations were used, HSCs selectively migrated to the EC capillary-like structures. This process was mediated, in part, by platelet-derived growth factor (PDGF) signaling. In addition, these HSCs firmly lined the outer surface of the EC capillary-like structures with long cytoplasmic processes (Kasuya et al. 2015) (Fig. 16.14). In the HSC-lined sinusoidal tissues, SHs retained higher levels of differentiated functions compared with those without ECs. This model provides a basis for the construction of functional, thick, vascularized liver tissues *in vitro*.
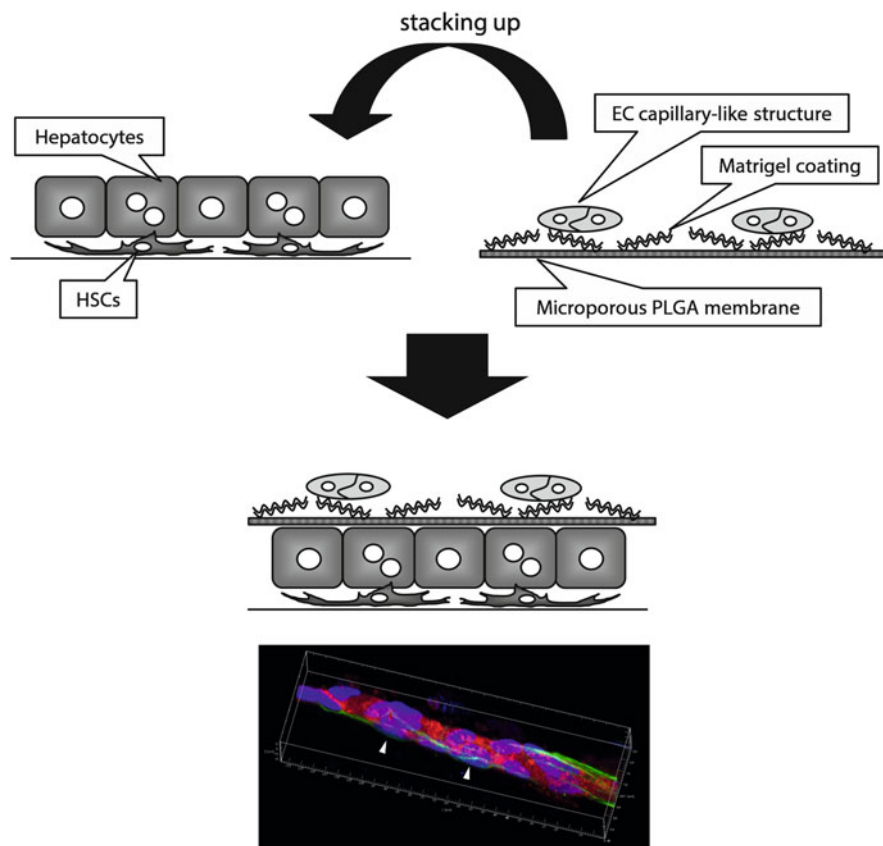
Fig. 16.14 *A triculture model using a biodegradable, PLGA microporous membrane*. SHs, including the HSC fraction, were cultured on a culture dish, while ECs were cultured on a Matrigel-coated microporous PLGA membrane to allow them to form capillary-like structures. Next, the membrane was stacked on the top of SH colonies grown in a culture dish, resulting in a 3D stacked-up triculture. Initially, HSCs were located between the SH layer and culture dish. However, HSCs passed through the microporous membrane and finally covered the outer surface of the capillary-like structures

16.5 Summary

16.5.1 Integrated Vascular Engineering

In this chapter, we introduced *in vitro* culture models for constructing microvascular networks, which are angiogenesis and vasculogenesis models. Because the control of microvascular formation is important both in the context of tissue engineering and cancer therapy, numerous studies have been performed to clarify the mechanism as to how we can construct or regress the microvasculature using

these *in vitro* models. Although "conventional" culture models are still useful in investigating vascular formation, recent advances in microfluidic technologies have allowed studying vascular formation under more controlled culture conditions. Because microfluidic devices have advantages in regulating the spatial and temporal control of cellular distributions, we can create increasingly physiological *in vitro* culture models that more precisely mimic capillary morphogenesis *in vivo*. In this context, the integration of culture microenvironments, such as biochemical and biophysical factors and cell-to-cell interactions both with temporal and spatial variations, is important for achieving more functional vascular formation.

16.5.2 Future Perspectives

Because previous studies have focused on individual factors, such as one biochemical or biophysical factor, combinations of these factors need to be investigated further, which may result in the discovery of nonlinear, synergistic, and system-level effects. Heterotypic cellular interactions are also important aspects. Because microvessels always interact with other tissues, ECs interact with other epithelial tissues and cells. Such interactions will be characterized once vascularized 3D tissues are constructed *in vitro*. Recent advances in microfluidic technologies have opened a new field exploring multiple organ-level functions, often referred to as "human-on-a-chip" or "organs-on-a-chip" (Zhang et al. 2009; Luni et al. 2014; Bhatia and Ingber 2014; Huh et al. 2011). Because organs are connected with the vascular networks of our body, methods for vascular formation described in this chapter also have the potential to be integrated into human-on-a-chip or organs-on-a-chip devices.

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