5

VASCULARIZATION OF ARTIFICIAL TISSUE

Learning Objectives

After completing this chapter, students should be able to:

1. Explain the need for vascularization during tissue fabrication and development.
2. Describe and discuss seminal publications in angiogenesis research by Dr. Judah Folkman.
3. Define vasculogenesis, angiogenesis, and arteriogenesis and understand differences between these three processes.
4. Identify triggers that drive vasculogenesis, angiogenesis and arteriogenesis.
5. Describe molecular mechanisms of vasculogenesis, angiogenesis, and arteriogenesis.
6. Discuss the process of therapeutic angiogenesis as it relates to regenerative medicine.
7. Explain why vascularization is important during tissue fabrication.
8. Describe strategies that have been used to incorporate vasculature within artificial tissue.
9. Explain differences between biologically replicated, biologically mediated, and biologically inspired strategies for vascularization of 3D artificial tissue.

Introduction to Tissue Engineering: Applications and Challenges, First Edition. Ravi Birla.

10. Give specific examples of vascularization strategies based on biologically replicated, biologically mediated, and biologically inspired models.
11. Design a process to engineer vasculature within artificial tissue.

CHAPTER OVERVIEW

We begin this chapter with a discussion centered on the need for neovascularization during 3D tissue fabrication. We next move on to describe seminal work in the field of angiogenesis by Dr. Judah Folkman and how this work relates to tissue engineering. During human development and growth, there are three distinct mechanisms that give rise to blood vessels: vasculogenesis, angiogenesis, and arteriogenesis. During the course of this chapter, we describe these three processes and the molecular mechanisms that lead to blood vessel formation. We also describe how these three processes can be used to support vascularization of 3D artificial tissue. Next, we look at one specific application of angiogenesis, therapeutic angiogenesis, which is a clinical strategy designed to support revascularization of diseased or injured avascular tissue. Using this framework of blood vessel formation during human development and growth, we discuss ways in which this information can be relayed back to the tissue fabrication process. We next compare *in vitro* models of vascularization with *in vivo* models and provide a discussion on the relative advantages and disadvantages of each strategy. After this, we present an idealized case for inducing vascularization within 3D artificial tissue. We next provide a framework for vascularization strategies in tissue engineering and present a flow chart to aid in the decision making process. Next, we describe vascularization strategies in tissue engineering, which fall into one of three categories: biologically replicated, biologically mediated, and biologically inspired. For each of the three vascularization strategies, we describe the underlying principles and provide examples from the literature. Finally, we conclude this chapter with a discussion of scientific and technological challenges that need to be overcome to develop efficient vascularization strategies to support the fabrication of 3D artificial tissue.

5.1 INTRODUCTION

In the human body, the vasculature serves as a distribution network to deliver oxygen, glucose, and other nutrients to various tissue and cells and remove waste products like carbon dioxide and lactic acid. The smallest blood vessels are known as capillaries and consist of endothelial cells supported by a basement membrane. These capillaries are found in close proximity to cells, with no cells being more than 100–200 μm away from a capillary. Cells can be supported via diffusion as a delivery mechanism for oxygen and other nutrients over short distances (~100–200 μm); vascularization is required beyond this critical range. Capillaries are a part of the circulatory system, which consists of arterioles feeding into the capillaries and venules receiving throughput from capillaries. The capillary

network couples with larger arteries and veins that then feed into the heart and complete the circulatory system.

Cells need to be in close proximity to a vascular network to support viability, and this scenario applies to tissue engineering as well. The process of tissue fabrication begins with a few cells cultured in an appropriate 3D environment. During culture, these cells proliferate and support tissue growth and maturation, providing impetus for tissue development and growth. During early stages of tissue fabrication, cell viability can be supported by diffusion (Figure 5.1). However, as the tissue matures

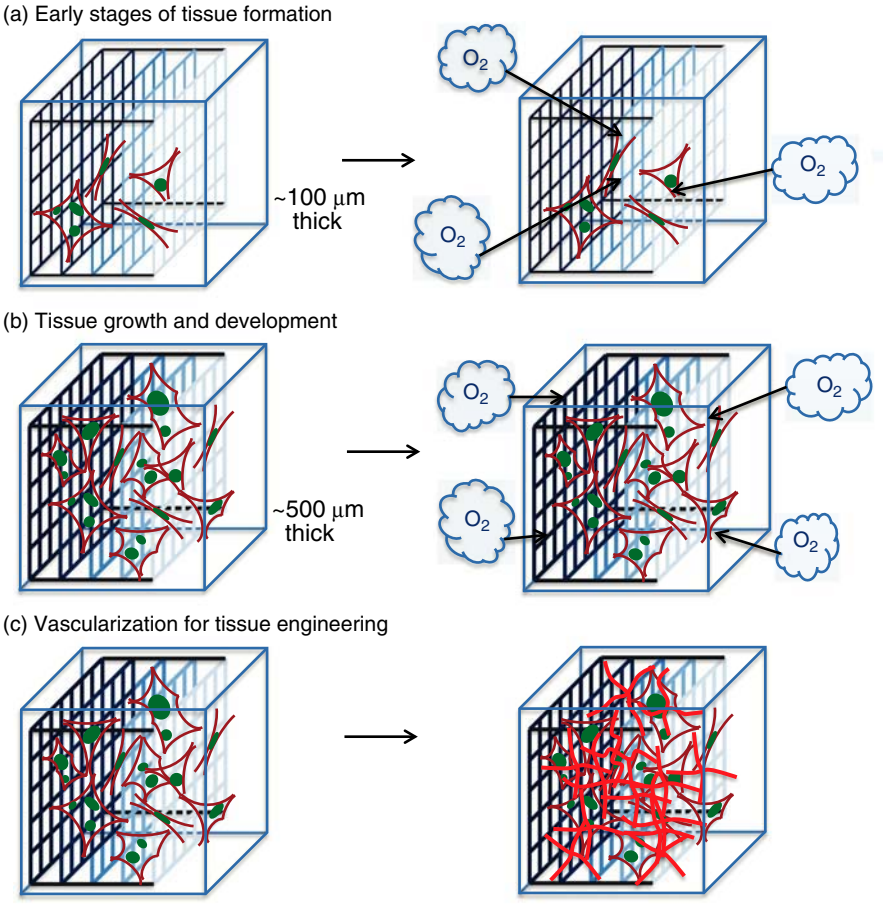

Figure 5.1 Vascularization for Tissue Engineering—(a) Early Stages of Tissue Development—Diffusion of oxygen is sufficient to support metabolic activity of cells. **(b) Tissue Growth and Development**—As the thickness of the artificial tissue increases, diffusion of oxygen is not able to support the metabolic activity of cells, particularly those within the inner core of the artificial tissue. **(c) Vascularization for Tissue Engineering**—As the thickness of the artificial tissue increases, vascularization is important to support the metabolic activity of cells within 3D artificial tissue.

and increases in thickness, diffusion is no longer sufficient to support the metabolic requirements of the cells. This is particularly the case when the thickness of the tissue-engineered constructs increases beyond 200 μm; at this stage, it is critical to engineer a vasculature within 3D artificial tissue.

5.2 SEMINAL PUBLICATIONS IN ANGIOGENESIS RESEARCH

There were a series of seminal studies in the early 1970s by Dr. Judah Folkman at Harvard Medical School that have paved the field of angiogenesis (1–11). In a 1971 publication, Dr. Folkman isolated and characterized tumor angiogenesis factor (TAF) and showed that TAF could support endothelial cell proliferation and formation of new capillaries (8). Based on his findings, Dr. Folkman proposed the following working hypothesis (8):

> "*It appears that most solid tumors, whether they originate from a cell transformed by virus or carcinogen, or whether they begin as a metastatic implant, must exist early as a small population of cells dependent upon nutrients which diffuse from the extravascular space. Such a pinpoint colony eventually expands to a size where simple diffusion of nutrients (and wastes) is insufficient. New capillaries are elicited and the tumor then enters a phase in which nutrients arrive by perfusion. It is possible that TAF is responsible for this final stage. It is tempting to suggest that tumor growth might be arrested at a very small size if the angiogenesis activity of this factor could be blocked.*"

The working hypothesis put forth by Dr. Folkman was that cancerous cells are proliferative and that during initial stages of growth, nutrient delivery takes place by diffusion. However, as the cell mass grows, diffusion is no longer able to support cell viability, and vascularization is important to support viability of the tissue mass. Dr. Folkman went on to suggest that blocking formation of new blood vessels could restrict the proliferation of cancerous cells and could be used for therapeutic purposes; Dr. Folkman demonstrated the feasibility of this approach in a later publication (10).

The publications by Dr. Folkman were seminal in the field of angiogenesis, as they provided a clear link between angiogenesis and cancer formation and proposed blocking angiogenesis as a therapeutic strategy for the treatment of cancer. This initial work has led to significant advancements in our understanding of our molecular mechanisms, angiogenesis, and the development of novel anti-angiogenic strategies. This work also has clear implications for tissue engineering. As cancer cells proliferate, vascularization is required for nutrient delivery to support tissue growth and development. Similarly, during tissue fabrication, cell proliferation is initially supported by nutrient diffusion until reaching a certain critical mass, after which vascularization is needed. In the case of cancerous cells, the objective is to block angiogenesis, whereas in the case of tissue fabrication, the objective is to induce angiogenesis.

5.3 VASCULARIZATION DEFINED

In this section, we provide the reader with important terminology associated with blood vessel formation during embryogenesis, human development, and in response to injury and other stimuli. The development and growth of vasculature is a very tightly regulated process and takes place in response to specific stimuli. In addition, different stimuli like hypoxia and fluid shear stress, initiate different signaling pathways, which in turn result in different mechanisms of blood vessel growth and remodeling. In this section, we provide an overview of these distinct processes for blood vessel development, and in subsequent sections, we provide a discussion of molecular mechanisms involved. We introduce the following concepts in this section: vasculogenesis, angiogenesis, and arteriogenesis (Figure 5.2).

During embryogenesis, fertilization of an oocyte by a sperm leads to zygote formation, which refers to the fertilized oocyte. A single cell then divides progressively to give rise to two cells and then four, eight, and so on until formation of an embryo occurs. During these early stages of embryogenesis, vascularization is absent, and nutrients are delivered to all cells via diffusion. However, as the embryo grows and increases in mass, diffusion is no longer able to supply nutrients to all of its cells, and vascularization becomes important. At critical time points during embryogenesis, mesodermal stem cells differentiate to form angioblasts and then endothelial cells, which in turn support capillary formation.

Vasculogenesis refers to initial events in vascular growth in which endothelial cell precursors (angioblasts) migrate to discrete locations, differentiate in situ, and assemble into solid endothelial cords, later forming a plexus with endothelial tubes (12–16). Vasculogenesis takes place very early during embryonesis and gives rise to the entire circulatory system by expansion and development of the vasculature.

	Vasculogenesis	Angiogenesis	Arteriologenesis
Definition	Blood vessel formation during embryogenesis	Formation of capillaries from existing ones	Maturation and development of blood vessels from existing ones
Starting from	Isolated stem cells	Capillaries	Mature vessels
Ending with	Capillaries	Capillaries	Mature vessels
When	Embryogenesis	Embryogenesis and adult life	Adult life
Trigger	VEGF expression	Hypoxia	Fluid shear stress

Figure 5.2 Vasculogenesis, Angiogenesis, and Arteriogenesis—The terms vasculogenseis, angiogenesis, and arteriogenesis have distinct meanings relating to blood vessel formation. Vasculogenesis refers to the process of blood vessel formation during embryogenesis. Angiogenesis refers to the formation of new blood vessels from existing ones. Arteriogenesis refers to the maturation and development of existing blood vessels.

In a sense, vasculogenesis can be viewed as embryogenesis for the vasculature—a very accurate description of the process. Figure 5.2 shows some characteristics of the process and highlights differences between vasculogenesis, angiogenesis, and arteriogenesis.

The process of vasculogenesis starts with very early stem cells: the mesodermal cells and hemangoblasts. The end results of vasculogenesis are early capillaries that do not have smooth muscle cells or vaso-contraction and vaso-relaxation properties. Since vasculogenesis is associated with embryogenesis and early human development, the triggers that initiate capillary formation are based on changes in the genetic profile of early embryonic stem cells. In particular, vascular endothelial growth factor (VEGF) has been associated with vasculogenesis and is the earliest known marker of vascular lineage. Commitment of mesodermal stem cells to a vascular lineage is defined on the basis of VEGF expression.

Angiogenesis refers to the growth, expansion, and remodeling of primitive blood vessels formed during vasculogenesis to form a mature vascular network (17,18). An important distinction can be made between angiogenesis and vasculogenesis: the former requires pre-existing vessels for formation of new ones, and the latter does not have this requirement. Another important distinction is based on time frame, as vasculogenesis occurs during embryogenesis, and angiogenesis occurs later on during development. In fact, angiogenesis succeeds vasculogenesis.

Sprouting angiogenesis is the process by which an existing blood vessel gives rise to another blood vessel by sprouting endothelial cells toward the angiogenic stimuli. This process increases the blood vessel count in tissue that is otherwise lacking vasculature. A second process by which angiogenesis occurs is known as intussusceptive angiogenesis, in which an existing blood vessel splits to form two blood vessels. This process is faster than sprouting angiogenesis as it does not require proliferation of endothelial cells and depends on reorganization of existing cells. Intussusceptive angiogenesis has only been recently discovered in 1986, and considerably less is known about it compared to sprouting angiogenesis, which has been known and studied for decades.

We have seen that vasculogenesis and angiogenesis are two steps in the process of blood vessel formation and organization. The third stage in this process is arteriogenesis, a process that results in an increase in the diameter of blood vessels, along with other functional modifications. Arteriogenesis is the process by which blood vessels increase in diameter to form muscular arteries and incorporate smooth muscle cells and vaso-contraction and vaso-relaxation properties (19–22). The primary stimulus for arteriogenesis is fluid stress, while the primary stimulus for angiogenesis is ischemia or the lack of oxygen.

5.4 MOLECULAR MECHANISM OF VASCULOGENESIS

During embryogenesis, there is a rapid expansion in cell mass from the single cell oocyte to two-, four-, and eight-cell structures. During this early expansion in cell mass, cell viability can be supported by diffusion of oxygen and other nutrients to

all cells. However, as cell masses increase beyond a certain critical limit, diffusion is no longer sufficient to support the metabolic activity of all cells. Neovascularization then becomes important, and it comes as no surprise that the circulatory system is the first system to be developed during embryogenesis, as early as embryonic day (E) 6.5–7. In this section, we present the molecular mechanisms of vasculogenesis in three steps, starting with a description of stem cells involved in the process, followed by morphological changes that take place, and finally, the molecular signals that regulate the vasculogenesis process.

We begin our discussion of vasculogenesis by looking at key stem cell sources (23,24). Early during embryogenesis, three germ layers that give rise to all cells in the human body are formed. These are known as the endoderm, mesoderm, and the ectoderm; they represent the inner, middle, and outer layers of the embryoblast (early cell mass), respectively, The mesodermal stem cells give rise to all cells within the circulatory system and can be considered as initial precursor cells for vasculogenesis. Mesodermal stem cells differentiate to form hemangioblasts, which are early progenitor stem cells that give rise to components of blood and the vascular system during early embryogenesis. Hemangioblasts have been defined as mesodermal progenitor cells committed to the generation of endothelial cells and blood cells, sometimes and perhaps always via a hematogenous endothelium intermediate. Hemangioblasts are multipotent and can differentiate to form endothelial cells or hematopoietic stem cells; hematopoietic stem cells give rise to other blood cells including monocytes, macrophages, neutrophils, basophils eosinophils, erythrocytes, megakaryocytes, platelets, T-cells, and B-cells. Hemangioblasts give rise to angioblasts, which are intermediate stem cells committed to endothelial cell differentiation. The differentiation pathway for the formation of vascular cells progresses from multipotent mesodermal stem cells to multipotent hemangioblasts, which later give rise to angioblasts, cells committed to forming endothelial cells, and multipotent hematopoietic stem cells.

We next explore the steps in capillary formation during vasculogenesis. The formation of blood vessels via vasculogenesis during embryogenesis can be divided into 5 stages (25):

Step One Differentiation of Mesodermal cells to Hemangioblasts—Very early during vasculogenesis, mesodermal cells differentiate to form hemangioblasts, early precursor cells that have the potential to be differentiated to all cells in the vasculature. The term hemangioblast is given to the common blood island precursor cell that eventually gives rise to both endothelial and hematopoietic cells.

Step Two Formation of Blood Islands—Hemangioblasts form clusters known as blood islands, which are the first semblance of capillaries.

Step Three Differentiation of Hemangioblasts—Once blood islands have formed, hemangioblasts differentiate to form angioblasts, precursor cells that give rise to endothelial cells. Angioblasts are organized on the outer side of blood islands. Hemangioblasts, positioned on the inside of blood islands, give rise to hematopoietic stem cells.

Step Four Blood Vessel Formation—Proliferation, migration, and association of angioblasts gives rise to the very first primitive blood vessels.

Step Five Lumenization—Lumenization is the process by which primitive vessels form organized capillaries. Angioblasts differentiate to form endothelial cells, while hematopoietic stem cells differentiate to more specialized functions. Tight junctions form between endothelial cells, basement membrane is deposited, and pericyte recruitment takes place.

The triggers that result in vasculogenesis are very different from the triggers that initiate angiogenesis and arteriogenesis. As we discuss in the next two sections, formation of new capillaries by angiogenesis is in response to hypoxia, while the formation of muscular blood vessels by arteriogenesis is in response to fluid shear stress. In both of these cases, a very specific external signal triggers new blood vessel formation. However, vasculogenesis is a developmentally regulated process and triggers are molecular and genetic, as opposed to environmental. Fibroblast growth factors, the hedgehog family of morphogens, vascular endothelial growth factors and their receptors, and transforming growth factors and their receptors have been indicated as important modulators of vasculogenesis (25).

5.5 MOLECULAR MECHANISM OF ANGIOGENESIS

Angiogenesis is the process of new capillary formation from existing capillaries in response to hypoxic conditions. Angiogenesis can occur during embryogenesis and adulthood. While the process is very specific, the word "angiogenesis" has often been used fairly loosely with reference to new blood vessel formation, particularly when applied to new blood vessel formation within 3D tissue constructs.

In this section, we will study the mechanism of new blood vessel formation via sprouting angiogenesis, a process in which new blood vessels are formed from existing ones. There are two important concepts that need to be defined here. First, endothelial cells are important for normal blood vessel function, as they line the luminal surface of all vessels, providing a non-thrombogenic surface during blood flow; endothelial cells are also important during angiogenesis. However, all endothelial cells that line the luminal surface of vessels are not the same, and at least three categories have been identified based on distinct cellular specifications: 1) tip, 2) stalk, and 3) phalanx cells (26,27). The second concept is the stimuli required to induce angiogenesis. Under normal physiological conditions, endothelial cells are not activated and do not participate in angiogenesis. The need for angiogenesis arises under hypoxic conditions, where tissue is deficient in oxygen and other nutrients. *How do endothelial cells know there is hypoxia tissue nearby and that they need to begin the process of angiogenesis?* Cells within hypoxic tissue release a protein known as VEGF, and the concentration of this protein is proportional to the degree of hypoxia. Endothelial cells have surface receptors for VEGF and can sense hypoxia tissue and initiate a cascade of intracellular

signaling events that leads to the formation of new blood vessels (26,27). This very elaborate signaling mechanism provides a basis for communication between hypoxic tissue and endothelial cells, which then respond by initiating a sequence of molecular changes leading to the formation of new blood vessels.

How exactly is a new blood vessel formed? As we have stated before, all endothelial cells are not the same. When an angiogenic signal like hypoxia is introduced via VEGF signaling, endothelial cells that are exposed to the highest concentration of VEGF are selected to become tip cells (27). Tip cells act at the forefront and lead the formation of new blood vessels in the direction of the angiogenic signal. In each sprout, a single tip cell determines the vessel. Tip cells are highly polarized and use filopodia to guide a sprouting vessel toward an angiogenic signal. The tip cells are non-proliferating. Endothelial stalk cells follow behind the tip cells, proliferate to form elongated stalks, and create a lumen. Further away from the tip cells are endothelial phalanx cells that are lumenized, non-proliferating cells that sense and regulate perfusion in the persistent sprout (27). As sprouting continues to be led by tip cells, two ends of sprouting vessels connect to form a luminal blood vessel. This results in initiation of blood flow through the newly formed blood vessel, thereby reducing the concentration of the angiogenic signal VEGF and causing a reduction in angiogenic sprouting. Pericyte recruitment is required for stabilization of the nascent blood vessel, and this stabilization is followed by extracellular matrix production and deposition (27).

5.6 MOLECULAR MECHANISM OF ARTERIOGENESIS

Arteriogenesis is the formation of new blood vessels from existing ones in response to specific physiological stimuli like changes in fluid shear stresses or pathological conditions like stenosis or blood vessel occlusion (28–33) (in cases of plaque formation occurs during atherosclerosis). Earlier in the chapter, we looked at vascuogenesis as a mechanism of blood vessel formation during embryogenesis, and we looked at angiogenesis as a mechanism of blood vessel formation from existing vessels. Compared to these mechanisms, arteriogenesis is different based on its initiation trigger, which is often a change in the fluid stress environment within existing blood vessels. There are similarities between arteriogenesis and angiogenesis. New blood vessels are formed by growth from existing ones; however, the triggers are different, with fluid shear stress for arteriogenesis and hypoxia for angiogenesis. Vasculogenesis is a different process altogether; it involves growth and development of blood vessels from early precursor stem cells during embryogenesis.

During arteriogenesis, blood vessel growth takes place in response to fluid shear stress or changes in the stress environment caused by pathological conditions like vessel occlusion (28–33). Upon blockage of a certain portion of an artery, redistribution of fluid stresses results in an increase of shear stresses within neighboring parts of the occluded vessel. In response to changes in the fluid shear stress, endothelial cells release growth factors such as TGF-β. This release in turn correlates to an increase in the rate of proliferation of other endothelial cells and

smooth muscle cells, a necessary prerequisite for arteriogenesis (increase in the rate of proliferation of endothelial cells (ECs) and smooth muscle cells (SMCs) is necessary for new blood vessel formation). Smooth muscle cell proliferation and remodeling is a very important component of arteriogenesis, as an increase in SMC number is a prerequisite for new blood vessel formation. There can be greater than a 20-fold increase in SMC numbers during arteriogenesis in humans. Another important component in the process of arteriogenesis is an increase in the rate of MMP synthesis, which is responsible for the degradation of ECM components, thereby "loosening" existing vessels to support growth and expansion, particularly for SMC proliferation and remodeling. An increase in activity of MMP-2 and MMP-9 has been observed during arteriogenesis along with an increase in degradation of elastin and other extracellular matrix components. Although exact cellular and molecular mechanisms for arteriogenesis have not been fully elucidated, the current knowledge base alludes to the following seven steps (28,33):

Step One Changes in the fluid shear stress environment due to occlusion or some other factor serve as the trigger for arteriogenesis.

Step Two Endothelial cells on the luminal surface of blood vessels sense these changes in shear stress environment using biological sensors, which may be cell surface integrins.

Step Three Endothelial cells respond to changes in fluid shear stress by an increase in the expression of adhesion molecules like monocyte chemoattractant protein-1 (MCP-1), intercellular adhesion molecule-1 (ICAM-1), and vascular cell adhesion molecule-1 (VCAM-1).

Step Four Increase in the expression of adhesion molecules by endothelial cells results in recruitment of circulating monocytes at the site of arteriogenesis; these monocytes are anchored to the adhesion molecules.

Step Five Once recruited to the site of arteriogenesis, monocytes produce proteases such as matrix metalloproteinase and uPA, which act to degrade extracellular matrix components. Degradation of ECM is important to "loosen" the tissue in order to support smooth muscle cell proliferation and migration.

Step Six The proteins released by monocytes serve to degrade vascular extracellular matrix components.

Step Seven Degradation products of the vascular extracellular matrix, particularly elastin, act as a stimulant for smooth muscle cell proliferation and migration toward the site of arteriogenesis.

While the detailed cellular and molecular mechanisms have not been fully elucidated for arteriogenesis, the seven steps outlined above provide an outline of the general outline that occurs in response to fluid stresses leading to the formation of muscular blood vessels.

5.7 THERAPEUTIC ANGIOGENESIS

Ischemia during Myocardial Infarction—Ischemic tissue is prevalent in the left ventricle after a myocardial infarction, and is due to plaque formation in the coronary artery that occludes the vessels and limits blood supply to heart muscle. This has detrimental consequences and leads to cardiac myocyte cell death and loss of left ventricular function. An experimental strategy to counter ischemia of heart muscle is therapeutic angiogenesis, designed to promote reperfusion (re-establishment of blood flow) through angiogenesis in ischemic tissue, thereby reducing cell death and restoring lost ventricular function (34–37).

What exactly is Therapeutic Angiogenesis? Vasculogenesis, angiogenesis, and arteriogenesis induce the formation of new blood vessels during embryogenesis and normal human development. The process of vascularization can also be stimulated in diseased or ischemic tissue as a therapeutic strategy. During myocardial infarction, blood supply to the left ventricle is compromised due to plaque formation in the coronary artery; this in turn causes cell death and loss of heart function. In theory, if we can develop a therapeutic strategy to increase blood supply to the left ventricle, this will reduce cardiac myocyte cell death and restore lost myocardial function. This strategy is known as therapeutic angiogenesis, which refers to the stimulation of angiogenesis for therapeutic purposes (34–37). New blood vessel formation is due to outgrowth from existing vessels. Hence, the process of angiogenesis occurs rather than vasculogenesis. Therapeutic angiogenesis has been defined as the use of angiogenic factors to induce formation of a collateral blood supply, effectively bypassing an occluded diseased blood vessel in patients with damaged coronary or peripheral myocardial tissue, or other types of damaged tissue.

Agents for Therapeutic Angiogenesis—Any agent that promotes vascularization during human development can be used for therapeutic angiogenesis (38–43). Molecular and developmental biology have provided many such potential candidates; a subset of these agents has been selected for experimental testing and validation. Commonly used agents for therapeutic angiogenesis include proteins like vascular endothelial growth factor (VEGF) (44–47) and fibroblast growth factor (FGF) (45,48). These angiogenic factors have been tested by direct delivery of the protein to the site of myocardial infarction or by delivering the gene that encodes the protein. In other words, protein and gene therapy have been used as modes of action to support therapeutic angiogenesis. Unsurprisingly, cell transplantation has also been used, particularly with endothelial progenitor cells (EPCs) (49–53) and bone marrow-derived mesenchymal cells (BMCs) (54–57).

Modes of Delivery—Intracoronary delivery or intramyocardial injection are two strategies for therapeutic agents (58–62). Intracoronary delivery involves the use of catheters to deliver therapeutic agents directly to the coronary artery, which then transports these agents to the heart muscle. In the case of intramyocardial delivery, the therapeutic agent is directly injected to the heart muscle using catheter-based methods.

Therapeutic Angiogenesis and Regenerative Medicine—In Chapter 1, we studied the field of regenerative medicine in terms of any stimulus that initiates the self-healing process in the human body. After studying therapeutic angiogenesis, it should be clear that therapeutic angiogenesis is one form of regenerative medicine. The therapeutic agent (protein, gene, or cell) delivered to the site of injury is designed to simulate a regenerative response in the host.

Mode of Action—Therapeutic angiogenesis involves delivery of genes, proteins, or cells to the site of injury to revascularize the ischemic tissue. *What exactly is the mechanism responsible for vascularization and improvement in functional outcome?* There are at least three potential mechanisms that have been proposed:

1. *Direct Angiogenesis*—When the therapeutic agent is an angiogenic factor like VEGF or FGF, the mode of action is direct angiogenesis, as these proteins act on existing endothelial cells to promote proliferation and capillary formation (48). Similarly, when the therapeutic agent is EPCs, it is thought that cells directly integrate within existing vasculature, supporting repair and/or new blood vessel formation.
2. *Paracrine Signaling*—If the therapeutic agent does not have a direct effect on angiogenesis (as in the case of bone marrow-derived mesenchymal stem cells), it is thought that these cells release soluble factor into the host environment (35,38–39). The soluble factor acts as a paracrine agent and communicates with endothelial cells to promote new blood vessel formation.
3. *Recruiting of Circulating EPCs*—The third hypothesis is also indirect. It suggests that transplanted agents, particularly non-angiogenic cells, recruit EPCs from the circulation, causing EPCs to home to the site of injury (49). Once circulating EPCs are recruited to the site of injury, they have a direct effect on angiogenesis by supporting formation of new blood vessels.

Assessment of Reperfusion—The success of therapeutic angiogenesis can be measured by many different metrics, though the most direct measure is the number and function of new blood vessels. This can be measured directly using histological techniques, which require tissue specimens stained with antibodies that bind to specific endothelial cell markers, like von Willibrand factor (vWF). These sections are used to obtain capillary counts and allow comparison of samples that have been treated with an angiogenic agent versus controls. This capillary count and comparison provides a very direct metric to measure reperfusion of ischemic tissue. A second approach is angiography, which involves delivery of a contract agent to the tissue, followed by x-ray imaging (63–65). Angiography can also provide a direct measure to blood vessels, in addition to assessment of flow.

5.8 TISSUE ENGINEERING AND VASCULARIZATION

We have looked at vasculogenesis, angiogenesis and related fields, and blood vessel development during embryonesis and human development. The critical question to

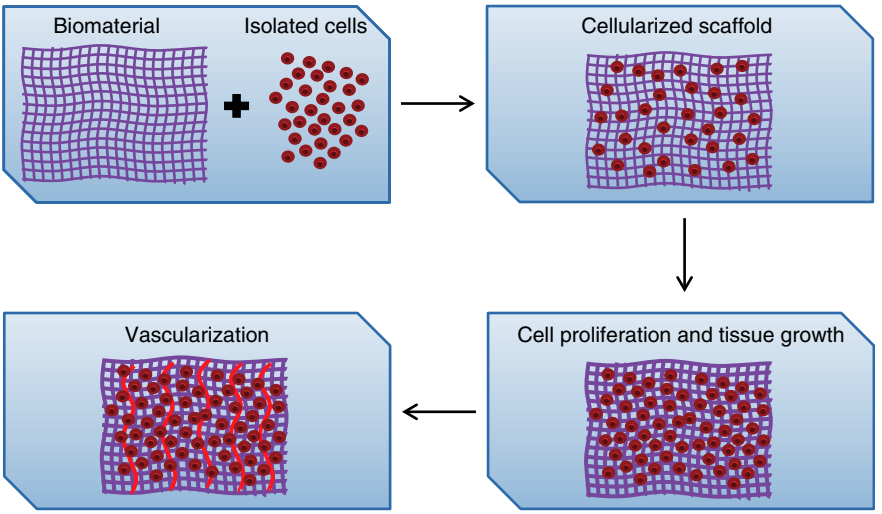

Figure 5.3 Vascularization During 3D Artificial Tissue Formation—During the tissue fabrication process, isolated cells are coupled with biomaterials to support fabrication of 3D artificial tissue. Cell proliferation and subsequent remodeling leads to artificial tissue development and maturation. During this process, induction of vascularization is a critical step in the development of 3D artificial tissue.

address is: *how is this related to tissue engineering?* We can answer this question by discussing the end point objective in tissue engineering, which is 3D tissue and organ fabrication. During 3D tissue development, we begin with isolated cells and develop strategies to fabricate artificial tissue and organs from these isolated cells. At some point during 3D tissue development and maturation, blood vessels are required to support the metabolic activity of artificial tissue, Figure 5.3.

This process is analogous to embryogenesis, in which fertilization leads to oocyte formation and subsequent cell division leads to an increase in cell mass. At some point along this pathway, vasculogenesis is initiated and is followed by angiogenesis and arteriogenesis. As we study the process of blood vessel formation from early embryogenesis through human development, we can apply this knowledge to tissue engineering. Important questions that need to be answered are:

- *During early embryogenesis, the cell mass is avascular and lacks any blood vessels. At some point during embryogenesis, vasculogenesis starts. At what point does vasculogenesis begin, and what are the signals that determine capillary formation?*
- *During tissue engineering, isolated cells come together to form 3D tissue. During early stages of 3D tissue development, there is no vasculature. What is the critical size of the 3D tissue that can remain viable in the absence of vasculature?*

- *As 3D tissue grows, the process of blood vessel development takes place. What are the critical signals that need to be created during 3D tissue development in order to support vasculature formation?*

Studying these questions will help understand the relationship between angiogenesis and tissue engineering and the interplay between the two disciplines. The objective is to understand the signals that determine blood vessel development during embryogenesis and human development, and translate this understanding toward the fabrication of 3D artificial tissue and organs.

5.9 CONCEPTUAL FRAMEWORK FOR VASCULARIZATION DURING ARTIFICIAL TISSUE FORMATION

In this section, we present a general overview of vascularization for tissue engineering. At some point during tissue fabrication, development, or maturation, we need to incorporate a vasculature. Based on what we have learned about vascularization and the specific molecular mechanisms involved, we need to ask the question: *what strategy can we adopt to engineer a vasculature within artificial tissue?*

We begin this section by addressing vascularization strategies on a conceptual and theoretical basis and then move on to look at specific methodologies that have been used to achieve these strategies. Before diving into the current methods of inducing vascularization, we need to take a step back and start from the beginning and ask one question: *based on our understanding of vascularization, if we had to develop a strategy to incorporate blood vessels within artificial tissue, where would we start?* Our discussion is centered on the premise that we have not reviewed the literature; therefore, our objective is to start with a fresh mind and an empty plate and explore the possibilities. *Where and how do we begin?*

A general scheme of vascularization for bioengineered tissue is present in Figure 5.4.

In this scheme, we present three options for the vascularization of 3D artificial tissue using strategies based on vasculogenesis, angiogenesis, and arteriogenesis. Our first case is based on vasculogenesis, and the general scheme begins with early progenitor stem cells like mesodermal stem cells (Figure 5.4a). The objective is to control external cues to promote differentiation of mesodermal cells to hemangioblasts and then to other cell types required for blood vessel formation (angioblasts, hematopoietic stem cells, and endothelial cells). Conceptually, this scheme is aligned with the objectives of tissue engineering—tissue fabrication being able to recreate embryogenesis coupled with vasculogenesis. Some challenges associated with this strategy are:

- *What will be the source of stem cells? Will the mesodermal cells be obtained by differentiation of human embryonic stem cells, and if so, are there ethical issues involved?*

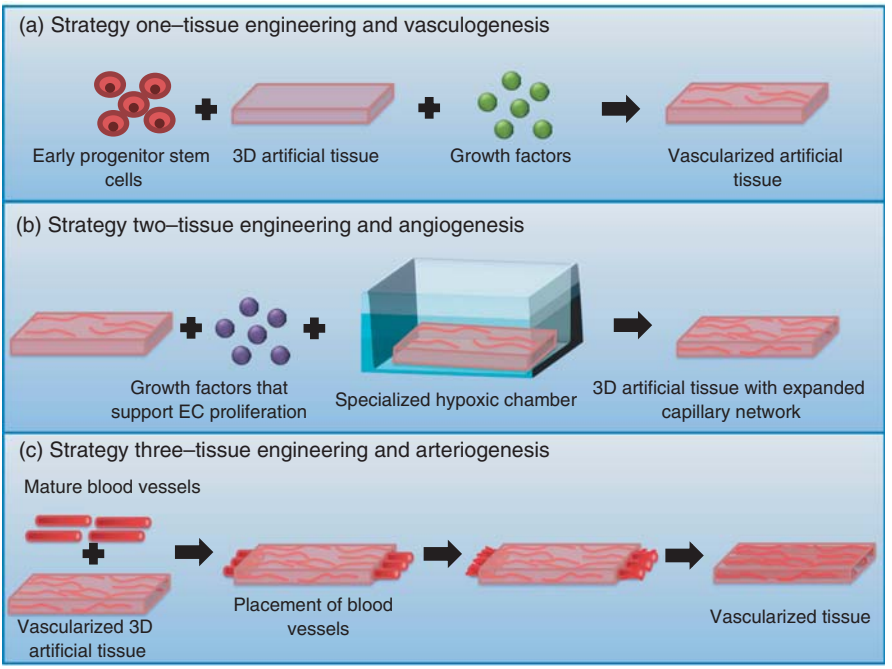

Figure 5.4 Tissue Engineering and Vascularization—**(a) Tissue Engineering and Vasculogenesis**—Early progenitor stem cells can be used to support the formation of capillaries during artificial tissue fabrication. **(b) Tissue Engineering and Angiogenesis**—Starting with vascularized artificial tissue, angiogenesis can be induced to promote formation of new blood vessels from existing ones. In this example, growth factors and hypoxia are used as drivers of angiogenesis. **(c) Tissue Engineering and Arteriogenesis**—Arteriogenesis is the formation and/or maturation of blood vessels from existing ones. In this example, blood vessels are positioned at specific locations in proximity to artificial tissue and are stimulated to support formation of new blood vessels. New blood vessels invade the artificial tissue and support vascularization of 3D artificial tissue.

- *What are the signals that drive the differentiation fate of early progenitor stem cells toward a vascular lineage, and how do we create these signals in vitro?*
- *If we are able to control the differentiation fate of progenitor cells toward a vascular lineage, how do we support the formation of capillaries and capillary networks? What if the cells stay in an isolated state and do not remodel to form complex vascular networks?*

The second strategy is to incorporate vasculature using methodology based on angiogenesis, which refers to the development of new capillaries from existing ones. As we have seen before, capillary formation during angiogenesis requires the presence of existing capillaries and requires a hypoxic culture environment. Therefore, from a tissue engineering standpoint, one can engineer capillaries using

vasculogenesis as described before and then subjecting the newly formed capillaries to hypoxic conditions in order to induce angiogenesis, as shown in Figure 5.4b. This process can, in theory, lead to the formation of an entire vascular network within 3D artificial tissue. There are many scientific and technological challenges in building an entire vasculature by recreating molecular mechanisms found during embryogenesis. Identification of a suitable stem cell source and defining signals that drive the differentiation fate of these stem cells toward a vascular lineage remain challenging. In addition, the following can be added to the list:

- *How do we create a hypoxic environment in vitro that replicates properties of an in vivo hypoxic microenvironment?*
- *What mechanisms will be implemented to support long term perfusion of the newly formed capillary network?*
- *What will be the orientation and alignment of newly formed capillaries relative to cells within the artificial tissue?*

We now explore the use of arteriogenesis to induce vasculature within artificial tissue. The starting point for arteriogenesis is the presence of muscular blood vessels, something which is clearly lacking in 3D artificial tissue (Figure 5.4c). In order to engineer a vasculature within artificial tissue using arteriogenesis, the starting substrate has to be muscular blood vessels. We can isolate and maintain blood vessels in culture using a closed loop perfusion system and anchor the vessels to both ends of 3D artificial tissue. We can then create occlusions by reducing the flow rate at specific points on the blood vessel; this reduction of flow rate will lead to changes in the fluid shear stress. This process will initiate arteriogenesis and can lead to the formation of a new vascular network within 3D artificial tissue; we then connect the vascular networks from both blood vessels to create a continuous fluid flow loop. Some of the scientific and technological challenges associated with this strategy include:

- *Long-term culture of muscular blood vessels in vitro is difficult, and the ability to engineer a perfusion loop and create an occlusion that represents pathological conditions is not trivial.*
- *Arteriogenesis requires participation of circulating monocytes, which are absent during in vitro culture.*
- *Interconnectivity of the two vascular beds that originate from the two source vessels is not a spontaneous event and requires complex interventional strategies.*

In this section, we have provided an interface between *in vivo* vascularization (what happens in nature) and *in vitro* vascularization strategies (what we want to accomplish in tissue engineering) for 3D artificial tissue. We presented three conceptual examples to translate our understanding of vasculogenesis, angiogenesis, and arteriogenesis toward the development of *in vitro* vascularization strategies. Our objective in presenting these examples is to demonstrate the relationship

between vascularization in nature and in tissue engineering. In addition, we provided some of the scientific and technological challenges in developing vasculature for 3D artificial tissue. This background is necessary as an entry point into the journey into neovascularization strategies for 3D artificial tissue that is described in the next few sections.

5.10 *IN VIVO* MODELS OF VASCULARIZATION

In the previous section, we looked at several *in vitro* approaches to engineer vasculature within artificial tissue. Our objective in all of the models was to develop *in vitro* conditions that replicate the process of vascularization as it occurs *in vivo*. Stated another way, the objective was to recapitulate *in vivo* conditions to support *in vitro* vascularization of 3D artificial tissue. This has been a very aggressive area of research with many different strategies being evaluated. However, if we look at this problem from a totally different perspective, we can envision a completely different strategy. Let us begin by re-evaluating our objective, which is to develop a vasculature within artificial tissue to recreate *in vivo* conditions under controlled *in vitro* conditions. *However, instead of recreating physiological conditions in vitro, what if we designed a system to engineer vasculature in vivo?* Such a strategy would require creating a custom chamber to house artificial tissue followed by implantation of this chamber *in vivo*; which will result in exposure to controlled physiological culture environment. This in turn will serve as a platform for vascularization of 3D artificial tissue. Since this is a fairly new concept and not as obvious as *in vitro* strategies, let us take a moment to explore this further by discussing the concept of *in vivo* vascularization, some of the advantages of this strategy, and the challenges of developing and implementing these strategies.

What exactly is in vivo vascularization? — The concept of *in vivo* vascularization revolves around culturing bioengineered tissue within specialized chambers that can be implanted to support the formation of new blood vessels within 3D artificial tissue (66–68) (Figure 5.5).

Once implanted, artificial tissue remains *in vivo* for a culture period of approximately 3–4 weeks, during which time the neovascularization of implanted tissue occurs due to host response. After implantation, artificial tissue is removed and separated from the chamber, leading to vascularization of implanted 3D artificial tissue.

Why would in vivo angiogenesis work? This strategy is based on the hypothesis that the *in vivo* culture environment has the right physiological cues for formation, development, and maturation of blood vessels. This involves presence of progenitor stem cells and the other cell types required for vascularization. In addition to cells, *in vivo* physiological conditions also have the right stimuli for blood vessel development, like fluid shear stress for arteriogenesis and hypoxia for angiogenesis, along with an abundance of growth factors like VEGF and FGF to support blood vessel formation. The microenvironment is also ideal for vascularization and consists of the right temperature, pH, ion concentration, oxygen saturation, and presence of

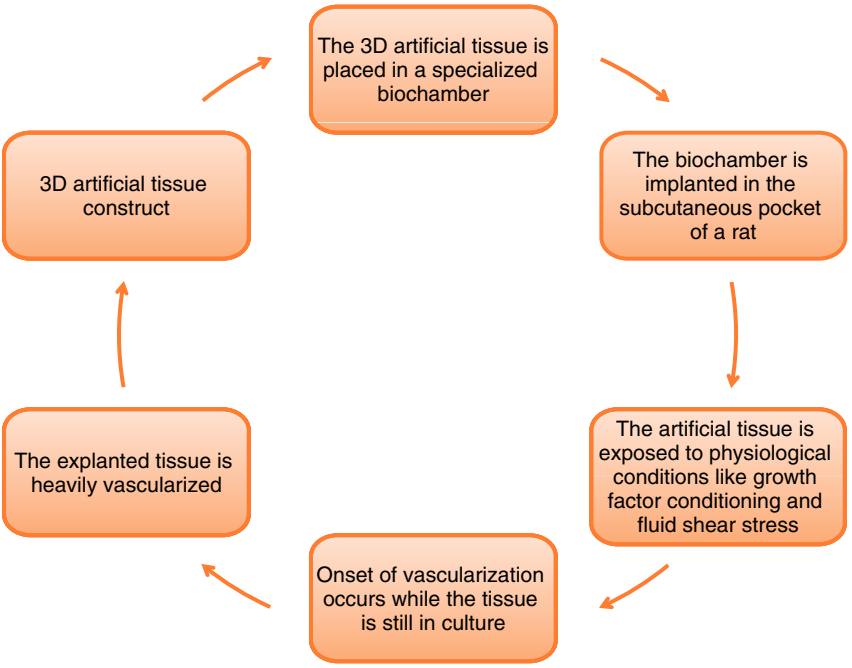

Figure 5.5 ***In Vivo*** **Models of Vascularization**—Artificial tissue can be secured within a custom biochamber and implanted *in vivo* to support vascularization of the 3D artificial tissue.

other components. Collectively, presence of all the required raw materials and presence of physiological stimulation cues, along with an optimized microenvironment, provides a strong rationale for blood vessel formation upon implantation.

What are some of the challenges with in vivo vascularization strategies? While *in vivo* vascularization strategies have many advantages, they are not without limitations. Development of a culture biochamber to support viability of artificial tissue during *in vivo* culture is challenging. The design of the biochamber has to support the culture and viability of 3D artificial tissue while protecting implanted tissue from host immune response and supporting physiological interaction with the host culture environment. In addition, the *in vivo* culture environment is harsh and can lead to damage of implanted artificial tissue. For example, fluid shear stresses resulting from arterial blood flow can be high. Artificial tissue may not be able to withstand high pressures, which can lead to physical damage of the tissue, cell death, and other adverse effects. Another challenge is the inability to regulate stimulation conditions during implantation; artificial tissue is exposed to the physiological culture environment without the ability to change variables. For example, if the arterial blood pressure is too high for artificial tissue, there is no way to bring it down to a lower level; this level of control can only be accomplished during *in vitro* culture. Finally, like with any other *in vivo* studies, there is significant variability

between experimental groups due to the inability to exhibit any control over physiological stimulation parameters; this reduces uniformity across experimental groups.

How does in vivo vascularization of artificial tissue compare with in vitro strategies? In vitro approaches are designed to replicate many of the conditions present during *in vivo* culture. At times, *in vitro* strategies are able to replicate some, though not all, of these conditions due to the complexity of the *in vivo* culture environment. This problem is solved by designing and implementing *in vivo* strategies for vascularization of 3D artificial tissue; these strategies have the right stimulation environment in place. *In vivo* strategies, however, do limit the amount of user control over the process of vascularization, which reduces the extent of customization of vascular networks within 3D artificial tissue.

What are some examples of potential implantation sites to support vascularization of artificial tissue? In theory, any implantation site that has been used for devices in the past can be used as a potential implantation site for vascularization. For proof of concept studies and experimental validation, subcutaneous implantation of artificial tissue has been used extensively (69). In such a case, the tissue is implanted in a subcutaneous pocket to support vascularization and explanted after a period of 3–4 weeks with a significant amount of neovascularization within the artificial tissue.

5.11 IDEALIZED VASCULARIZATION STRATEGY FOR TISSUE ENGINEERING

We started this chapter by looking at vasculature formation during normal human development and physiological conditions and studied molecular mechanisms responsible for vasculogenesis, angiogenesis, and arteriogenesis. We then looked at two general strategies for fabricating vasculature within 3D artificial tissue, including *in vitro* and *in vivo* methods. After exploring vascularization strategies in nature and in tissue engineering, we need to ask ourselves one question: *what is the best way to vascularize artificial tissue in an idealized system, and what are the scientific and technological challenges in order to address this?*

Our strategy is based on a biologically inspired approach designed to recreate blood vessel formation during human development and growth within 3D artificial tissue using early stem cells. Our idealized process is presented in Figure 5.6 and consists of four distinct phases: 1) formation of nascent capillary network, 2) expansion of capillary network, 3) growth of artificial tissue, and 4) perfusion of newly formed vascular network.

The first step in the process is to support formation of capillaries using early mesodermal stem cells. This requires isolation and expansion of mesodermal stem cells. This is followed by conditioning using a cocktail of growth factors to stimulate differentiation of mesodermal stem cells to endothelial cells and support organization of ECs for capillary formation. The composition and concentration of growth factors needs to be optimized to regulate differentiation of mesodermal stem cells; this part of the process is designed to recapitulate vasculogenesis during human

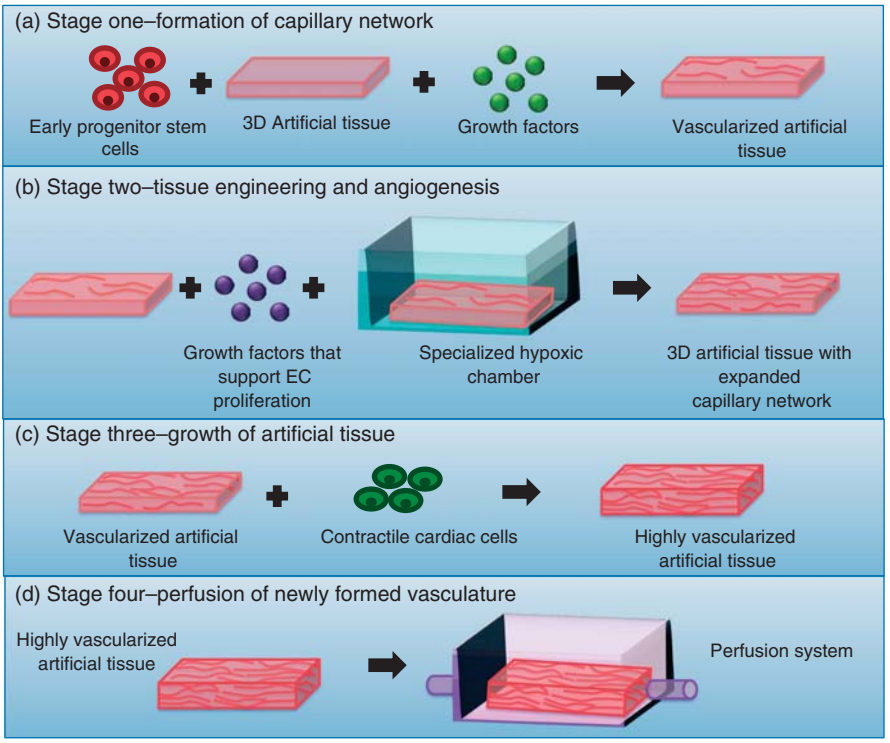

Figure 5.6 Idealized Strategy for Vascularization of 3D Artificial Tissue—(a) Formation of Capillary Network—The formation of new capillaries is promoted by the process of vasculogenesis. Early mesodermal stem cells are added to artificial tissue and stimulated with growth factors to support capillary formation. **(b) Expansion of Capillary Network**—The process of angiogenesis is used to expand the newly formed vasculature within 3D artificial tissue. Angiogenic factors and hypoxia are used as drivers of angiogenesis to support expansion of vasculature. **(c) Growth of Artificial Tissue**—Once a vascular network is in place, conditions are optimized to promote growth of 3D artificial tissue construct. **(d) Perfusion of Newly Formed Vasculature**—A perfusion system is developed to perfuse the newly formed vasculature.

development. This process will result in vascularized artificial tissue, though the capillary network will be rudimentary at this stage.

The second stage of the process is designed to expand the vascular network by creating *in vitro* conditions that replicate the angiogenic response during human development. Endothelial cells that have been obtained by differentiation of mesodermal stem cells will be used to support angiogenesis; these endothelial cells will be further stimulated by angiogenic factors like VEGF and FGF. The vascularized artificial tissue obtained from the studies of the first stage of the process will be coupled with angiogenic growth factors and incubated in a specialized hypoxic chamber designed to induce angiogenesis. After successfully completing

stage two of the process, the end product will be artificial tissue with an integrated vasculature.

During stages one and two of our idealized process, we have implemented strategies to support the formation of a capillary network within 3D artificial tissue. This provides a platform to support the growth of the tissue that has been limited in thickness due to lack of a vasculature. Once a capillary bed has been engineered, we can increase the thickness of the artificial tissue by adding layers of cells and extracellular matrix, as the newly formed capillary network can now support the increase in metabolic activity resulting from an increase in tissue mass. Stage three of our idealized process is designed to accomplish this: increase thickness of the 3D artificial tissue making use of the newly formed vasculature to support tissue viability.

The fourth stage of our process consists of fabricating a perfusion system to deliver continuous media flow through the newly formed vascular network. Continuous media perfusion is designed to support metabolic requirements of artificial tissue, apply intraluminal pressure to the vascular network to prevent the blood vessels from collapsing, and support stability of the newly formed vessels. Continuous pulsatile media flow will also have a positive effect on the functional performance of 3D artificial tissue.

The model presented is oversimplified and is laden with scientific and technological challenges, some of which include the ability to control the differentiation pathway of mesodermal stem cells to form endothelial cells, and the ability to induce an angiogenic response using a hypoxic trigger. In addition, engineering challenges of fabricating multi-layer tissue constructs and complex perfusion systems are no less intimidating. However, the idealized process does provide a strategy that has the potential to create vascularized artificial tissue and identifies some of the challenges in doing so.

5.12 FLOW CHART AND DECISION MAKING

Earlier in this book, we defined tissue engineering in terms of tissue fabrication. We also emphasized that tissue fabrication should be viewed in terms of process flow charts, decision making, and process optimization, like any other engineering problem. We have maintained this theme throughout this book and continue here with vascularization. In this section, we present a process flow chart for vascularization and identify critical decision points. In this chapter, we have presented the molecular mechanisms of vascularization during embryogenesis and human development and used this as a platform to develop vascularization strategies for artificial tissue. We have observed that tissue engineering strategies often draw inspiration from these vascularization methods found in nature; other times, vascularization strategies are based on novel innovative methods outside of those found in nature. In this section, we bring this information together and present a process flow chart that can be used as a decision making tool for the development of vascularization strategies in tissue engineering. Our scheme is presented in Figure 5.7. The process

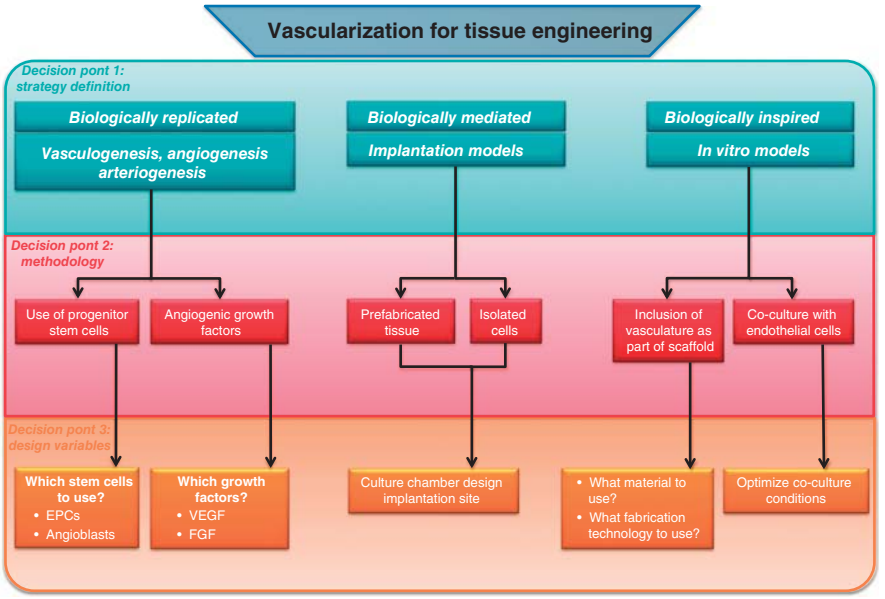

Figure 5.7 Process Flow Chart for Vascularization in Tissue Engineering—Three critical decision points are defined. The first step in the process is defining the vascularization strategy, which can be biologically replicated, biologically mediated, or biologically inspired. Once the vascularization strategy has been defined, the next two steps are focused on defining the specific methodology and statement of design variables.

flow chart is based on critical decision making points at three hierarchical levels, starting with defining a broad strategy and moving toward a specific methodology.

When presented with the challenge of fabricating vasculature within 3D artificial tissue, defining a starting point is vague, and the most difficult question to answer is—*where do I start?* The first decision point in our process flow chart is focused on addressing this issue by providing options for defining a broad vascularization strategy. The process flow chart for vascularization identifies three potential starting points, which are identified in the first level of decision making hierarchy. Based on current technology, there are three broad strategies for vascularization: biologically replicated, biologically mediated, and biologically inspired. In the first case, biologically replicated strategies are designed to replicate *in vivo* processes of vascularization *in vitro*. Biologically replicated processes are influenced by molecular biology, with the objective being understanding biological phenomena and defining controlled laboratory conditions to replicate these processes. These strategies are focused on defining *in vitro* conditions used to drive vasculogenesis, angiogenesis, and arteriogenesis; examples include the use of early progenitor stem cells and angiogenic growth factors to support capillary formation.

The second strategy is referred to as biologically mediated and includes vascularization models that are based on implantation of 3D artificial tissue. The term

"biologically mediated" refers to the notion that successful implementation of these strategies requires intervention and mediation from recipient of the implanted tissue. Mediation of the vascularization process is a result of implantation of cells or artificial tissue.

The third strategy is referred to as "biologically inspired" and in this case, inspiration is drawn from biological process with an objective to replicate these processes using innovative *in vitro* strategies. The goal is not to replicate the biological process, but replicate functionality.

Once we have identified a broad strategy for the specific application, the next step is to identify a specific methodology. Specific examples of methodologies for each of the three strategies are provided in the process flow chart. For example, if we select "biologically replicated", we will develop a method that replicates vascularization as it occurs during human development. This can be done in one of two ways: use of early progenitor stem cells or use of angiogenic growth factors.

If we select "biologically mediated" as our vascularization strategy, our objective is to develop methods that support neovascularization of 3D artificial tissue by the host upon tissue implantation. This can be done one of two ways. In the first case, artificial tissue is fabricated under controlled *in vitro* laboratory conditions and then prefabricated tissue is implanted to support vascularization; tissue fabrication and vascularization are sequential processes. In the second case, isolated cells are placed within a culture chamber and then the culture chamber is implanted; fabrication of artificial tissue and vascularization occur simultaneously.

The third strategy is "biologically inspired", and in this case, we draw inspiration from nature and biological processes, and then develop strategies to replicate these biological processes. One example of this is the design of novel scaffold with pre-engineered vasculature; to achieve this objective, we will use prototyping techniques to fabricate blood vessels as a core component of the 3D scaffold, which is then populated with cells to support formation of vascularized 3D artificial tissue. A second example is the development of novel co-culture systems that recapitulate blood vessel formation with multiple cell types in culture.

The third and final step in the process is to define specific test variables and experimental conditions to evaluate these variables; this step involves specific experimentation and validation stage of the vascularization process. For example, if we decide to use a biologically replicated process based on progenitor stem cells, our experimentation strategy will need to focus on identifying stem cell type and source along with differentiation strategies to drive the phenotype of selected cells to a vascular lineage. Similarly, if our biologically replicated process is based on angiogenic growth factors, we need to identify specific growth factors, characterize the dose-dependent and time-course relationship, and develop strategies for temporal and spatial variations in growth factor delivery.

If we decide to go with a biologically mediated process based on implantation models, using either cells or artificial tissue, we need to select the implantation model, including the implantation site, *in vivo* culture time, and design of the implantation chamber. We could also go with a biologically inspired strategy for vascularization based on prevascularization of scaffolds. Specific experimental

variables that need to be optimized would include the type of polymer and processing conditions and specific fabrication technologies for engineering vascular networks.

In summary, the process flow chart presented in Figure 5.7 provides a tool to assist in the decision making process for vascularization strategies in tissue engineering. There are several scientific and technological challenges that need to be addressed at every stage of the vascularization process. With this background, we proceed to give specific examples in tissue engineering in which many of these strategies have been implemented to fabricate vascularized 3D artificial tissue.

5.13 BIOLOGICALLY REPLICATED VASCULARIZATION STRATEGIES

As we have seen before, vascularization during embryogenesis and human development is through three distinct mechanisms: vasculogenesis, angiogenesis, and arteriogenesis. Tissue engineering strategies have been developed to replicate these vascularization strategies *in vitro* by culturing artificial tissue in controlled conditions that replicate these processes and promote vascularization. These strategies aim to replicate *in vivo* conditions *in vitro* and are therefore termed "biologically replicated vascularization strategies." There have been two areas of research that have been aggressively pursued. The first is focused on replicating vasculogenesis *in vitro*, starting with progenitor stem cells and culturing these cells under controlled conditions that trigger capillary formation. The second strategy is focused on recapitulating angiogenesis, starting with endothelial cells and using growth factors to promote capillary formation. In the following sections, we provide three examples of biologically replicated vasculature strategies for tissue engineering (Figure 5.8).

The first strategy is based on scaffold-guided differentiation of endothelial progenitor stem cells (70). In this method, endothelial progenitor cells were used and the trigger for capillary formation was provided by a custom fabricated scaffold. The steps in this process are outlined below (70):

Step One Custom scaffolds were fabricated using three extracellular matrix proteins—fibrinogen, fibronectin, and laminin at concentrations of 50 mg/ml, 5 μg/ml and 5 μg/ml respectively.

Step Two Primary mesenchymal stem cells were obtained from the bone marrow of rats and maintained in tissue culture flasks. The MSCs were then selected for EPCs, which were enriched and selectively grown from bulk culture of the MSCs. Using this process, a large number of EPCs were readily available for studies.

Step Three EPCs were seeded on the scaffold and cultured under controlled *in vitro* conditions for 21 days. At various time intervals, EPCs were analyzed to assess the differentiation fate of the EPCs to an endothelial lineage.

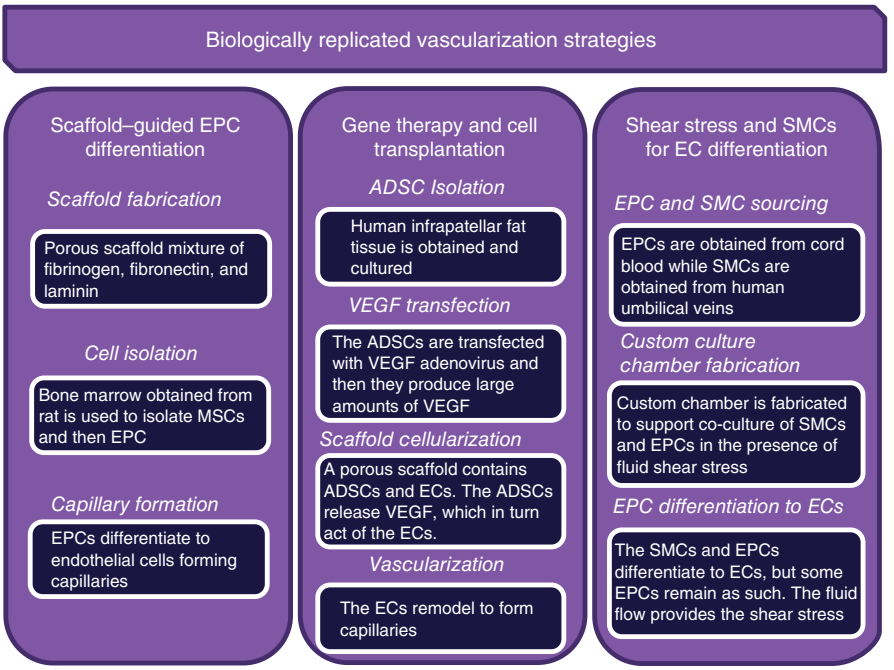

Figure 5.8 Biologically Replicated Vascularization Strategies for Tissue Engineering—Three examples of biologically replicated vascularization strategies: (a) Scaffold-Guided EPC Differentiation, (b) Gene therapy to deliver VEGF to drive the differentiation of adipose-derived stromal cells to form endothelial cells, and (c) Shear stress and contact with SMCs used for EPC differentiation to form endothelial cells.

The results of this study demonstrated that the EPCs were differentiating to form endothelial cells, and newly formed endothelial cells remodeled to form capillaries, resulting in vascularization of the scaffold. The trigger was engineered into the scaffold by using extracellular matrix components, which supported EPC differentiation to endothelial cells. This process was designed to recapitulate vasculogenesis by differentiation of early progenitor stem cells to a vascular lineage and use the differentiated cells to support capillary formation.

In the second approach, endothelial cells were stimulated using vascular endothelial growth factor to support capillary formation (71). The novelty of this strategy was that VEGF was released into the culture environment by adipose-derived stromal cells (ADSCs) using a genetic engineering approach (71). The details of this process are outlined below (71):

Step One Adipose tissue was obtained from human donors from infrapatellar tissue and was subjected to an enzymatic digestion process to isolate ADSCs, which were then cultured and expanded *in vitro*.

Step Two ADSCs were transfected with VEGF-containing adenovirus constructed by using cotransfection of 293 cells. This process is an example of genetic engineering and is designed to increase the rate of production and release of VEGF in ADSCs.

Step Three Transfected ADSCs were co-cultured with endothelial cells in custom poly(lactide-co-glycolide) (PLAGA) scaffolds. The ADSCs served as a source of VEGF to stimulate capillary formation by endothelial cells.

Step Four In response to VEGF stimulation, endothelial cells remodeled to form capillaries, resulting in vascularization of the 3D scaffold.

The strategy was designed to promote vascularization of 3D artificial scaffolds based on remodeling of endothelial cells in response to VEGF as the trigger, a strategy aimed to replicate vasculogenesis. The use of genetically engineered ADSCs as the source of VEGF added to the novelty of this strategy.

A third strategy was based on the differentiation of EPCs to endothelial cells, which can then be used to vascularize artificial tissue (72). The trigger for EPC differentiation was fluid shear stress and co-culture with SMCs, a process aimed at replicating arteriogenesis to guide EPC differentiation (72). The steps in the process were (72):

Step One EPCs were obtained from a human source and isolated from cord blood while SMCs were obtained from human umbilical veins.

Step Two A custom culture chamber was fabricated to support co-culture of SMCs and EPCs in the presence of fluid shear stress. The SMCs and the EPCs were in direct contact with each other, and EPCs were also exposed to fluid shear stress. The SMCs were not exposed to fluid shear stresses.

Step Three EPCs differentiated to form ECs in response to fluid shear stress and direct cellular contact with SMCs.

In this model, the objective was to replicate the process of arteriogenesis. The study was focused on differentiation strategies required to drive EPC differentiation toward vascular lineage; differentiated cells were not used to support vascular formation. It was demonstrated that direct contact of EPCs with SMCs or exposure to fluid shear stress resulted in differentiation to ECs; furthermore, the coupled effect of fluid shear stress and contact of EPCs with SMCs increased the differentiation efficiency of EPCs to form endothelial cells.

5.14 BIOLOGICALLY MEDIATED VASCULARIZATION STRATEGIES

Biologically mediated vascularization strategies are based on implantation models and depend on the host to promote and support vascularization. This means that

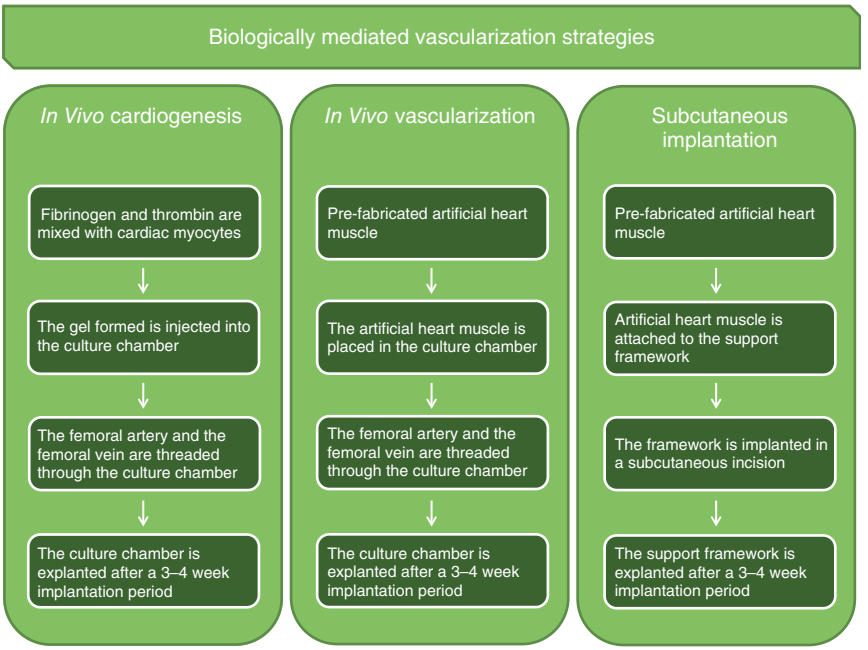

Figure 5.9 Biologically Mediated Vascularization Strategies for Tissue Engineering—Biologically mediated vascularization strategies rely upon implantation models for vascularization of 3D artificial tissue. Examples include *in vivo* cardiogenesis, *in vivo* vascularization, and subcutaneous implantation.

vascularization is mediated or facilitated by the host. While there have been several models published in the recent literature that showcase this methodology, we present three specific examples for illustrative purposes (Figure 5.9).

One approach has been to promote artificial tissue fabrication while vascularization takes place in a controlled culture chamber, all *in vivo* (73). This process has been used to engineer vascularized heart muscle using the following four step process (73):

Step One Primary cardiac myocytes were isolated and suspended in a complex 3D fibrin gel to provide support for artificial tissue formation.

Step Two The fibrin gel, with primary cardiac myocytes, was secured within a custom culture vessel designed to support artificial heart muscle formation during *in vivo* culture.

Step Three The culture chamber was implanted in close proximity to the femoral artery and femoral vein in recipient rats. The vascular pedicle serves as a source of nutrients for the cells as they remodel to form 3D tissue while providing pulsatile conditioning to enhance function of the cardiac myocytes. In addition, the vascular

pedicle serves as a site for angiogenic sprouting, and the newly formed blood vessels are incorporated within the artificial heart muscle.

Step Four After a 3–4 week implantation, the culture chamber was explanted from the recipient animal. The culture chamber was separated from the fibrin gel, which, by the end of the implantation period, had remodeled to form vascularized 3D artificial heart muscle.

The four-step process leads to the formation of highly vascularized artificial heart muscle. During *in vivo* culture in the chamber, isolated cells remodel to form artificial heart muscle while the femoral artery and femoral vein serve as sites for vascular sprouting; both of these processes occur simultaneously. The femoral artery and femoral vein are retained as a part of the tissue construct. The novelty of this model lies in the fact that 3D tissue fabrication and vascularization happen at the same time and cells remodel around the newly formed vasculature. This process does not aim at replicating any *in vivo* vascularization strategy, but rather to develop a novel tissue engineering technology around angiogenic sprouting.

A second strategy, somewhat related to the previous one, was to fabricate artificial heart muscle *in vitro* and then implant the tissue *in vivo* to support vascularization. In this case, complete fabrication of 3D artificial tissue was conducted *in vitro* and then implanted *in vivo* to support vascularization. In this approach, vascularization takes place after tissue fabrication in contrast to the previous approach in which both processes occurred in tandem. This is a four-step process:

Step One Artificial heart muscle is fabricated *in vitro* using a published model to support the self-organization of primary cardiac myocytes to form scaffold-free tissue, known as cardioids.

Step Two Cardioids are secured within a custom culture chamber that has been designed to house 3D artificial tissue during implantation.

Step Three The culture chamber is implanted in close proximity to the femoral artery and femoral vein in recipient rats. The vascular pedicle serves the same functions described in the previous case, which include nutrient delivery to the cardioids, pulsatile conditioning to enhance cardioid function, and as a source for angiogenic sprouting to support vascularization.

Step Four After a 3–4 week implantation period, the culture chamber is explanted from the recipient animal and, as in the previous case, separated from cardioids. Implantation resulted in extensive vascularization of cardioids.

We have presented two models for vascularization. While the two strategies have similarities, there are significant differences between the two. In the first case,

artificial tissue formation takes place in the culture chamber implanted *in vivo* while vascularization occurs simultaneously; tissue fabrication is in response to *in vivo* conditions. In the second case, artificial heart muscle is fabricated *in vitro* under controlled laboratory conditions; artificial tissue is transferred to a culture chamber and implanted *in vivo*, where vascularization takes place.

The third model presented for biologically mediated vascularization is based on the subcutaneous implantation of cardioids that have been fabricated under controlled *in vitro* conditions (69). In this case, artificial tissue is implanted in a subcutaneous pocket (69). The steps in this process are:

Step One Cardioids were fabricated under controlled *in vitro* conditions, as described for the previous model.

Step Two A custom support framework was fabricated to anchor cardioids during subcutaneous implantation. This support framework was different from the culture chamber described for the first two models. The function of the support framework was to provide attachment points for cardioids, which otherwise form a tissue mass due to spontaneous contractions.

Step Three The support framework with cardioids was implanted in a subcutaneous pocket in recipient animals.

Step Four After a 3–4 week implantation period, the support framework was removed from the site of implantation and the cardioids were separated; at the time of implantation, cardioids were extensively vascularized.

The third model is a variant of the first two, as it does not depend on direct interaction with a vascular pedicle for vascularization. Instead, the host response promotes vascularization of implanted artificial heart muscle.

5.15 BIOLOGICALLY INSPIRED VASCULARIZATION STRATEGIES

Thus far, we have looked at methods that are designed to replicate normal biological processes of vascularization *in vitro* or rely upon implantation models to induce vascularization. The third category is focused on developing technology inspired from nature; these processes draw inspiration from biological processes and use this inspiration to design novel strategies to induce vascularization (Figure 5.10). We will present three examples in the following sections.

The first strategy is based on technology to support scaffold fabrication with specific characteristics to support vascularization. In this approach, scaffolds were fabricated using a sugar leaching process, resulting in an average pore diameter of 100 μm (74). The pores served as sites for vascularization to support culture and proliferation of endothelial cells (74). The specific steps in the process are (74):

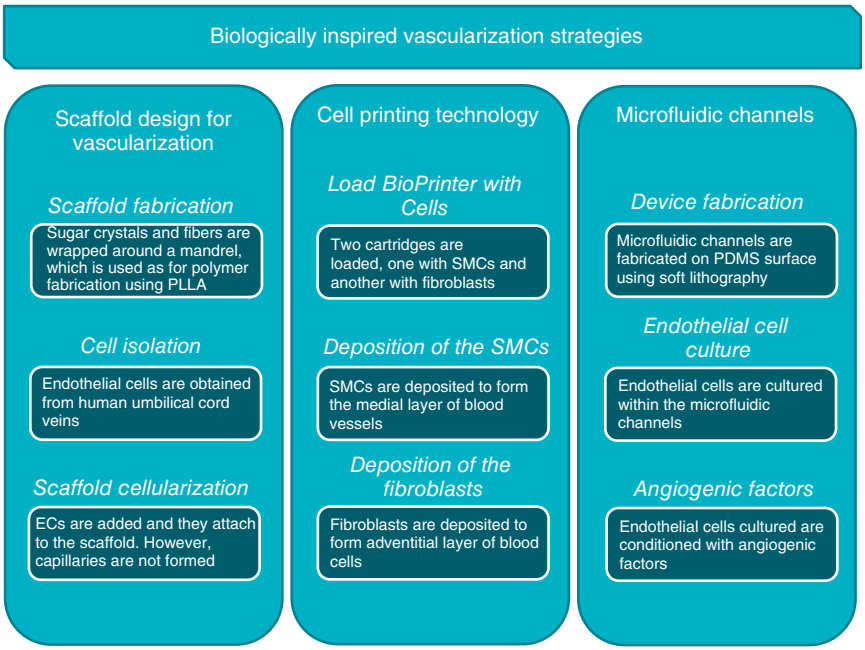

Figure 5.10 Biologically Inspired Vascularization Strategies for Tissue Engineering—These strategies draw inspiration from vascularization in nature; some examples are presented in the figure.

Step One Sugar crystals were solubilized and then heated and fibers wrapped concentrically around a mandrel. The mandrel was used as a framework for polymer fabrication using PLLA. PLLA polymer formed a 3D structure around the fibers and the 3D structure contained sugar crystals, which were solubilized to leave behind a highly porous polymer scaffold. These newly formed pores provided microchannels to support vascularization.

Step Two ECs were obtained from human umbilical veins and used to populate the porous scaffold.

Step Three The study demonstrated viability, attachment, and proliferation of the ECs within the pores of the scaffold. However, the study did not demonstrate capillary formation, which would require concentric organization of the ECs on the luminal surface of the pores within the scaffold.

A second strategy for engineering vascularization made use of cell printing techniques, which deposit droplets of cells to fabricate layers of tissue; individual layers can be combined to form complex artificial tissue (75). This strategy overcomes

the need for synthetic scaffolds, as the cells produce their own components. This strategy has been used to fabricate 3D blood vessels consisting of the medial and advential layers, using steps described here (75):

Step One Cartridges were loaded with SMCs and fibroblasts.

Step Two SMCs were deposited to form the medial layer of blood vessels.

Step Three Fibroblasts were deposited to form the adventitial layer of the blood vessel.

This process resulted in fabrication of bi-layer blood vessels with expansion capability to add ECs to form and complete tri-layer blood vessels.

The third process was based on microfluidic channels fabricated using PDMS surfaces. These surfaces can be designed to form complex vascular networks and can also be seeded with angiogenic factors to support capillary formation (76). The steps in the process are (76):

Step One Microfluidic channels were fabricated on PDMS surface using soft lithography.

Step Two Endothelial cells were cultured within the microfluidic channels.

Step Three Endothelial cells that were cultured within the microfluidic channels were conditioned with angiogenic factors.

The steps described above were shown to support culture and proliferation of ECs, while angiogenic factors were shown to increase the rate of proliferation of ECs.

SUMMARY

Current State of the Art—During early stages of tissue fabrication, nutrient delivery to cells is supported by diffusion. As the cell mass increases, nutrient delivery by diffusion is not adequate to support cell viability, and vascularization is required. Seminal work in the field of angiogenesis research was conducted by Dr. Judah Folkman and lead to significant advancements in the field of angiogenesis. While vascularization is a generic term that refers to blood vessel formation, vasculogenesis, angiogenesis, and arteriogenesis refer to specific processes during human development and growth. Understanding molecular mechanisms of vascularization has allowed researchers to develop novel strategies to integrate blood vessels within the 3D architecture of artificial tissue. There are three categories of vascularization strategies for tissue engineering: biologically replicated, biologically mediated, and biologically inspired.

Thoughts for the Future—While there have been several interesting approaches to support vascularization of 3D artificial tissue, the ability to fabricate vascularized 3D tissue remains challenging and requires significant attention.

The most important area that needs to be developed is to carefully understand the triggers that drive vascularization during normal human development and growth (vasculogenesis, angiogenesis, and arteriogenesis) and use these triggers in tissue engineering. As we gain a better understanding of these triggers, this information can be translated to fabricate vascularized 3D artificial tissue.

PRACTICE QUESTIONS

1. Why is vascularization important for the development of artificial tissue?
2. Discuss the seminal work by Dr. Folkman in the area of angiogenesis.
3. Describe the terms vasculogenesis, angiogenesis, and arteriogenesis. What is the difference between these three processes?
4. Vasculogenesis, angiogenesis, and arteriogenesis are distinct processes for vascularization. Compare the relative advantages and disadvantages of each of these processes for vascularization of 3D artificial tissue.
5. Describe the molecular mechanism of vasculogenesis. What are the participating stem cells, processes for capillary formation, and molecular signals that guide the process of vasculogenesis?
6. Describe the molecular mechanism for angiogenesis. What are the participating stem cells, processes of capillary formation, and molecular signals that guide the process of angiogenesis?
7. Describe the molecular mechanism for arteriogenesis. What are the participating stem cells, processes of capillary formation, and molecular signals that guide the process of arteriogenesis?
8. Explain the concept of therapeutic angiogenesis. What are some potential therapeutic agents that can be used for therapeutic angiogenesis? What are the proposed mechanisms by which therapeutic angiogenesis provides a functional benefit to injured tissue?
9. Identify any clinical condition and explain how therapeutic angiogenesis may be used as a potential therapeutic strategy.
10. What is the difference between *in vivo* and *in vitro* vascularization models for artificial tissue? Compare the relative advantages and disadvantages of *in vivo* and *in vitro* vascularization models for artificial tissue.
11. What are some of the scientific and technological challenges associated with *in vivo* strategies for vascularization of 3D artificial tissue?
12. Develop an *in vivo* vascularization strategy for 3D artificial heart muscle.
13. Select any tissue engineering application and explain why *in vivo* or *in vitro* vascularization strategies would be better suited for your selected application.

14. Describe the idealized process for vascularization of 3D artificial tissue, as described in the chapter.

15. For any given tissue engineering application, develop your own idealized process for vascularization of 3D artificial tissue.

16. Describe the process flow chart used for development of vascularization strategies for artificial tissue.

17. Use the process flow chart for any selected tissue engineering application.

18. Explain the following terms: biologically replicated vascularization strategies, biologically mediated vascularization strategies, and biologically inspired vascularization strategies. What are the relative advantages and disadvantages of these strategies for vascularization of 3D artificial tissue?

19. Pick any tissue fabrication application. Develop three vascularization strategies for your selected application using biologically replicated, mediated, and inspired vascularization strategies.

20. Identify three critical challenges in the field of vascularization as it applies to vascularization for tissue engineering. What can be done to overcome these challenges?

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