

# Chapter 4

## Hypoxia and Matrix Manipulation for Vascular Engineering



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### 4.1 Introduction

The permanency of multicellular organisms on Earth is stringently reliant on the ability of cells to respond and adapt to their surrounding milieu. Cells respond to a complex array of biological, biophysical, and biochemical cues to develop and regenerate functional tissues. Alterations to these properties can catastrophically result in impaired development or healing, as well as tumor development and

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metastasis. At the most basic level, two of the most critical components of the cellular microenvironment are oxygen ( $O_2$ ) and the composition of the extracellular matrix (ECM).

According to the most recent findings,  $O_2$  reached sufficient levels (estimated to be 0.2–2%  $O_2$ ) for aerobic organisms to be able to survive between 2.45 and 2.2 billion years ago in the oceans [19, 154] and between 540 and 600 million years ago in Earth's atmosphere [72, 154, 194]. Ever since,  $O_2$  has been a highly available potential source of energy for multicellular organisms to commence, survive, and multiply. Multicellular organisms require specialized systems to enable sufficient amounts of  $O_2$  to reach their cells. For instance, insects regulate the transport of  $O_2$  into their tissues with a special respiratory system consisting of spiracles and trachea. Around their tissues, they retain the relatively low  $O_2$  levels (1.4 mmHg) thought to be equivalent to the atmospheric  $O_2$  concentrations at the time of their evolution [161, 162]. In vertebrates,  $O_2$  is carried by proteins in the blood, particularly by hemoglobin, and is transported to tissues through endothelial cells (ECs). The cells throughout the body are highly dependent on the dynamics of  $O_2$ . In humans,  $O_2$  concentrations vary between 1 and 10% in tissues (other than the lungs) and between 5 and 13% in blood vessels [126, 155]. Therefore, the ability for cells to sense and respond to  $O_2$  is critical, and  $O_2$  acts as a signaling molecule for cells, regulating their metabolism, survival, cell-cell interactions, migration, and differentiation.

Besides  $O_2$  availability, the transition from unicellular to multicellular organisms requires that cells be connected together in a way that allows them to interact with each other as parts of the same system. This interconnectedness could happen either by having junctions at the cell peripheries or by having connecting cement between the cells. Many multicellular organisms connect their cells in both ways: they use cellular junctions to allow direct signaling between cells, and they use the ECM to regulate the transport of molecules (e.g.,  $O_2$ , glucose, and signaling proteins) between the cells by remodeling the components of the ECM. Thus, both cell-cell and cell-ECM interactions are significant for determining the fate of cells in tissues. Transmembrane proteins known as integrins are responsible for signaling from the ECM to the cell. Therefore, cells have different responses with respect to the composition and structure of the surrounding ECM. In particular, vascular morphogenesis is regulated by endothelial cell (EC) interactions with the ECM through integrins and is highly dependent on the ECM context [49].

In the field of vascular engineering, the effects of both  $O_2$  tension and the ECM on blood vessel formation continue to be extensively investigated. Blood vessel formation essentially occurs by angiogenesis or vasculogenesis. Angiogenesis is the formation of blood vessels from pre-existing vasculature, orchestrated with the proliferation, migration, and assembly of ECs, as well as the remodeling of the ECM [127, 213]. Most of the ECs comprising the blood vessel walls are in a state of quiescence in physiological conditions. A stimulus is required for ECs to switch from their resting state to their navigating state, where they are activated to produce angiogenesis-promoting proteins [70]. Angiogenesis occurs in several situations, such as wound healing, arthritis, cardiovascular ischemia, and solid tumor

growth [43, 199]. In all of these situations, the tissue or vasculature is deprived of oxygen, leading to hypoxic conditions that promote angiogenesis. For vessel sprouting, the ECM surrounding the vasculature needs to be degraded so that ECs can easily navigate into the tissue and proliferate. Hypoxia is known to promote the production of ECM-degrading enzymes via secretion from activated ECs [21, 61, 65]. Thus, EC sprouting is more favorable toward hypoxic regions in the ECM through which the secretion of enzymes is upregulated by hypoxia, whereas the invasion of vessels into the ECM is not favored in the direction of sufficiently oxygenated regions.

An oxygen gradient emerges in early development, which guides cellular differentiation and morphogenesis [141]. While the  $O_2$  uptake of early embryonic cells relies on the simple diffusion of oxygen, hypoxia starts to be observed in different regions as the embryo expands [5, 141]. The initial vascularization, vasculogenesis, starts with the differentiation of angioblasts (embryonic progenitors of ECs), which surround hemopoietic cells to form blood islands. Blood islands ultimately fuse as angioblasts differentiate into endothelial cells to form the primary capillary plexus and then undergo further tubulogenesis and vascular network formation throughout the yolk sac [156, 185, 230]. This process of vasculogenesis has been suggested to occur in hypoxic conditions [141, 156]. Hypoxia also stimulates microvascularization and the capillary network to form around the developing organs. Vasculogenesis in adult organs has been demonstrated to originate from endothelial progenitor cells (EPCs) circulating in the blood [225]. The migration of EPCs and their recruitment to the appropriate sites to induce the formation of new blood vessels depends on complex cell signaling. Circulating EPCs home to hypoxic regions along both  $O_2$  and growth factor gradients, in particular gradients of stromal-derived factor 1 (SDF-1) [33, 51]. Investigations of tumor growth and wound healing have revealed that hypoxia occurs in both situations, inducing EPCs to migrate from the circulating blood through the ECM. Hypoxia also plays a role in the recruitment of EPCs by promoting receptor expression on the tissue that recognizes EPCs [242], as well as on the EPCs themselves [35], which is followed by their differentiation into mature ECs [32]. Moreover, vascular endothelial growth factor (VEGF), a key regulatory protein known to induce vasculogenesis and angiogenesis, was found to be upregulated in hypoxia [159]. These processes take place in the milieu of the ECM, which is mostly composed of fibronectin during early development [49, 148]. In adult tissue, on the other hand, collagen becomes abundant and controls the cellular fate.

The formation of new blood vessels through angiogenesis or vasculogenesis depends on the dynamic effects and interplay between the ECM and oxygen tension. A thorough understanding of the mechanisms involving the ECM and  $O_2$  during angiogenesis and vasculogenesis is essential for the fundamental understanding that can be harnessed for developing vascular engineering applications. Indeed, the effects of these two factors on vascular cells are being investigated extensively. The *in vitro* vascularization of primary vascular cells has been studied using many different biomaterials [10, 24, 88] as three-dimensional (3D) matrix components, and

they were shown to influence various aspects of angiogenesis and vasculogenesis. Similarly, a considerable amount of work has focused on the effects of hypoxia-inducible factors (HIF1 $\alpha$ , HIF2 $\alpha$ , HIF3 $\alpha$ ) on the regulation of genes that induce vasculature network formation [68, 159, 241]. In addition, some researchers have also investigated the effects of hypoxia and the ECM context together [170, 177]. Success in engineering blood vessels from primary vascular cells or stem cells relies on understanding the influence of all critical parameters and controlling them in targeted directions.

The main focus of this chapter is a review and discussion of how the cells in the body respond to variations in oxygen tension and ECM components, leading to new vasculature formation.

## **4.2 Concepts in the Regulation of the Vasculature by Oxygen and the ECM**

### ***4.2.1 The Influence of Oxygen Tension on Vascularization***

Variations in oxygen concentrations at every stage of embryogenesis and in different regions of adult tissues lead to diverse vascular responses, depending on the cell type and microenvironment. Many cell types respond differently, but also collectively, to the changes in O<sub>2</sub> equilibrium through specialized sensing mechanisms and effectors in order to maintain homeostasis. In this section, we will first discuss the formation and location of poorly oxygenated regions in the body, as well as the mechanisms that cells utilize to sense changes in oxygen levels. Then, we will then focus on several responses of pluripotent and vascular cells to low O<sub>2</sub> tensions in terms of gene regulation, differentiation, oxygen consumption, and cell survival.

#### **4.2.1.1 The In Vivo Consequences of Oxygen Gradients**

##### **Oxygen Availability in the Body**

In vertebrates, O<sub>2</sub> transport to the tissues relies on three main processes: the oxygenation of the blood in the alveoli in the lungs, the convective transport of oxygen in the blood along the veins, and the diffusion of oxygen across the vessel walls followed by penetration of O<sub>2</sub> to the deeper tissues. There are three distinct resistances to the mass transfer of the O<sub>2</sub> molecule, which result in O<sub>2</sub> gradients throughout the body.

O<sub>2</sub> deprivation has been observed early in the development of mouse embryos [141]. Additionally, polarographic oxygen measurements in the human placenta have shown that O<sub>2</sub> levels are 1.3–3.5% in the first 8–10 weeks and reach between 7.2 and 9.5% in weeks 12–13 of pregnancy [188, 202]. Oxygen levels measured in

the gestational sac revealed even lower  $O_2$  levels in earlier stages of embryogenesis, where  $O_2$  is only transported by simple diffusion [115]. Diffusion, as opposed to convection, transports nutrients between cells very slowly. Before vasculogenesis begins, the maximum diameter that a spherical embryo can reach without having any anoxic cells was calculated to be 2 mm [29]. This value varies with the embryo's geometry and, most importantly, with the  $O_2$  consumption of the animal cells. The results of *in vivo* imaging of various animal embryos show that the maximum diameter remains below 1 mm, which agrees with the theoretically estimated value [29, 230].

Vasculogenesis is crucial to facilitate cell proliferation and for the embryo to grow larger. In mouse embryos, vasculogenesis commences after day 7, with the differentiation of the mesoderm into angioblasts, which then assemble to form a simple circulatory system consisting of a heart, dorsal aorta, and yolk sac by day 8 [56, 107]. Afterward, spatial increases are observed in  $O_2$  levels throughout the course of embryonic development [141]. These profound spatiotemporal  $O_2$  level changes in the embryo can be accepted as evidence for vascular formation during embryogenesis. The large existing vasculature then sprouts and proliferates to supply  $O_2$  and nutrients to cells located in poorly oxygenated regions. Hypoxia, considered the most critical factor controlling the angiogenesis process, works via numerous protein-signaling pathways. The mechanism determining the directionality of angiogenesis and the complex networking of endothelial capillaries around the tissues is manipulated by several other parameters, including hemodynamic forces and cytokines; this mechanism will be discussed later in the chapter [150].

Once embryonic development is complete and sufficient concentrations of  $O_2$  and nutrients are supplied to the tissues, the oxygen gradient still persists in some tissues, providing several benefits to specific cell types. In adults,  $O_2$  distribution ranges from 1 to 13 in normal tissues. Although the formation of blood vessels and capillary networking is complete, some tissues still lack vasculature, such as the bone marrow niche [91, 132, 175]. In such tissues, diffusion is the controlling mechanism for nutrient transport, thus resulting in a wide range of  $O_2$  distributions from the internal hypoxic region to the external regions, which remains at physiological  $O_2$ .

The discovery of circulating EPCs in blood vessels revealed that neovascularization in adults is directed not only by angiogenesis but also by the vasculogenesis process, which depends on the renewal, mobility, recruitment, and differentiation of EPCs [12, 13, 96, 219]. The bone marrow (BM) provides a host microenvironment for a variety of cells, including hematopoietic stem cells (HSCs), mesenchymal stem cells (MSCs), and EPCs. The development of EPCs occurs in the BM, which has a unique structure that allows severe hypoxic regions to exist. Although the BM is inaccessible for noninvasive oxygen measurements, both simulation studies and qualitative measurements have demonstrated the existence of hypoxic regions. Several theoretical models have been developed in order to simulate the distribution of oxygen throughout the BM [41, 132, 133]. Chow et al. used homogeneous Kroghian models to estimate oxygen levels in the BM [41]. Their simulations suggested that both HSCs and EPCs are exposed to low  $O_2$  tensions in the BM. There are various BM architectural organizations possible depending on parameters such

as the spatial arrangement of vasculature and the distribution of many different cell types populating the BM. Therefore, in the absence of supporting evidence from *in vivo* quantitative measurements, model predictions must be used to assess the effects of different parameters on the  $O_2$  tension distribution in the BM. The model described by Kumar et al. considered three possible vessel arrangements to simulate oxygen level variations under various conditions [132]. They suggested that hypoxic, and even anoxic, regions could be found in the BM, assuming that the cells' oxygen consumption is constant and that the density of arterioles in the BM is low.

On the other hand, qualitative observations in the study by Parmar et al. demonstrated that HSCs are distributed according to oxygen availability in the BM [175]. Staining with pimonidazole and sectioning revealed the oxygen gradient throughout the BM, showing that HSCs more likely reside at the lower end of the gradient. These results are in agreement with other *in vitro* studies suggesting that hypoxia supports the maintenance of stemness [45, 64, 69]. Moreover, BM transplantation studies have shown that BM-derived EPCs enhance neovascularization and the formation of arteries [231, 235]. The renewal of EPCs in the BM depends on the differentiation dynamics of HSCs, which are regulated by the microenvironments (i.e., the niches) they reside in. Osteoblasts, bone cell progenitors, bind to each other and to HSCs via adhesion molecules to form the osteoblastic niche that is located far from the sinusoidal arteries. Researchers have discovered the existence of another type of niche within the BM, the vascular niche, which is located closer to the sinusoidal arteries than the osteoblastic niche. The differences in physicochemical factors within the various niches play fundamental roles in controlling the dynamics of HSC migration and differentiation. Since the vascular niche's close proximity to arteries means that it is richer in  $O_2$  than the osteoblastic niche, Heissnig's group hypothesized that HSCs are in a quiescent state in the osteoblastic niche's severe hypoxic conditions [92]. When vasculogenesis is necessary in neighboring tissues, specific cell signaling stimulates the migration of HSCs from the osteoblastic niche to the more oxygenated vascular niche, where HSCs can switch from their quiescent state to a proliferative state. The proliferation and differentiation of HSCs reconstitute the EPC pool in the vascular niche before they enter the circulation.

Wound healing, another situation where tissue hypoxia is prevalent, consists of a series of events that includes new vasculature formation, which is regulated by varying  $O_2$  levels. Platelets interfere with microcirculation in the wounded tissue, followed by the release of coagulation factors to reinforce the clotting process. Histamine and bradykinin, secreted by mast cells, also influence the microcirculation by enhancing vascular permeability and arteriolar vasodilation, thereby increasing the blood flow rate [11, 102]. Recruitment of leukocytes and macrophages into the damaged tissue is followed by their activation in response to several growth factors (GFs) and integrins. High rates of  $O_2$  consumption in activated macrophages, along with perturbation of the microcirculation, lead to a further decrease in  $O_2$  levels and result in hypoxia [204], which leads to the accumulation of HIF1 $\alpha$  at the wound site [246]. Albina et al. [9] showed that the HIF1 $\alpha$  mRNA of inflammatory cells peaks about 6 h after injury. On the other hand, HIF1 $\alpha$  protein levels could be detected between 1 and 5 days after wounding. More recently, Zhang et al. [246] demon-

strated that, during the burn wound-healing process, the accumulation of HIF1 $\alpha$  increases the number of circulating angiogenic cells, as well as smooth muscle actin-positive cells, in the wounded tissue. These hypoxic conditions—either directly or indirectly through the accumulation of HIF—stimulate angiogenesis during wound healing.

Ischemic tissues, including those affected by myocardial infarction or peripheral artery occlusion (e.g., limb ischemia), have also been a hotbed for study of the effects of low O<sub>2</sub> on cellular recruitment and tissue regeneration. In particular, lack of O<sub>2</sub> delivery to these diseased tissues results in HIF stabilization and subsequent upregulation of recruitment chemokines, perhaps most importantly SDF-1, as well as transmembrane proteins integrin  $\beta$ 2 and ICAM-1 that facilitate adhesion of circulating cells to the damaged endothelium [33, 35, 51, 242]. Importantly, hypoxic conditions also facilitate ECM remodeling through upregulation of proteases, such as cathepsins and matrix metalloproteinases [4, 106, 226]. These factors, in concert with HIF-induced production of other pro-angiogenic factors, such as VEGF, lead to robust formation of neovasculature.

### Oxygen-Sensing Mechanisms of Vascular Cells

Most cell types in the body respond to variations in O<sub>2</sub> tensions [233]. Gene expression, viability, metabolism, and the oxygen uptake rate of the cells change with alterations in O<sub>2</sub> levels, in order to maintain homeostasis. When cells experience a change in extracellular O<sub>2</sub> levels, they adapt to the new conditions, which may occur rapidly. Hence, O<sub>2</sub> sensing in cells is expected to be controlled by well-organized, highly sensitive mechanisms.

Several mechanisms have been proposed in the literature to account for O<sub>2</sub> sensing in cells. Although their sensitivities may differ from one another, more than one such mechanism can coexist in a cell, resulting in various cellular responses. Within the cell, the O<sub>2</sub> molecule mainly engages in two distinct processes: it is involved directly in biosynthesis reactions; or it participates in metabolic processes, such as the electron transport chain occurring in mitochondria. Any change in the concentration of O<sub>2</sub> extensively perturbs these processes and, following a sequence of events, may have a number of different effects on the cell. Therefore, O<sub>2</sub> sensors in cells can be mainly categorized as mitochondria-related sensors (bioenergetic) and biosynthesis-related sensors (biosynthetic)—although they can be linked to each other in some cases, making the distinction not completely clear [233].

Among the several effectors of O<sub>2</sub>-sensing mechanisms, HIFs are the most essential in terms of the diversity of their influences. The family of HIF $\alpha$  subunits (HIF1 $\alpha$ , HIF2 $\alpha$ , and HIF3 $\alpha$ ) has been shown to be responsible for regulating expression of a large number of genes, including those coding for key regulatory proteins of angiogenesis and vasculogenesis. Although HIF $\alpha$  is expressed at every oxygen tension, it is rapidly ubiquitinated in normoxic conditions, resulting in its degradation. Thus, the amount of intracellular HIF $\alpha$  protein depends on the balance between its expression and degradation. In conditions of low O<sub>2</sub> availability, all HIF $\alpha$  proteins heterodi-

merize with HIF $\beta$  (ARNT) and form a transcriptional complex which regulates the transcription of numerous genes [159]. Stabilization of HIF $\alpha$  in the cell is controlled by two main O<sub>2</sub> sensing proteins, prolyl hydroxylase domain (PHD) and factor-inhibiting HIF $\alpha$  (FIH), which belong to the previously mentioned biosynthetic sensors category. Three isoforms of PHDs are present in all mammals [28]. Specific proline residues on the oxygen-dependent domain of HIF $\alpha$  are hydroxylated by PHDs at separate hydroxylation sites, leading to HIF $\alpha$  degradation. The activity of PHDs in the cytoplasm is controlled by various O<sub>2</sub>-dependent molecular events and, directly, by the concentration of the O<sub>2</sub> molecule [68]. All three PHDs remain partially active in normoxia. PHD activity is expected to be very sensitive to small changes in cytoplasmic O<sub>2</sub> levels since K<sub>m</sub>, the Michaelis-Menten parameter for the activation of PHDs, is approximately 230–250  $\mu$ M, which is much higher than physiological oxygen concentration (approximately 60  $\mu$ M) [98]. Besides, mitochondria are also involved in the PHD activation process through their consumption of O<sub>2</sub>, regulation of reactive oxygen species (ROS), and production of nitric oxide (NO). While the stabilization of HIF $\alpha$  depends on PHD activity, the expression of HIF $\alpha$  is controlled by FIHs. Therefore, when O<sub>2</sub> levels are lowered, both the stabilization and transactivation of HIF $\alpha$  increase, resulting in several angiogenic responses that will be discussed in the following section.

NO and ROS not only contribute to the HIF $\alpha$  stabilization process, but they also have several direct effects on vascular cells and blood vessels. A number of studies have shown that NO induces angiogenesis, hyperpermeability, and vasodilation [73]. Moreover, NO also perturbs EC respiration through the inhibition of cytochrome c oxidase, which causes lower mitochondrial O<sub>2</sub> consumption [118]. Mitochondrial ROS are also increased as a consequence of electron transport chain inhibition, which then contributes to the deactivation of PHDs via oxidizing cofactor Fe (II) and helps to stabilize HIF $\alpha$ . ROS production, in respect to hypoxia, is proportional to the concentrations of intracellular O<sub>2</sub> and electron donors. Under hypoxia, the amount of O<sub>2</sub> required to form superoxides is decreased, whereas the concentration of the electron donors increases as a consequence of the reduction in the proximal electron transport chain. Therefore, ROS production can change in both manners, depending on the variations in these molecules' concentrations [233]. Ushia-Fukari et al. [227] showed that ROS influence the expression of surface adhesion molecules of ECs and stimulate EC proliferation and vessel permeability. Moreover, the hypoxia-induced decrease in ROS production leads to the inhibition of K<sup>+</sup> channels of pulmonary artery smooth muscle cells (SMCs), whereas an increase in ROS production leads to intracellular Ca<sup>+</sup> release from ryanodine-sensitive stores [233]. Another molecular path found between mitochondrial energy generation and K<sup>+</sup> channel inhibition occurs through AMP kinases. The energy of the cell is generated by the conversion of ADP to one molecule of ATP and AMP. Hence, AMP kinase becomes highly dependent on the ADP/ATP ratio, which is very sensitive to changes in cytoplasmic O<sub>2</sub> concentrations. AMP kinases were shown to inhibit K<sup>+</sup> channels through the regulation of Ca<sup>+</sup> release in pulmonary arterial SMCs and also to induce cellular survival in tumor cells when exposed to severe hypoxia [63, 171].



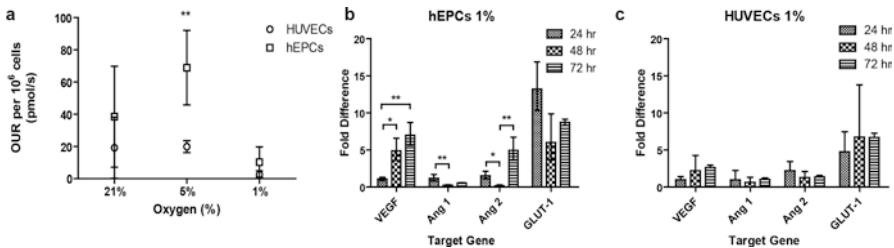
Moreover, heme oxygenases (HOs) and NADPH oxidases (NOXs) play important roles in the biosynthetic oxygen sensing of cells. NOX-2, one of the three isoforms of NOX, is used for superoxide production from molecular  $O_2$ . Hypoxic conditions can cause a decrease in NOX-2-derived ROS concentrations, due to the low  $K_m$  values (18  $\mu M$ ) of NOX-2; this helps  $Ca^{2+}$  release in pulmonary artery SMCs [239]. However, some studies also suggest that hypoxia increases NOX-2 activity, therefore causing the generation of a greater amount of ROS [233]. On the other hand,  $Ca^{2+}$ -activated  $K^+$  channels in glomus cells were shown to be related to the activity of HO-2, an isoform of HO which can convert heme to CO, biliverdin, and Fe(II) using  $O_2$  and NADPH [237].

The effectiveness of an oxygen sensor can be determined by evaluating (a) its sensitivity to small changes in intracellular  $O_2$  levels and (b) the subsequent diversity of triggered cellular responses. Taking these considerations into account, PHDs and FIHs appear to be the most critical oxygen sensors responsible for controlling HIF activity [94]. Deactivation of these two sensors leads to HIF $\alpha$  stabilization, initiating the regulation of hundreds of different genes. Using  $O_2$  as a controlling parameter to engineer vascular tissues demands a clear understanding of the biochemical events that follow changes in  $O_2$  tension, as well as the net response of the cells and how  $O_2$  affects their collective behaviors.

#### 4.2.1.2 Cellular Responses to Different Oxygen Concentrations

##### Metabolism and Oxygen Uptake Rate

Several studies have observed that the  $O_2$  consumption of cells depends on  $O_2$  availability [1, 26, 171, 210]. We have recently shown that the  $O_2$  uptake rates (OURs) of EPCs and human umbilical vein endothelial cells (HUVECs) are similar, but not identical, to each other and that both decrease when  $O_2$  availability is lowered (Fig. 4.1a) [1]. Many mechanisms have been proposed to explain the relationship between mitochondrial  $O_2$  consumption and variations in  $O_2$  levels. HIF1 $\alpha$  was found to be responsible for inducing the enzymes required for glycolysis [171]. It also plays a role in activating pyruvate dehydrogenase kinase-1, which reduces the



**Fig. 4.1**  $O_2$  tension regulates vascular cell responses. Comparison of EPCs and HUVECs at three different  $O_2$  tensions in terms of (a) oxygen uptake rate (OUR) and (b, c) gene regulation [1]

amount of pyruvate that flows into the TCA cycle and therefore decreases aerobic respiration in mitochondria [171]. In addition, the increases in both the transcription and expression of glucose transporter protein 1 (GLUT-1) were shown to be HIF1 $\alpha$ -dependent in hypoxic conditions [7]. In other words, deactivation of PHDs and FIHs at low O<sub>2</sub> levels leads to the stabilization of HIF $\alpha$ , which then reduces O<sub>2</sub> aerobic respiration by inducing pyruvate degradation while, at the same time, promoting glycolysis by increasing the expression of glucose transporter proteins. Another proposed mechanism involves the inhibition of cytochrome oxidase by NO, which is known to be regulated by shear stress and O<sub>2</sub> tension. NO influences mitochondrial respiration by the competitive inhibition of cytochrome oxidase with O<sub>2</sub> and by inhibiting electron transfer between cytochrome b and c, therefore increasing ROS production [26].

The effects of blood flow and O<sub>2</sub> tension are crucially important for the ECs comprising the vessel walls, since these conditions can be perturbed in many pathophysiological situations in the body. Some studies have shown mitochondrial respiration of ECs to be lower than other cell types, suggesting that most of the O<sub>2</sub> consumption is non-mitochondrial [78, 223]. Helmlinger et al. demonstrated that ECs consume O<sub>2</sub> during capillary formation, whereas they also preserve and expand the capillary structures, even under severe hypoxia (about 0.6% O<sub>2</sub>), by upregulating VEGF expression [93]. It is not surprising that ECs possess a special type of metabolism— aerobic glycolysis in their resting state (physiological conditions) and anaerobic glycolysis in their navigating state (hypoxic conditions)—since O<sub>2</sub> is transported through ECs to other tissues, and thus they must possess the ability to survive and commence angiogenesis under hypoxic conditions [70].

Moreover, when ECs are exposed to excess glucose, their ATP generation shifts to glycolysis, and lactate levels, increased as a by-product of glycolysis, contribute to the inactivation of PHDs and, therefore, the stabilization of HIF $\alpha$  [240]. Where blood flow is perturbed, such as in ischemia and wound healing, both NO and O<sub>2</sub> levels are changed in blood vessels, and all of the metabolic variations discussed become more important.

### Transcription of Angiogenic Genes

Manalo et al. showed in their study of ECs that 245 genes are upregulated and 325 genes are downregulated at least 1.5-fold in response to hypoxia and HIF1 $\alpha$ . These genes are responsible for the expression of collagens, GFs, receptors, and transcription factors, all of which are significant for the processes of angiogenesis and vasculogenesis. This wide range of hypoxia-related transcription factors also indirectly affects HIF1 $\alpha$ . The genes directly regulated by HIF1 $\alpha$  include VEGF-A, VEGFR-1, Flt1-1, and erythropoietin (EPO). Examples of indirectly regulated genes include fibroblast growth factor (FGF), placental growth factor (PLGF), platelet-derived growth factor (PDGF), angiopoietins (ANG-1 and ANG-2), and Tie-2, the receptor of ANGs [68]. Although VEGF is the major GF that stimulates blood vessel formation, when it alone was transgenically overexpressed in mice, defective blood

vessels formed, which then led to tissue edema and inflammation [184]. On the other hand, overexpressing both VEGF and ANG-1, which is important for maintaining vascular integrity, has been shown to induce hypervascularity without imperfections in mice [218]. ANG-2 is responsible for EC apoptosis and vascular regression in the absence of VEGF, whereas, when combined with VEGF expression, it enhances angiogenic responses by destabilizing the blood vessels [100, 101]. More recently, ANG-4 was shown to function similarly to ANG-1 and to induce angiogenesis by binding the ANG receptor TIE-2, which is also upregulated by HIF1 $\alpha$  [241]. We have recently shown that VEGF and ANG-2 genes are upregulated in hypoxic (1% O<sub>2</sub>) cultures of EPCs and HUVECs [1], and the fold differences in upregulation levels of VEGF and ANG-2 in EPCs were shown to vary during the 3-day exposure period (Fig. 4.1b), where no significant change was observed for HUVECs (Fig. 4.1c). How hypoxia affects the regulation of these angiogenic genes depends on the cell type; for instance, VEGF is upregulated in ECs, SMCs, cardiac fibroblasts, and myocardiocytes, whereas ANG-2 is induced only in ECs [159]. Therefore, from a tissue engineering perspective, co-culturing of different cell types under controlled hypoxic conditions should be considered, since a combination of hypoxia-induced angiogenic proteins is required to obtain vascular formation without excessive permeability.

### Cell Death and Survival

Hypoxia influences the proliferation and viability of many cell types [1, 69, 172, 245]. The wide spectrum of HIF1 $\alpha$ -dependent genes also includes proapoptotic and prosurvival genes. BH3-only proapoptotic genes, a subfamily of BCL-2 that includes BNIP3, BNIP3L, NOXA, RTP801, and HGTP-P, are directly activated by HIF1 $\alpha$  [234]. Although these genes play important roles in cellular apoptosis, a growing body of evidence suggests that hypoxia mediates cellular survival in many cell types [160, 172, 245]. Programmed cell death is, of course, a very critical step for cells and is most likely taken only after all possible survival mechanisms have been exhausted. One of these mechanisms, autophagy, is a cellular catabolic process where cytoplasmic organelles are degraded to provide ATP generation in nutrient deprivation. Hypoxia was found to induce mitochondrial autophagy via both HIF1 $\alpha$ -dependent and HIF1 $\alpha$ -independent pathways [172, 245]. Small interfering RNA silencing of BNIP3 and BNIP3L together suppresses autophagy to a greater extent than silencing only one of them at a time [20]. Zhang et al. have shown that mitochondrial autophagy is induced by HIF1 $\alpha$ -dependent upregulation of BNIP3 incorporated into the constitutive expression of BECLIN-1 and ATG-5 [245]. On the other hand, the neuron-derived orphan receptor (NOR-1), which is overexpressed in ECs exposed to hypoxia, mediates cellular survival as a downstream effector of HIF1 $\alpha$  signaling [160]. CD105, one of the EC markers also shown to play a role in cellular survival, is significantly upregulated under hypoxia [146]. In vivo studies of rats subjected to hypoxia also found the induction of mitochondrial autophagy by overexpression of BNIP3 [17]. In addition, Papandreou et al. propose

that hypoxia induces autophagy in tumor cells through AMP kinase, which is activated by hypoxia independently of HIF1 $\alpha$ , as discussed previously in the O<sub>2</sub> sensing section [172].

#### 4.2.1.3 Cell Pluripotency and Differentiation

Vasculogenesis takes place in low O<sub>2</sub> environments, such as the early development of the embryo, EPC regeneration in the BM, or EPC attachment and differentiation into mature ECs at neovascularization sites. All of these processes rely on pluripotent/unipotent cells differentiating into the endothelium, where O<sub>2</sub> tension is a crucial parameter regulating their differentiation characteristics. As already discussed, EPC regeneration in the BM depends on cellular dynamics between the osteoblastic niche (low O<sub>2</sub>) and vascular niche (high O<sub>2</sub>); HSCs are quiescent in the osteoblastic niche and differentiate into EPCs in the vascular niche before joining the circulation [111]. Therefore, it is important to understand the effect of O<sub>2</sub> tension on the differentiation of cells into EPCs/ECs as a primary step of vasculogenesis. Hypoxia enhances human embryonic stem cell (hESC) pluripotency via the upregulation of Oct-4, NANOG, and SOX-2, which are pluripotent markers [45, 64, 69, 116]. HIF2 $\alpha$  is responsible for the overexpression of Oct-4, SOX-2, and NANOG, while HIF3 $\alpha$  also plays a role in the process by inducing HIF2 $\alpha$  transcription [45, 69]. Prasad et al. demonstrated that hypoxic conditions (5% O<sub>2</sub>) prevent the spontaneous differentiation of hESCs, whereas the inhibition of Notch activation revoked this effect, suggesting that hypoxia-induced pluripotency occurs via Notch signaling [182]. On the other hand, the efficiency of the process of reprogramming mouse and human somatic cells into induced pluripotent stem cells (iPSC) was shown to be improved in 5% O<sub>2</sub> cultures, compared to atmospheric O<sub>2</sub> cultures [243]. In contrast, other studies have demonstrated that hypoxia induces the expression of early cardiac genes in spontaneously differentiating embryoid bodies (EBs) [125, 168]. In a more recent study, Lopez et al. showed that EPCs/ECs can be obtained from hESCs more efficiently when cultured in 5% O<sub>2</sub>, compared to previous methods that induce EB formation in atmospheric O<sub>2</sub> [181]. Additionally, simply priming EBs in hypoxic conditions mediated suppression of pluripotent marker Oct-4 and upregulated VEGF [140]. When hPSCs are differentiated toward an endothelial lineage, hypoxia has also been shown to enhance EC differentiation through changes in the early stages (mesodermal specification) of EC lineage commitment. Interestingly this affect, which was dependent on low O<sub>2</sub> tension, was driven by production of ROS [135]. More in-depth investigations have uncovered the specific role of NADPH oxidase 2 (Nox2)-produced ROS in upregulating Notch signaling to facilitate differentiation toward arterial endothelial cells [119]. Another group showed a biphasic regulation of EC fate specification via a HIF1 $\alpha$ -mediated pathway in mESCs. Hypoxia led to upregulation of the transcription factor Etv2 in the early stages of differentiation, which resulted in development of endothelial progenitor cells. Continued exposure to hypoxia led to HIF1 $\alpha$ -induced upregulation of Notch1 signaling and formation of functional arterial endothelial cells, with

the capacity to contribute to revascularization of ischemic tissues [224]. Similarly, HIF1 $\alpha$  also induces the differentiation of peripheral blood mononuclear cells into EPCs, and hypoxia stimulates the further differentiation of EPCs into mature ECs [8, 117].

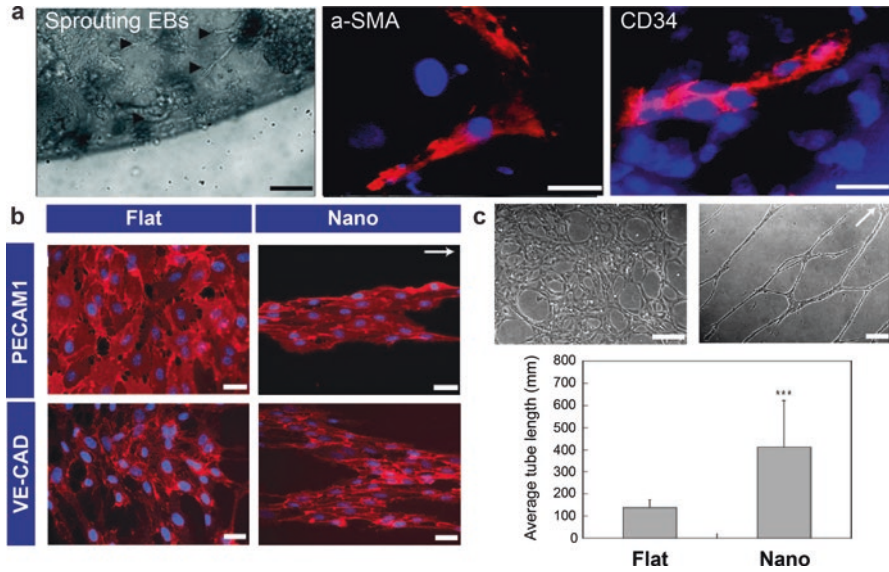
All of these findings highlight the significance of O<sub>2</sub> tension as a critical parameter to control vascular differentiation of pluripotent or multipotent cells. Although some of these studies suggest contrary hypotheses, the importance of O<sub>2</sub> considerations in cell culture environments cannot be overstated, as O<sub>2</sub> tension can be manipulated to prevent spontaneous differentiation of pluripotent cells and to enhance the efficiency of the differentiation into EPCs and mature ECs.

## ***4.2.2 Vascular Responses to ECM***

In the human body, vascular cells are surrounded by diverse components of the ECM, the unique spatial and temporal distribution of which affects GF availability and matrix properties which, in turn, regulate vasculogenesis and angiogenesis. Just like oxygen tension, which varies throughout vascular development, ECM components are also uniquely distributed; for example, hyaluronic acid (HA; also known as hyaluronan) levels were found to be highest during embryogenesis and to be replaced by fibronectin and then collagen, which remains abundant throughout adulthood. In this section we will discuss ECM distribution and its effects on vascular development and maintenance. Then, we will discuss various ECM components that affect vascular morphogenesis. Lastly, we will describe strategies for manipulating the ECM using synthetic biomaterials and emerging technology.

### **4.2.2.1 Types of ECM Found Participating in Vascularization**

The ECM surrounding blood vessels contributes significantly to their diverse functions and complexity. This ECM diversity encompasses different vascular development periods (i.e., embryonic versus adult) and specialized vessels at various locations in the body (i.e., capillary, arteriole, and venule) or tissues in the body (i.e., heart, kidney, lung, etc.). During early vascular development, the ECM provides informational cues to the vascular cells, thus regulating their differentiation, proliferation, and migration. Fibronectin and HA, which are major components of the embryonic ECM, have been shown to be vital regulators for vascularization during embryogenesis [222]. Fibronectin, a unique glycoprotein, contains cell adhesion and heparin-binding sites that synergistically modulate the activity of VEGF to enhance angiogenesis [236]. Various lineage studies have found developmental abnormalities in embryonic hearts and vessels in fibronectin-null mice, suggesting its crucial role in mediating EC interactions [14, 71]. The levels of hyaluronan, a nonsulfated linear polysaccharide, are greatest during embryogenesis and then decrease at the onset of differentiation [221], where it plays a crucial role in



**Fig. 4.2** Matrix composition and orientation affect vasculogenesis. (a) Hyaluronic acid microenvironment for vasculogenesis. Human ESC colonies were cultured in conditioned medium for 1 week, followed by the replacement of medium containing 50 ng/ml VEGF<sub>165</sub>. Left: Cell sprouting was observed after 48 h of culture in medium containing VEGF (indicated by arrowheads). Middle and right: After 1 week of differentiation, sprouting elongating cells were mainly positive for alpha-smooth muscle actin (a-SMA) (middle), while some were positive for the early-stage endothelial marker CD34 (right). Scale bars—left, 100  $\mu$ m; middle and right, 25  $\mu$ m. Printed with permission [79]. (b) Nanotopography induces the formation of supercellular band structures in long-term EPC culture. EPCs cultured on flat substrates began forming confluent layers of cells after 6 days of culture. In contrast, EPCs cultured on nanotopography began to form supercellular band structures aligned in the direction of the features (as indicated by the arrow) after 6 days of culture. These morphological differences are evident through staining of PECAM-1 and VE-CAD. Scale bars are 50  $\mu$ m. Printed with permission [23]. (c) Organized capillary tube formation in vitro. Capillary-like structures (CLSs) were induced by the addition of Matrigel after 6 days. EPCs cultured on flat substrates (upper left) formed low-density unorganized structures, while EPCs cultured on nanotopographic substrates (upper right) formed extensive networks of organized structures with (lower panel) longer average tube lengths than EPCs cultured on flat substrates (\*\*\*)  $p < 0.001$ ). The direction of the linear nanotopographic features is indicated by the arrow. Scale bars are 200  $\mu$ m. Printed with permission [23]

regulating vascular development [18]. Hyaluronan and its receptor, CD44, have been shown to be essential in the formation and remodeling of blood vessels [18, 30, 62]. We have previously reported that a completely synthetic HA hydrogel can maintain the self-renewal and pluripotency of hESCs [79, 86, 87]. Interestingly, when VEGF is introduced into the culture media, this unique HA microenvironment can direct the differentiation of hESCs into vascular cells, as indicated by positive staining for  $\alpha$ -smooth muscle actin and an early stage of the endothelial cell marker, CD34 (Fig. 4.2a). More recent studies have employed higher throughput methods to explore the effects of ECM composition on EC fate. As the ECM of the developing

embryo consists of multiple components, culturing ECs on combinatorial ECM arrays revealed optimal conditions for EC survival, in response to low O<sub>2</sub> and low nutrient availability [104], as well as enhancements in EC fate which were regulated by ECM composition, at least partially through upregulation of integrin  $\beta$ 3 and its associated signaling pathway [105].

In contrast, the adult ECM consists mostly of a laminin-rich basement membrane, which maintains the integrity of the mature endothelium, and interstitial collagen I, which promotes capillary morphogenesis [50]. Although collagen I is present during development, its role becomes increasingly important in postnatal angiogenesis, after its reactive groups have been cross-linked to further stabilize the interstitial matrix [186]. EC integrins, which interact with collagens and fibrin, are key receptors in EC activation, proliferation, and tubular morphogenesis. The collagen-I-mediated activation of Src and Rho and the suppression of PKA promote the formation of prominent actin stress fibers, which mediate EC retraction and capillary morphogenesis. Moreover, the activation of Src also disrupts VE-cadherin from cell junction and cell-cell contact which, in turn, facilitates multicellular reorganization. Conversely, basement membrane laminin-1 is responsible for maintaining the mature endothelium. During the proliferative stage of morphogenesis, the laminin-rich basal lamina is degraded, exposing the tips of sprouting ECs to the underlying interstitial collagens and activating signaling pathways that drive cytoskeletal reorganization and vascular morphogenesis. This sharp difference in how ECM components affect capillary morphogenesis is responsible for controlling the delicate balance between vascular sprouting and maturation.

Once nascent vessels are formed, ECM components regulate their maturation and specialization into capillaries, arteries, and veins. Capillaries, the most abundant vessels in our body, consist of ECs surrounded by pericytes and basement membrane. Exchanges of nutrients and oxygen occur through diffusion between blood and tissue in these regions, due to the capillary's thin wall structure and large surface-area-to-volume-ratio. Maturation of the vessel wall involves the recruitment of mural cells, development of the surrounding matrix, and organ-specific specialization [113]. ECM distribution in various tissues dictates the specialization of these capillaries to support the functions of specific organs. The capillary endothelial layer is continuous in most tissues (e.g., muscle), while it is fenestrated in exocrine and endocrine glands (e.g., kidney and pancreas). Moreover, the enlarged sinusoidal capillaries of the liver, spleen, and BM are discontinuous, allowing increased exchange of hormones and metabolites between the blood and the surrounding tissues. In contrast, where the excess exchange of molecules is not desirable, such as at the blood-brain barrier and the blood-retina barrier, the interendothelial connection is further reinforced with tight junctions, such as occludin and ZO-1 [238].

Compared with capillaries, arterioles and venules have an increased coverage of mural cells and ECM components. Arterioles are completely surrounded with vascular SMCs that form a closely packed basement membrane. The walls of larger vessels are composed of three layers: the tunica intima, the tunica media, and the tunica adventitia. The EC layer of blood vessels is anchored to a basement membrane, which is the major component of the tunica intima [57]. The basement mem-

brane contains network-organizing proteins, such as collagen IV, collagen XVIII, laminin, nidogen, entactin, and the proteoglycan perlecan. The tunica media contains vascular SMCs (v-SMCs) and elastic tissue composed of elastin, fibrillins, fibulins, emilins, and microfibril-associated proteins. The tunica adventitia contains fibroblasts and elastic laminae and has its own blood supply, known as the vasa vasorum [57]. SMCs and elastic laminae contribute to the vessel tone and regulate vessel diameter and blood flow. This generic blood vessel architecture is modified with various ECM components to fulfill their individual tasks. Arteries, which function to deliver oxygenated blood, usually have a thick tunica media with numerous concentric layers of v-SMCs, whereas veins have a thick tunica adventitia layer enriched in ECM components with elastic properties, such as elastin and fibrillin.

As described, the composition of the ECM is inherently dynamic throughout development as well as vascular regeneration, positing the importance of remodeling and deposition of new ECM as these processes progress. Additionally, stability of mature vessels requires a different ECM composition than developing or regenerating vasculature. Several studies have highlighted these changes in ECM deposition and have identified regulators of these important mechanisms. Much of the work to date has established the role of perivascular cells, including pericytes and smooth muscle cells, in ECM production [232]. Crucially, ECs also produce ECM as blood vessels form. Of particular interest, endothelial progenitor populations and mature ECs produce ECM differently; EPCs produce collagen IV, fibronectin, and laminin, while mature ECs have limited ECM production in standard cell culture conditions. However, when subjected to hypoxic conditions, mature ECs adopt an ECM secretome similar to the pro-regenerative EPCs, wherein they secrete collagen IV, fibronectin, and laminin at low  $O_2$  (1%). At moderate hypoxia (5%  $O_2$ ), both cell types produce collagen I [134]. When developing engineered vasculature, these factors are critical to consider to obtain mature, long-lasting blood vessels, as ECM composition is an important parameter governing vascular stability.

#### 4.2.2.2 Properties of the ECM that Affect Vascular Morphogenesis

Recent decades have vastly expanded our understanding of how ECM properties affect vascular assembly, primarily due to newly available, well-defined *in vitro* models. The most common models are cultures of ECs in gels made of different ECM components, such as collagen, fibrin, fibronectin, and Matrigel. These ECM components contain instructive physical and chemical cues that direct vascular morphogenesis, which involves several steps: (1) proteolytic degradation of basement membrane proteins by both soluble and membrane-bound matrix metalloproteinases (MMPs); (2) cell activation, proliferation, and migration; (3) vacuole and lumen assembly into a tube with tight junctions at cell-cell contacts; (4) branching and sprouting; (5) synthesis of basement membrane proteins to support the formation of capillary tube networks; and (6) tube maturation and stabilization by pericytes. These complex processes require a delicate balance between various immobilized and soluble GFs, as well as endothelial and perivascular cell



interactions. Gels made from ECM components, engineered to have properties resembling those of native tissues, have been widely explored as a tool to study the molecular regulation underlying vascular development [49] and as a scaffold to transplant vascular progenitor cells [15, 46, 164]. However, their manipulation for vascular tissue engineering has been narrowly limited by their inherent chemical and physical properties. Therefore, a great need exists to chemically modify these ECM components [40, 130] or to utilize biomaterials to form scaffolds from hydrogels, which are xeno-free and instructive for vascular tissue engineering [152]. Hydrogels are cross-linked polymer networks which can store a large amount of fluid and which have biophysical properties similar to many soft tissues [138]. Hydrogels can be engineered from natural biomaterials (including ECM components), artificial protein polymers, self-assembling peptides, and synthetic polymers to form scaffolds which mimic the native ECM. For example, dextran and chitosan, natural biomaterials with similar structures, do not possess any inherent cross-linking ability [214, 215]. However, a simple chemical modification, such as introducing double bonds into the repeating unit, allows the cross-linking of these polysaccharides to form hydrogels. Alginate is another natural material which can be physically cross-linked by adding cations (e.g.,  $\text{Ca}^{2+}$  or  $\text{Mg}^{2+}$ ) [80]. Another approach utilizes a purely synthetic polymer, like polyethylene glycol (PEG) or poly-[lactic-co-glycolic acid] (PLGA), whose physical and chemical properties can be easily manipulated. A simple modification can turn PEG, a cell-resistant material, into an instructive scaffold designed to promote vascularization [58, 59, 166, 176]. Furthermore, the synthetic material of choice must be biodegradable and biocompatible, and such physical properties as pore size, degradation kinetics, and matrix mechanical properties must be easily tunable to favor vascular morphogenesis. Bioactive molecules—like GFs, cell adhesion motifs such as arginine-glycine-aspartic acid (RGD), and MMP-sensitive peptides—must be presented with correct spatial and temporal distributions within the synthetic biomaterials. Next, we will discuss several strategies for manipulating the chemical and physical properties of synthetic biomaterials.

### Cell Adhesion Regulates Neovascularization

In order to support vascular cells and instruct them to undergo vascular morphogenesis, synthetic biomaterials must first be able to provide cell adhesion. Instead of incorporating ECM components to make such materials bioactive, certain synthetic peptides important for vascular morphogenesis can be incorporated into these inert synthetic materials. The most common template is the integrin-binding domain of fibronectin, RGD [178], and the laminin-derived peptide IKVAV [203]. The first crucial step in vascular morphogenesis occurs when vascular cells utilize integrin receptors to sense their surrounding microenvironments. Integrins are transmembrane receptors which not only maintain cell adhesion to ECM but also control cell proliferation, migration, differentiation, and cytoskeletal organization. Since blood vessels must be able to assemble in diverse tissue environments (e.g., adult versus

embryo and muscle versus kidney), which have different distributions of ECM components (as discussed in the previous section), it is evident that both  $\beta_1$  and  $\alpha_v$  integrins can support vascular morphogenesis. For example,  $\alpha_v\beta_3$  and  $\alpha_2\beta_1$  integrins associate with vascular morphogenesis in collagen-rich ECM, like adult tissue, while  $\alpha_5\beta_1$  and  $\alpha_6\beta_1$  integrins involve fibronectin- and fibrin-rich ECM, like in embryonic tissue and healing wounds [50]. The binding of integrins onto RGD triggers several downstream signaling events mediated by Rho GTPase, particularly Rac1 and Cdc42 [49]. Extensive work by Davis and his colleagues revealed the molecular mechanism that regulates this EC morphogenesis in fibrin and collagen gels (an excellent review of their work can be found in Chap. 20 of this book). This mechanism has also been observed and controlled in synthetic (HA-based) hydrogels [89].

To further substantiate the role of cell-ECM interactions, particularly those mediated by integrin engagement, several groups have identified the importance of integrin specificity in vascular regeneration. In tumor vessels,  $\alpha_v\beta_3$  is preferentially expressed, leading to formation of new, albeit disorganized, leaky vasculature [53]. In order to establish organized, mature neovessels, engagement of  $\alpha_3/\alpha_5\beta_1$ , rather than  $\alpha_v\beta_3$ , was necessary [147]. While RGD peptides facilitate cell adhesion in synthetic matrices, it is important to consider the non-specific integrin engagement potential of these peptides, which may influence vascular regeneration.

The number of RGD adhesion sites and the method of their presentation to the vascular cells are also crucial in affecting cell migration [82] and vascular morphogenesis [110]. Using an in vitro angiogenesis model, Folkman and Ingber were able to show that, when cultured on a moderate coating density that only partially resisted cell traction forces, ECs could retract and differentiate into branching capillary networks [67, 110]. High ECM density was saturated with RGD adhesion peptide, which allowed the ECs to spread and proliferate, while low ECM density resulted in rounded and apoptotic cells. Interestingly, in medium ECM density, with the appropriate RGD adhesion peptide, ECs collectively retracted and differentiated into branching capillary networks with hollow tubular structures. It is evident that the ECs exerted mechanical forces on the surrounding ECM to create a pathway for migration and branching in forming vascular structures [48]. Hence, both the quantity of RGD peptide and the method of presentation within the engineered synthetic biomaterials determine the initial morphogenetic events in angiogenesis.

### Scaffold Degradation Regulates Vascular Morphogenesis

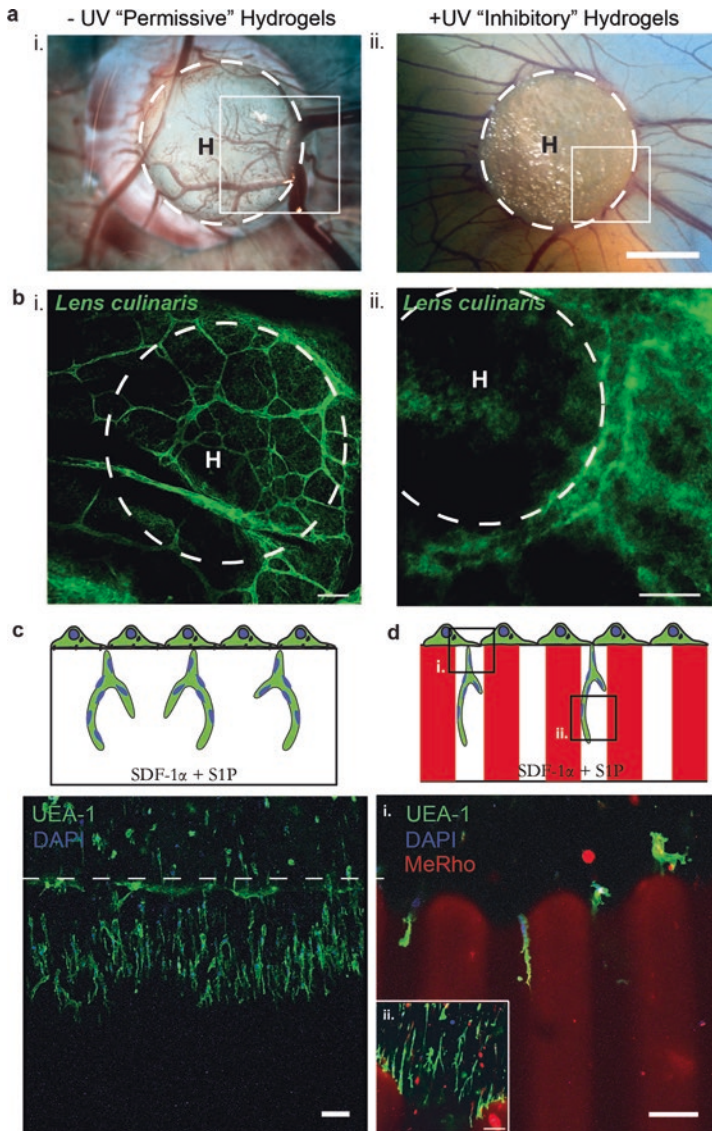
Scaffolds made from ECM components, like collagen and fibrin gels, contain proteolytic degradable sequences which can be degraded by the MMPs and other proteases (e.g., cathepsins) secreted by vascular cells. This cell-mediated degradation controls both structural integrity and temporal mechanical properties, which dictate the presentation of chemical and mechanical cues at various stages of angiogenesis. However, the degradation kinetics of these ECM-based scaffolds is determined by their inherent cross-linking density which, in turn, limits their manipulation for vascular tissue engineering. In contrast, synthetic biomaterials can be engineered to

have degradation profiles ranging from days to months, in order to suit the specific needs of the engineered vascularized tissue constructs [215]. The polymer backbone can be cross-linked using a nondegradable cross-linker that provides structural integrity and/or a degradable cross-linker that allows directed cell migration and vascular morphogenesis. Hydrolytic degradation by the body fluid can break down the ester bonds within the polymer backbone, allowing tissue infiltration over time [214, 215]. MMP-sensitive peptides can also be used to cross-link hydrogels, allowing cell-mediated degradation, leading to a rapid response of vascular growth. Overall, by adjusting the percentages of nondegradable and degradable cross-linkers, scaffold degradation can be tuned to allow cellular infiltration, lumen formation, and ECM synthesis and distribution.

In order for the intracellular vacuoles to coalesce into a lumen, ECs require adhesive ligands for traction [152] and utilize membrane-type-1 MMPs (MT1-MMPs) to create physical spaces which facilitate the directed migration of cells to align with neighboring cells [48, 192, 212]. Therefore, ECs can only invade this synthetic scaffold if the minimal pore size is larger than the cell diameter (e.g., a soft self-assembling peptide) [201] or if the scaffold bears an MMP-degradable sequence [153]. The Hubbell research group has pioneered this approach by incorporating an MMP-degradable sequence as a cross-linker into PEG scaffolds to promote vascular healing and therapeutic angiogenesis [196, 249]. When grafted *in vivo*, ECs were able to invade, remodel, and vascularize this MMP-sensitive scaffold [248, 249]. Using concepts from this work, synthetic (HA-based) biomaterials utilized spatial control of degradation through photopatterning to organize vascular morphogenesis (Fig. 4.3) [90]. Hence, incorporating MMP-degradable peptides is essential for directing vascular morphogenesis in 3D synthetic biomaterials.

### Physical Orientation of the ECM

The native ECM provides an instructive template for ECs and perivascular cells to orient, interact, and organize into tubular structures. Studies have demonstrated that a stable vasculature could be achieved by co-transplantation of ECs and perivascular cells, such as MSCs or SMCs [15, 16, 128, 142, 164]. Recent studies showed that engineering a stable vascularized tissue construct requires the triculture of ECs, fibroblasts, and tissue-specific cells, such as cardiac or skeletal muscle cells [31, 142]. Perivascular cells, such as fibroblasts, stabilize the developing vascular tube through physical support, by differentiating into v-SMCs and wrapping around the nascent tube [114, 229], and chemical support, by secreting Ang-1, PDGF-BB, and tissue inhibitor of metalloproteinase-3 (TIMP-3) [95, 97]. These perivascular cells are also responsible for laying down ECM components in early embryogenesis and continue to do so throughout adulthood. Many studies using fibroblast-derived matrices have further revealed the 3D complexity of these ECM networks [206–208]. A study by Soucy and Romer showed that fibroblast-derived matrix alone is sufficient to induce HUVECs to undergo vascular morphogenesis, independent of any angiogenic factors. Further analysis of protein colocalization suggested that



**Fig. 4.3** Spatial control of vascular morphogenesis in synthetic hydrogels. (a, b) Uniform -UV (permit cell-mediated degradation) and +UV (inhibit cell-mediated degradation) hyaluronic acid (HA) hydrogels are grafted onto the CAM membrane. (a) LM imaging and (b) confocal analysis of the boxed regions in a shows CAM vessels penetrating into the -UV but not into the +UV hydrogels. CAM vessels are stained with Fluorescein-conjugated *Lens culinaris* lectin. Scale bars in a, 20 mm, and b, 100  $\mu$ m. H = hydrogels. Dotted white lines indicate the boundaries of the hydrogels. (c) ECFCs are seeded on top of a uniform -UV (c) and 100  $\mu$ m stripe photopatterned (d) HA hydrogels for an angiogenesis assay. After 3 days in culture, ECFCs invade and sprout into the 3D hydrogels (c). When photopatterned HA hydrogels are used, invasion and sprouting are observed only within the -UV regions and not within the inhibitory +UV region (d). Confluent monolayer of ECFCs sprouts and invades the -UV region (i) and further branches along the -UV regions (ii). The +UV regions are labeled using MeRho (red); ECFCs are stained with fluorescein-conjugated UEA-1 lectin (green) and DAPI (blue). Scale bars are 100  $\mu$ m. Reproduced and reformatted with permission [90]

fibronectin with a distinct structure and organization was uniquely distributed among other secreted matrix components, such as collagen, tenascin C, versican, and decorin. Cell matrix adhesions and MT1-MMP activities were reported to orient and localize within this fibrous fibronectin, which is indicative of integrin-mediated vascular morphogenesis [190]. In fact, ECs initiate neovascularization by unfolding soluble fibronectin and depositing a pericellular network of fibrils that serve as a structural scaffolding on a mechanically ideal substratum for vessel development [247]. We have studied how such fibronectin organization influences endothelial tube formation by patterning fibronectin on cell culture surfaces to optimize vasculogenic potential and understand how microstructure influences vascular tube formation [54]. Alignment of other important ECM proteins, such as collagen, has also been shown to guide vascular regeneration by enhancing EC organization and migration [136]. Interestingly, similar effects in vascular organization are observed when tensile forces are incurred upon vascular fibrin-based constructs, where vascular network alignment was induced by application of force. Aligned microvasculature was shown to enhance vascular integration upon implantation in abdominal muscle [191]. This last study suggests a potential mechanism for organization of ECM components to guide vascular network organization through force-induced remodeling. It is likely such a mechanism is coupled with ECM degradation and secretion of new ECM components to establish a microenvironment amenable to the formation of new blood vessels.

The unique orientation, organization, and nanotopography of fibrous fibronectin represent features that can be integrated into synthetic scaffolds. Synthetic polymers, like PLGA and polycaprolactone (PCL), can be electrospun to produce various fiber sizes with micro- to nanoscale features that resemble fibrous fibronectin. We previously showed that surface nanotopography enhanced the formation of capillary-like structures (CLSs) *in vitro* [23]. Growing EPCs on grooves that were 600 nm wide reduced their proliferation and enhanced their migration without changing the expression of EC markers. Moreover, after 6 days of culture, the EPCs organized into superstructures along the nanogrooves, in significant contrast to the EPCs grown on planar surfaces (Fig. 4.2b). The addition of Matrigel further induced the formation of CLSs, with enhanced alignment, organization, and tube length compared to a flat surface (Fig. 4.2c). This underscores the increasingly important role of nanotopography in guiding and orienting vascular assembly. When integrated into the tissue-engineered construct—for instance, using filamentous scaffold geometry [75] and micropatterning [55, 108, 165]—the orientation and structure of the engineered vasculature can be controlled.

### Regulating Matrix Mechanics

It has become increasingly evident that the biomechanical properties of the ECM, such as matrix orientation and mechanics, profoundly influence the control of vascular morphogenesis. Due to their versatility with respect to mechanical properties (e.g., cross-linking density, pore sizes, and topography), synthetic biomaterials have

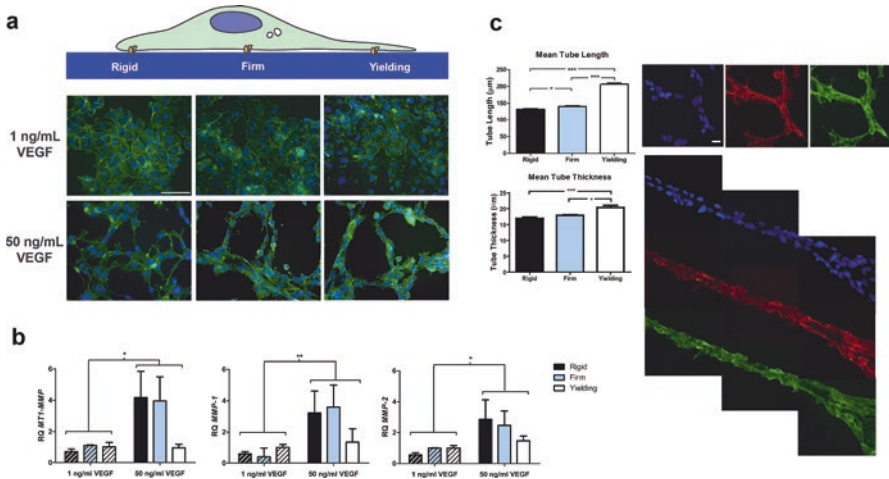
powerful features that can be exploited to further direct vascularization. Changes in ECM mechanics can lead to changes in GF availability [40, 110], drive capillary morphogenesis [109], and stimulate angiogenesis *in vivo* [122]. By altering matrix adhesive characteristics and mechanics, Ingber and Folkman illustrated how bFGF-stimulated ECs can be switched between growth and differentiation during angiogenesis [110]. Recently, biomechanical cues from the ECM and signals from GF receptors have been implicated in regulating the balance of activity between TFII-I and GATA2 transcription factors, which govern the expression of VEGFR2 to instigate angiogenesis [158]. Matrix stiffness regulates not only the cell's response to soluble GFs but also cell morphogenesis during angiogenic sprouting. Primarily due to MMP activity, the tip of a new capillary sprout becomes thinner, locally degrading the basement membrane proteins. This region, with its high rate of ECM turnover and thin basement membrane, becomes more compliant and stretches more than the neighboring tissue. Consequently, the decrease in matrix stiffness changes the balance of forces across the cell integrin receptors, increases cell tension, and results in cytoskeletal arrangement to form branching patterns that are characteristic of all growing vascular networks [109].

The pioneering work by Deroanne et al. showed that a decrease of matrix stiffness increased capillary branching and the elongation of tubes. A reduced tension between ECs and ECM, accompanied by a profound remodeling of the actin-FAP complex, is sufficient to trigger an intracellular signaling cascade leading to tubulogenesis [52]. This observation has been further confirmed in collagen gels [52, 200], fibrin gels [211], self-assembling peptides, and HA-gelatin hydrogels.

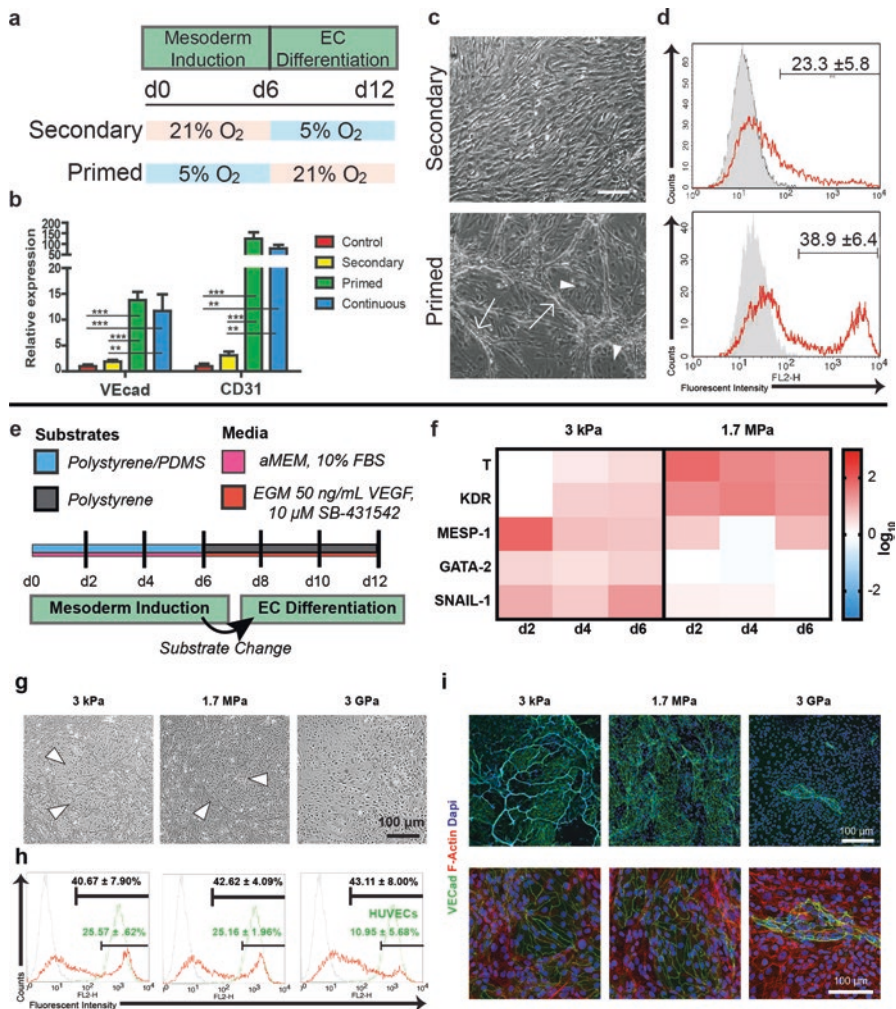
Although ECM-based gels, such as collagen, fibrin, and Matrigel, have been widely used in angiogenesis assays, their inherent physical properties have limited their usage when studying the effects of matrix mechanics on angiogenesis. The stiffness of ECM-based gels can be increased either by increasing their concentration, which also alters their ligand and fibril density [189], or by altering the cross-linking of ECM proteins in a narrow range using a microbial transglutaminase [244]. Therefore, examining the effects of matrix stiffness alone on angiogenesis requires the use of synthetic hydrogels, the stiffness of which can be easily adjusted over a wide range of moduli without altering other chemical properties. Unlike naturally available ECM-based gels, the elasticity of which is limited to their inherent cross-linking density, synthetic HA hydrogels can be used to study a physiologically relevant range of matrix elasticity [88]. When the cross-linking density of the HA-gelatin hydrogels was further reduced, the matrix elasticity became relatively compliant, resulting in an increase of capillary branching, elongated tubes, and enlarged lumen structures [88]. On a relatively compliant matrix, EPCs can produce fewer MMPs than a stiffer matrix would require and still degrade, exert mechanical tension on, and contract the matrix to enable vascular morphogenesis. On the other hand, EPCs must produce more MMPs on a stiffer matrix, to overcome the extra mechanical barriers; even then, this local decrease in substrate stiffness cannot support vascular morphogenesis (Fig. 4.4). This model also explains the rapid appearance of large functional vessels in granulation tissue, as a response to the wound-healing mechanism [122].

In addition to the effects of matrix stiffness on postnatal vascular regeneration, matrix stiffness has been probed as an important regulator of stem cell fate. Beginning with the pioneering work of Engler et al. [60], studies examining the effects of substrate stiffness and mechanical signaling transduction pathways on stem cell fate have proven instrumental in enhancing our collective knowledge of differentiation schema. To this end, our group has shown that substrate stiffness can govern EC fate through alterations in mesodermal precursors. Similar to the enhancements in EC fate observed upon culture in low  $O_2$  environments (Fig. 4.5a–d) [135], compliant substrates enhance mesodermal differentiation, which results in robust EC differentiation (Fig. 4.5e–i) [205].

A recent illuminating study identified stress relaxation as an important, yet understudied, regulator of mechanical signal transduction. Specifically, in alginate-based hydrogels with the same matrix stiffness and pore size, altering stress relaxation modulated MSC cell fate [34]. While studies of the effect of stress relaxation on EC fate and vascular morphogenesis have not been published, stress relaxation is an important parameter to bear in mind for biomaterial design, particularly because covalently cross-linked hydrogels do not exhibit stress relaxation behavior similar to that of the native ECM.



**Fig. 4.4** Mechanoregulation of vascularization. **(a)** EPCs were seeded on rigid, firm, and yielding substrates for 12 h, supplemented with 1 ng/ml (low) VEGF (upper panel) and formed CLSs when supplemented with 50 ng/ml (high) VEGF (lower panel), as demonstrated by fluorescence microscopy of F-actin (green) and nuclei (blue). **(b)** Real-time RT-PCR revealed a significantly increased expression of (1) MT1-MMP, (2) MMP-1, and (3) MMP-2 in response to 50 ng/ml VEGF (high) concentration for EPCs cultured on the rigid, firm, and yielding substrates, respectively. As the matrix substrate was reduced, EPCs cultured in medium supplemented with 50 ng/ml (high) VEGF showed a decrease in expression of these MMPs. **(c)** Metamorph analysis of CLSs revealed a significant increase of mean tube length and mean tube area, as substrate stiffness decreased. Confocal analysis of nuclei (blue), VE-CAD (red), and lectin (green) further revealed that branching and hollow tubular structures formed on the yielding substrate. Significance levels were set at  $*p < 0.05$ ,  $**p < 0.01$ , and  $***p < 0.001$ . Scale bars **(a)** 100  $\mu\text{m}$  and **(c)** 20  $\mu\text{m}$ . Printed with permission [88]



**Fig. 4.5** Low O<sub>2</sub> and compliant substrates enhance induction of mesodermal precursor populations, thereby improving EC fate specification. **(a)** Schematic of manipulated O<sub>2</sub> environments studied during differentiation. **(b)** RT-PCR analysis of VECad and CD31 expression of EVCs differentiated under the four studied oxygen conditions. Comparison of secondary and primed 5% O<sub>2</sub> conditions demonstrated by **(c)** light microscopy images (arrows indicate elongated cell bundles; arrowheads indicate cobblestone area-forming cells; scale bar is 100 μm) and **(d)** flow cytometry for VECad expression. Isotype control in gray. \**p*<0.05; \*\**p*<0.01; \*\*\**p*<0.001. **(e)** Schematic of stiffness-primed mesoderm induction followed by EC differentiation on E ~ 3 GPa substrates. a-MEM, a-minimum essential medium; FBS, fetal bovine serum; EGM, endothelial growth medium. **(f)** Gene expression of mesodermal markers for cells differentiated on soft 3-kPa substrates and stiff 1.7-MPa substrates, normalized to expression from E ~ 3 GPa surfaces. Color key is presented in log<sub>10</sub> scale. **(g)** Bright-field images of cobblestone endothelial colonies (white arrows) on day 12 EVCs. **(h)** Day 12 EVC flow cytometry plots of VECad expression in red, with corresponding HUVEC VECad expression in green. Black font, VECad+ cells; green font, highly expressing VECad+ cells. Data are presented as means ± SEM. **(i)** Representative immunofluorescence images of VECad expression on day 12 EVCs: low-magnification (top) and high-magnification (bottom) images are shown (green, VECad; red, phalloidin; blue, nuclei). Reproduced and re-formatted with permission [135, 205]



These studies underline the importance of engineering a tissue construct with a matrix stiffness amenable to promote *in vivo* vascularization. However, investigating how matrix stiffness may affect *in vivo* vascularization remains challenging due to the complexity of the system, which involves matrix remodeling, host capillary ingrowth, as well as anastomosis of the vascular construct and contributions from other cell types. For example, *in vivo* vascular ingrowth into Matrigel scaffolds was found to be optimal at intermediate matrix stiffness, in sharp contrast to the observed *in vitro* ingrowth [158]. Elegant work by Yoder's research group also found that increasing the collagen concentration yielded stiffer scaffolds, which in turn promoted host capillary ingrowth *in vivo*. Compared to stiffer scaffolds, softer scaffolds might have experienced excessive *in vivo* remodeling and failed to retain the vascular constructs. Moreover, *in vitro* angiogenesis studies have found that ECM-based gels produce a much narrower range of stiffness [46, 158] than synthetic hydrogels [88, 158]. Future investigations are needed to evaluate vascularization by both the host capillary and the engineered vascular construct over a wider range of physiologically relevant matrix elasticities. Despite the differences in scaffold composition (ECM-based gels versus synthetic hydrogels), culture conditions (*in vitro* versus *in vivo*), assay type (2D versus 3D), and ranges of matrix stiffness, all of these studies highlight the relevance of engineering scaffolds with mechanical elasticity suited to the specific needs of tissue vascularization.

### ***4.2.3 The Effects of Oxygen Availability and the ECM***

In this section, we will consider  $O_2$  tension and the ECM as two interdependent factors determining the efficiency of vasculature formation. We will review currently available  $O_2$  measurement techniques and challenges, along with the mathematical modeling approaches used to overcome some of these challenges in describing  $O_2$  gradients in 3D environments. Then, we will discuss cellular adaptations and responses to  $O_2$  availability in 3D ECM constructs and the possible outcomes of variations in  $O_2$  distribution in 3D cultures of vascular cells.

#### **4.2.3.1 Varying Oxygen Tensions in the ECM of Tissue and Matrix Scaffolds: Measuring and Modeling**

##### Oxygen Measurement Techniques and Challenges

Manipulation of oxygen, in order to direct pluripotent or vascular cells to form blood vessels, requires knowing the precise  $O_2$  tension that the cells are exposed to under varying conditions. Many different  $O_2$  measurement techniques have been used *in vitro* and *in vivo*. The accuracy of these measurements is fundamental to confidently describe the cellular responses under various  $O_2$  availabilities, as well as to controlling the  $O_2$  tension in order to direct angiogenesis and vasculogenesis. An

O<sub>2</sub> measurement method needs several properties to be considered superior, including accuracy, sensitivity, repeatability, rapidity, and noninvasiveness. Although some methods are used more commonly in a broader range of applications, no “gold standard” exists for all applications, since the method chosen usually depends on the purpose of the measurement. In vivo O<sub>2</sub> measurement methods can be divided into two main categories: (1) direct measurements, where the concentration or the partial pressure of O<sub>2</sub> is directly measured, and (2) indirect measurements, where levels of O<sub>2</sub>-indicative molecules (e.g., hemoglobin, cytochrome) are detected and correlated to relative O<sub>2</sub> concentrations.

The most common direct measurements are electrodes, phosphorescent probes, electron paramagnetic resonance (EPR) oximetry, and nuclear magnetic resonance (NMR). Some of the indirect measurement methods involve monitoring of hemoglobin/myoglobin, mitochondrial cytochromes, and NADH/FADH [209]. Springett’s paper thoroughly reviews the benefits and limitations of the most recent methods [209].

In addition, in vitro studies have applied these currently available methods to monitor O<sub>2</sub> levels quantitatively, such as by measuring O<sub>2</sub> tensions at the cellular level in 2D monolayer cell cultures or O<sub>2</sub> gradients in 3D gels or scaffolds. Two major methods used to measure O<sub>2</sub> levels during in vitro cultures are polarographic and fluorescence quenching techniques. The latter has been shown to surpass the polarographic technique, which consumes O<sub>2</sub> during the measurements [197]. When an implemented measurement technique, like the polarographic technique, consumes O<sub>2</sub>, it more likely generates even greater inaccuracies and leads to incorrect conclusions in low O<sub>2</sub> environments, as occurred in studies investigating the effect of hypoxia in 3D scaffolds [36, 120, 145]. Fluorescence quenching technology is available both for invasive applications, using an electrode probe with a very thin (approximately 5 μm) tip, and for noninvasive applications, using a sensor patch composed of a ruthenium-based metal complex that can be excited by an external fluorescent light source.

### *Modeling Oxygen Transport in Tissues*

The limitations of these measurement techniques, caused mostly by the difficulties in measuring spatial O<sub>2</sub> concentrations in tissues or scaffolds, raise a need for predictive mathematical models. Transport of O<sub>2</sub> in vivo is controlled by several parameters, including blood flow rate, degree of vascularization in the tissue, physiological distance of the cells from the microvasculature, and, depending on cell type, the cells’ rate of O<sub>2</sub> consumption. These factors affect O<sub>2</sub> distribution in the tissue, and some can also have an impact on O<sub>2</sub> transport in 3D in vitro cultures of pluripotent or vascular cells. Additional factors that in vitro studies should consider are the geometry of the scaffold, the available surface area for O<sub>2</sub> transport from the environment to the system, and controlled dissolved O<sub>2</sub> levels in the culture media.

In general, fundamental mathematical models estimating O<sub>2</sub> distribution in 3D constructs can be classified into: (1) static models, where O<sub>2</sub> is only transported via diffusion, and (2) dynamic models, where convective transport of O<sub>2</sub> is also incor-

porated using perfusion systems, such as microfluidic devices, or microcirculation in the tissues.

### Static Models

In tissues cultivated under static conditions within 3D scaffolds, using different types of biomaterials, spatial  $O_2$  concentration can be defined with a one-dimensional (1D), unsteady-state species continuity equation:

$$\frac{\partial C_{O_2}}{\partial t} = D_{O_2} \frac{\partial^2 C_{O_2}}{\partial z^2} - R \quad (4.1)$$

where  $C_{O_2}$  is the spatial  $O_2$  concentration in the scaffold changing with time ( $t$ ) and axial position ( $z$ ),  $D_{O_2}$  is the diffusion coefficient of  $O_2$  in the scaffold material, and  $R$  is the oxygen consumption rate of cells. This form of the transport equation has been used in many studies attempting to predict the  $O_2$  gradients in 3D scaffolds [27, 76, 137]. The equation implies that  $O_2$  changes both with time and depth, while being consumed by the cells as it diffuses from the environment into the scaffold. Boundary conditions, which are critical for  $O_2$  distribution, depend on the  $O_2$  equilibrium between the environment (media/air) and the boundaries of the scaffold. Therefore, for a 3D scaffold with a depth of  $L$  and open boundaries from both sides, the boundary conditions can be given as:

$$\text{At } z = 0 \text{ and } z = L, C_{O_2} = S \cdot P_{O_2} \quad (4.2)$$

Thus, the solubility ( $S$ ) of  $O_2$  in the scaffold material is one of the determining parameters of  $O_2$  distribution. Although the diffusion coefficient can also be considered a critical factor in relatively stiff scaffolds of the sort usually used for cartilage and cardiomyocyte tissues [27], it has been shown to be less significant for the natural hydrogel scaffolds commonly used for vascular tissues, such as collagen and HA. For instance, the diffusion coefficient of  $O_2$  in collagen gels was found to be 99% of that in water [83]. Therefore, modeling studies usually assumed that it has the same  $O_2$  diffusion coefficient as water or cell media ( $3.3 \times 10^{-5} \text{ cm}^2/\text{s}$  at  $37^\circ\text{C}$ ) [157]. The consumption rate of  $O_2$  ( $R$ ) given in Eq. (4.1) is a function of both  $C_{O_2}$  and  $\rho_{\text{cell}}$  and is governed by the Michaelis-Menten equation, which states that the  $O_2$  uptake rate of each cell increases with  $O_2$  availability, reaching a maximum at a point,  $V_{\text{max}}$ :

$$R = \rho_{\text{cell}} \frac{V_{\text{max}} C_{O_2}}{K_m + C_{O_2}} \quad (4.3)$$

where  $K_m$  is the  $O_2$  concentration at which the  $O_2$  uptake rate is half of its maximum value and  $\rho_{\text{cell}}$  is the cell density as a function of time and position. Different groups have reported the  $V_{\text{max}}$  and  $K_m$  parameters of many vascular cell types at various cell seeding densities [78, 163]. For example, the  $V_{\text{max}}$  and  $K_m$  of HUVECs, at a density

of  $1 \times 10^6$  cells/ml, are found to be  $22.05 \pm 1.92$  (pmol s<sup>-1</sup> 10<sup>-6</sup> cells) and  $0.55 \pm 0.02$  (μM), respectively [78]. It should be noted that these parameters are estimated as to mitochondrial consumption of O<sub>2</sub>. However, as already discussed, ECs also consume O<sub>2</sub> for ROS production, and the theoretical models should also take this additional O<sub>2</sub> consumption into account by, for example, including a linear correlation in the O<sub>2</sub> consumption rate equation (Eq. (4.3)). Besides, all estimations of the  $V_{\max}$  and  $K_m$  parameters for the O<sub>2</sub> consumption of different cell types are carried out in 2D cultures. The literature currently lacks studies investigating whether or not, depending on the composition of the extracellular matrix, encapsulating cells in 3D gels changes their consumption of O<sub>2</sub>.

Vascular cells proliferate, die, migrate, and assemble during 3D cultivation, which affects their spatial and temporal density and, therefore, O<sub>2</sub> distribution. Models developed for 3D cultures of cardiomyocytes take into account the cellular proliferation and changes in the dimensions of the cells during nutrient transport in scaffolds [76, 77]. However, we need more detailed models, which consider how capillary formation affects O<sub>2</sub> transport, to achieve more reliable estimations of spatial O<sub>2</sub> concentration. Tube formation and the networking of ECs in 3D gels have been simulated by more complicated numerical models [47, 139], although the effects of O<sub>2</sub> concentrations on tube formation dynamics still need to be incorporated.

#### *Dynamic and In Vivo Models*

The models used for static cultures in 3D scaffolds can also be used to describe O<sub>2</sub> distributions in vivo when combined with a fluid perfusion model that considers the convective O<sub>2</sub> transfer to the tissues. The velocity profile of a fluid in capillaries or in an engineered microchannel system can be calculated using the simplified Navier-Stokes equation with cylindrical coordinates given for a laminar, one-dimensional, steady-state, and fully developed flow of an incompressible fluid:

$$\frac{dP}{dz} = \mu \left[ \frac{1}{r} \frac{d}{dr} \left( r \frac{dV_z}{dr} \right) \right] \quad (4.4)$$

where  $P$  is the total pressure in the fluid changing in an axial direction,  $\mu$  is the viscosity of the fluid, and  $V_z$  is the axial velocity of the fluid changing in a radial direction. After estimating the blood velocity profile, the species continuity equation, which involves both diffusional and convective transfers of O<sub>2</sub>, can be used to obtain the O<sub>2</sub> distribution inside the capillary or microchannel:

$$V_z \frac{\partial C_{O_2}}{\partial z} = D_{O_2}^{\text{Blood}} \left[ \frac{1}{r} \frac{\partial}{\partial r} \left( r \frac{\partial C_{O_2}}{\partial r} \right) + \frac{\partial^2 C_{O_2}}{\partial r^2} \right] \quad (4.5)$$

The technical difficulties of making quantitative O<sub>2</sub> measurements in BM have led many researchers to develop mathematical models to describe BM O<sub>2</sub> distribu-

tion [41, 131, 133]. Additional parameters that need to be considered *in vivo* are the vascularization of the tissue and the transport of  $O_2$  via hemoglobin proteins, making the concentration of hemoglobin another essential factor for determining the oxygenation of the tissue. Studies take these additional factors into account using the following equation:

$$D_{O_2}^j [\nabla^2 C_{O_2}^j] = V_z \frac{\partial}{\partial z} [C_{O_2}^j + N\phi_{O_2}] \quad (4.6)$$

The superscript  $j$  denotes each sinusoid/arteriole around the BM.  $N$  is the  $O_2$  carrying capacity of the blood and is the concentration of  $O_2$  bound to hemoglobin, which depends on the plasma  $O_2$  concentrations [180]. Finally, spatial and temporal  $C_{O_2}$  in tissue can be estimated in a similar manner to the *in vitro* models, using Eq. (4.1) incorporated with the continuity of fluxes assumption at the ECM-vessel interface as a boundary condition.

#### 4.2.3.2 Targeted Cellular Responses to $O_2$ Availability in Matrix Hydrogels

Engineering vascular tissues in a 3D ECM is well-orchestrated process combining proliferation, apoptosis, migration, activation, and assembly of vascular or precursor cells inside the construct. As discussed in the previous section, the composition of the biomaterial used to encapsulate the cells is critical for cellular fate and vessel formation. In addition to the effects of the chemical and physical properties of the ECM material on blood vessel formation, temporal and spatial levels of  $O_2$  and other nutrients are also crucial for various targeted cellular responses. A number of studies have investigated the effects of matrix content and stiffness on angiogenesis/vasculogenesis [109], and many others have proposed using different types of bio-synthetic materials to develop more precise blood vessels [10, 152]. However, only a few studies have highlighted how  $O_2$  gradients occurring in the matrix contribute to the angiogenic process [93, 170, 177]. The availability of  $O_2$  and other nutrients decreases at the center of the gel compared to the periphery, especially in engineered vascular tissues, which require a high cell seeding density for sufficient vascular tissue generation or repair. Hence, cells that reside along various layers of the matrix respond differently to the nonuniform distribution of  $O_2$  and nutrients. For primary vascular cells to form blood vessels, they require survival, activation, and the induction of angiogenesis by GFs, cell signaling, and regression. All of these responses, necessary for blood vessel formation, are controlled by ECM properties, as well as by  $O_2$  availability. Therefore, the influences of both the ECM and dissolved  $O_2$  distribution should be considered simultaneously.

Cell assembly and tube formation in the ECM require a sufficient cell density. Deprived of  $O_2$  and nutrients, vascular cells can undergo apoptosis or necrosis [21]. These two cellular death mechanisms should be distinguished; apoptosis contributes to the process of angiogenesis at any  $O_2$  tension, whereas necrosis usually

results in the collapsing and deformation of tubes [195]. The critical issues to consider to prevent cellular necrosis during 3D vascular cell cultures are the permeability of the ECM material to  $O_2$  and glucose, the cell seeding density, and the thickness of the gel. Thus, cell seeding density is constrained by an upper limit, above which the cells undergo necrosis due to nutrient deprivation, and a lower limit, below which the cells cannot assemble sufficiently to form tube-like structures. Both limits depend on the equilibrium  $O_2$  levels in the environment.

Cells may also die as a result of apoptosis after their encapsulation in the gel. Interestingly, some groups have demonstrated that programmed cellular death is necessary for angiogenesis/vasculogenesis [195]. Segura et al., having studied tube formation of ECs in both 2D Matrigel and 3D collagen, concluded that a considerable number of cells undergo apoptosis at the initial stages of cultivation and that, once angiogenesis is induced and tube formation has started, no further apoptosis occurs throughout the process. Inhibition of proapoptotic proteins has been shown to correlate with defective tube formations, suggesting that apoptosis is important for avoiding imperfections during blood vessel growth. Hypoxia, as already discussed, induces angiogenic responses and also regulates proapoptotic gene expressions. Thus, spatial variations in  $O_2$  levels may alter the apoptotic responses in the gel and therefore regulate vascular tube morphogenesis.

MMPs are promoted by integrin-ligand interactions between cells and the ECM, leading to the degradation of the ECM and facilitating the migration of the cells [81]. It is hypothesized that ECM fragmentation, orchestrated by the secretion of MMPs, can mediate caspase activity through the rebinding of ECM protein fragments to unligated integrins, namely, death receptors [38]. Therefore, the survival of ECs depends on the balance between cell survival promoters, such as FAK, Src, and Raf, and cellular apoptosis promoters, such as caspase 8 and caspase 3. Hypoxia may again play a critical role here, affecting both sides of the equilibrium, by upregulating MMPs and VEGF at the same time [21, 93]. Hypoxia, accompanied by nonuniform distribution of  $O_2$  throughout the gel, can result in spatial differences of cellular viability, which may subsequently disrupt vascular networking.

Overall, blood vessel growth requires remodeling of the ECM, which is based on two distinct mechanisms: (1) degradation of the ECM by secreted proteases, and (2) production of new ECM to support the invading vasculature. Many studies have shown that hypoxia can regulate the degradation, maintenance, and synthesis of the ECM [61, 179]. ECM degradation is important for cellular migration into and blood vessel invasion of tissue. MMPs, as mentioned above, are a major family of proteinases that participate in the degradation of the ECM during angiogenesis. In particular, MMP-2 and MMP-9, both members of the gelatinase subgroup of MMPs, have been shown to contribute to the process of angiogenesis [85]. MMP-2 secreted by the cells is activated through membrane MT1-MMPs where the activation can be avoided in the presence of tissue inhibitor of MMP-2 (TIMP-2) at high levels [99]. Furthermore, hypoxia was shown to influence the expression of MMP-2, as well as of MT1-MMP and TIMP-2, in ECs [21]. Lahat's group demonstrated the upregulation of MMP-2 expression in hypoxic (0.3%  $O_2$ ) cultures of HUVECs, whereas MT1-MMP and TIMP-2 are downregulated, enhancing migration and tube formation [21].

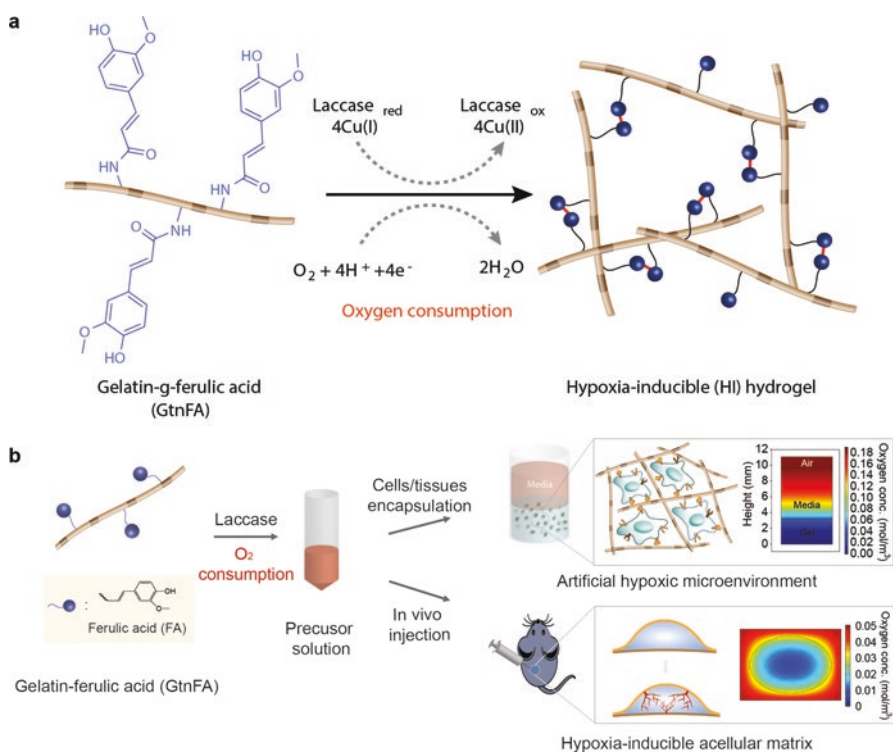
ECM degradation is accompanied by ECM production and the secretion of cells. Once the quiescent state of the ECs composing the blood vessel walls is perturbed and angiogenesis is induced, ECs start to proliferate and invade the neighboring ECM by using proteinases. At the same time, they start to remodel the existing ECM by synthesizing new ECM. In healing wounds, ECs produce transitional ECM proteins, including fibrinogen and fibronectin, and temporarily deposit them in the ECM in order to provide available ligands during vessel growth [38]. Moreover, ECs also produce such matricellular proteins as tenascin C and SPARC in the ECM to mediate angiogenesis [38]. Clearly, the new ECM synthesis of cells is crucial for angiogenesis, and hypoxia, through HIF1 $\alpha$ , has been shown to regulate the expressions of many different types of ECM proteins [167]. For example, many *in vivo* and *in vitro* studies have shown that hypoxia enhanced the synthesis of collagen, the most abundant protein in mammalian tissues [22, 103, 217].

Moreover, proliferation of the cells during angiogenesis/vasculogenesis in 3D scaffolds is regulated by basic fibroblast growth factor (bFGF) and VEGF, which are known to be hypoxia-dependent proteins [177]. In most studies of the vascularization of 3D scaffolds, both GFs are broadly used as soluble factors that supplement cell growth medium to induce proliferation and migration [127, 193]. In addition, Shen et al. demonstrated that immobilization of VEGF into a 3D collagen scaffold promotes EC viability, proliferation, and vascularization [198]. VEGF has been shown to promote blood vessel formation, not only by inducing cellular proliferation and migration but also by directly regulating elongation and capillary networking in 3D ECM constructs deprived of O<sub>2</sub> and nutrients [93]. Helminger et al. used a sandwich system to seed HUVECs inside a collagen gel [93]. The transfer of O<sub>2</sub> and nutrients was accomplished with only simple diffusion through the edges of the collagen, so that O<sub>2</sub> and nutrient levels decreased toward the center. They found that, in a short time period (about nine hours), VEGF intensity increased in the interior regions deprived of O<sub>2</sub>, which correlates well with cell elongation and branching. VEGF promoted capillary networking independently of proliferation, highlighting the role of autocrine VEGF in the reorganization of vascular networks in hypoxic regions of solid tumors. Another study focusing on quantitative measurements of O<sub>2</sub> gradients in 3D collagen also showed that increased VEGF concentrations correlated well to decreasing O<sub>2</sub> levels throughout the 3D constructs during a ten day period of cultivation [36].

A few studies considering the induction of angiogenesis by hypoxia in 3D scaffolds have emphasized that lowering the O<sub>2</sub> tension in 3D gels improved cellular branching and tube formation of ECs [170, 177]. As made abundantly clear throughout this chapter, both O<sub>2</sub> and matrix mechanics act as potent upstream regulators of a variety of signaling pathways that contribute significantly to the regulation of vascular differentiation as well as morphogenesis. To better understand these processes, engineers and biologists together have built an impressive library of materials and assays to examine an array of signaling cascades guiding the formation of blood vessels. There are several interesting platforms that can be used to study the effect of hypoxia and O<sub>2</sub> gradients on cellular function, including hydrogels (Fig. 4.6) [143, 144, 173, 187] and microfabrication or microfluidic devices [2,

3, 6, 37, 74, 169]. Stiffness gradients can also be controlled to study ranges of viscoelasticity and their effect on cell fate, migration, and other parameters [84]. Finally,  $O_2$  and matrix mechanics can be independently controlled in the same system using gelatin- and dextran-based hydrogels. These polymers can be both enzymatically cross-linked by an  $O_2$ -consuming reaction to create hypoxic conditions and cross-linked by a secondary non- $O_2$ -consuming reaction, to create a stiffer microenvironment without appreciably affecting microenvironmental  $O_2$  [25].

A growing body of publications have both investigated the influence of the ECM composition on angiogenesis/vasculogenesis and suggested the crucial roles of hypoxia in blood vessel formation. In addition, the evidence discussed above is sufficient to suggest that the ECM composition and  $O_2$  tension are coupled factors that need to be taken into account concurrently when developing and repairing vascular tissues in 3D microenvironments.



**Fig. 4.6**  $O_2$  controllable hydrogels can be used to study the effects of hypoxic gradients both in vitro and in vivo. **(a)** Synthesis of Gtn-FA, which can form a hydrogel network via a laccase-mediated cross-linking reaction with  $O_2$  consumption. **(b)** The Gtn-FA precursor solution can be mixed with either cells or tissues to provide artificial hypoxic microenvironments with  $O_2$  gradients (see inserted computer simulation of  $O_2$  tension and gradients). The precursor solution can also be directly injected into the animal as a hypoxia-inducible acellular matrix that induces temporal hypoxia and an  $O_2$  gradient in the body (see inserted computer simulation of oxygen tension and gradients). Reproduced and re-formatted with permission [144]

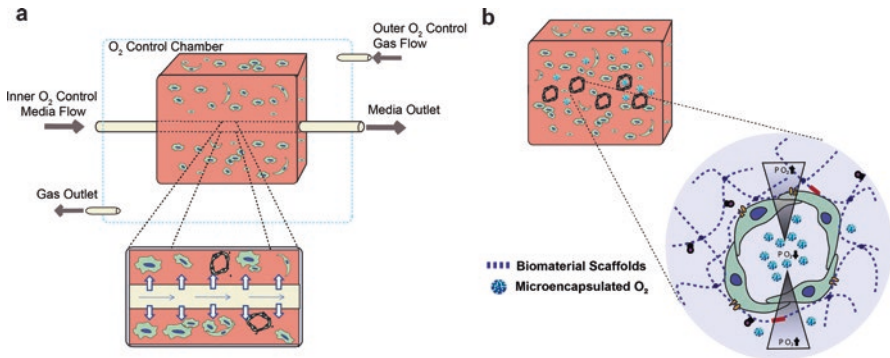


### 4.3 Future Directions

Understanding the simultaneous effects of the ECM and  $O_2$  tension on the processes of angiogenesis/vasculogenesis will enable researchers to control these two factors and thereby manipulate cellular responses in desired directions. Recent developments in many different fields of research, such as smart biomaterials and microfluidics, have made it possible to design and construct novel in vitro microenvironments for cells. Smart biomaterials have been developed that can dynamically respond to external stimuli, such as light [79], pH [174], temperature [216], and cytokines [124]; these materials can truly mimic the complexity of a native ECM environment. The ability to control the physical and chemical properties of the gels at different spaces and times will provide better control over different stages of angiogenesis. Light-sensitive hydrogels can be used to create biomaterials with distinct cross-linking densities to promote and inhibit cell spreading and migration [121], which in turn can be used to pattern complex vascular networks. Since vascular morphogenesis is sensitive to tissue stiffness [52], orientation [23], and polarity [42, 149], researchers could also induce vascular assembly into a tube by creating elasticity, GFs, adhesion peptide, and oxygen gradients along the 3D scaffold [144, 151, 228]. The development of photodegradable hydrogels, as well as the control of cell-mediated degradation in synthetic hydrogels, whose mechanical and chemical properties are controllable during the timescale of cellular development [123], has enabled control of vascular assembly [89, 90]. On the other hand, creating smart biomaterials that can shrink, swell, or degrade in response to oxygen tension would also be desirable to prevent the formation of anoxic regions inside the gels. More precise temporal control of  $O_2$  gradients inside the constructs could also be beneficial to explain various phenomena taking place in the body, such as EPC regeneration in the BM and embryonic development, where the  $O_2$  gradient plays a critical role in differentiation and migration dynamics.

Figure 4.7 illustrates two proposed approaches for controlling oxygen distribution and ECM properties. One proposal for regulating  $O_2$  gradients inside the gel would be to incorporate microfluidic technology [66, 129, 163, 220]. Although this approach provides better  $O_2$  control over 3D microenvironments, the problem of spatial variations in  $O_2$  levels throughout the gel, due to the cells'  $O_2$  consumption, must still be addressed. Advancements in microfluidic technology could enable spatial  $O_2$  control over 3D microenvironments; for instance, the gel could be prepared around a microtube, which would supply  $O_2$  by flushing growth media containing a desired amount of  $O_2$  (Fig. 4.7a). Hence, different  $O_2$  gradients could be generated via the manipulation of  $O_2$  concentrations in the outside environment and inside the microtube.

Another method for controlling and improving  $O_2$  transport in the gel would be to microencapsulate  $O_2$  carrier liquids, such as perfluorocarbons (PFCs). Due to their high capacity to dissolve  $O_2$ , PFCs have been used as a blood replacement to improve  $O_2$  delivery to tissues [112, 183]. Based on the high oxygen-carrying capacity of PFCs, Radisic et al. [183] developed a PFC-perfused system to supply sufficient



**Fig. 4.7** Controlling ECM and  $O_2$  in vitro by (a) 3D gel prepared around a microtube supplying  $O_2$  (insert: white arrows indicate oxygen transport; blue arrows indicate the direction of airflow) and (b) microencapsulated  $O_2$  carriers, such as PFCs, embedded within 3D gel. Drawing not to scale

levels of oxygen to 3D cardiomyocyte cultures. A study by Chin et al. [39] made a similar attempt, developing hydrogel-PFC composite scaffolds to improve oxygenation throughout the gel. In a similar manner, taking advantage of the high  $O_2$  solubility of PFCs, controlled release of  $O_2$  in 3D microenvironments could be improved via microencapsulation of PFCs (Fig. 4.7b). Polymeric microspheres loaded with  $O_2$  have also been shown to be effective in enhancing cell viability in anoxic microenvironments [44].

The continued development of novel biomaterial technologies, which enable the study of human cells in highly biomimetic settings in vitro, will continue to guide discovery of cell behavior and vascular morphogenesis. These powerful systems, coupled with continued innovation throughout all areas of biotechnology, including gene editing and stem cell technology, have positioned the field of vascular tissue engineering in a fascinating arena, where new therapeutic targets and more robust vascularized constructs continue to be discovered and translated to the clinic. Combining expertise in biology, materials science, engineering, and medicine will continue to inform our understanding of the complex cell-cell and cell-matrix interactions that drive tissue formation and regeneration.

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