Disease-inspired tissue engineering: Investigation of cardiovascular pathologies

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Abstract

Once focused exclusively on the creation of tissues to repair or replace diseased or damaged organs, the field of tissue engineering has undergone an important evolution in recent years. Namely, tissue engineering techniques are increasingly being applied to intentionally generate pathological conditions. Motivated in part by the wide gap between 2D cultures and animal models in the current disease modeling continuum, disease-inspired tissue-engineered platforms have numerous potential applications, and may serve to advance our understanding and clinical treatment of various diseases. This review will focus on recent progress toward generating tissue-engineered models of cardiovascular diseases, including cardiac hypertrophy, fibrosis, and ischemia reperfusion injury, atherosclerosis, and calcific aortic valve disease, with an emphasis on how these disease-inspired platforms can be used to decipher disease etiology. Each pathology is discussed in the context of generating both disease-specific cells as well as disease-specific extracellular environments, with an eye toward future opportunities to integrate different tools to yield more complex and physiologically relevant culture platforms. Ultimately, the development of effective disease treatments relies upon our ability to develop appropriate experimental models; as cardiovascular diseases are the leading cause of death worldwide, the insights yielded by improved *in vitro* disease modeling could have substantial ramifications for public health and clinical care.

Graphical Abstract

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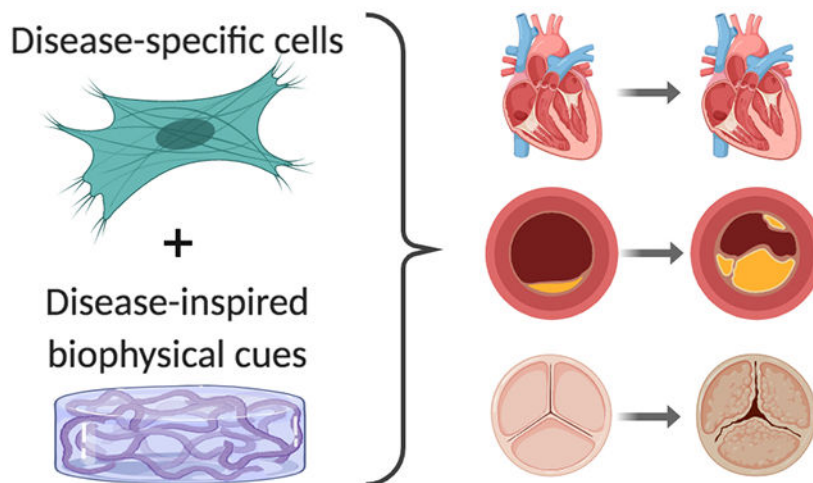
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Introduction

From its inception, tissue engineering has promised to yield a source of healthy neo-tissues targeted at replacing, repairing, or regenerating diseased or damaged tissues[1]. Myriad tools have been developed to advance this pursuit, including generation/identification of new cell sources[2–3], controlled delivery of biophysical and biochemical stimuli[4–5], and the synthesis of increasingly complex 3D scaffolds to mimic the extracellular environment[6–8]. Successful products have been developed[1], with the most recent market evaluation identifying 21 U.S. companies with commercialized tissue-engineered products, yielding $9 billion in sales in 2017[9]. Despite these successes and advancements, the development of plentiful replacement organs has been a much slower and winding road than was predicted for the field[10].

Thus, the field of tissue engineering has been experiencing a subtle, but important, evolution. Although it remains a common assumption that one's goal in performing tissue engineering is to create healthy tissues - even in 2019, encyclopedias, journal articles, and the NIH still define "tissue engineering" as creating materials for "restoration or replacement of a damaged or diseased body part"[9, 11–12] - an increasing share of tissue engineering researchers have turned their attention toward the intentional creation of diseased tissues. The purpose of these tissue-engineered disease models varies with the specific application and tissue, and may range from interrogating specific pathological pathways to examining patient-specific responses to a drug. In some cases, the shift to disease tissue engineering creates a somewhat ironic scenario where one is "doing tissue engineering to avoid doing tissue engineering" – i.e., if a disease treatment can be elucidated using a tissue-engineered disease model, it may obviate the need to ultimately replace the tissue (theoretically, with an engineered substitute).

While tissue engineering's original goal of creating healthy tissue replacements remains a necessary and important pursuit, the potential impact of tissue-engineered disease models is significant, and has the ability to yield advancements in the clinic on a shorter time scale than 'traditional' tissue engineering. These platforms are poised to occupy an important and unique position on the disease model continuum, which typically spans from 2D studies to animals and then humans. The limitations of 2D cultures have been reviewed at length[13], and researchers continue to discover how much of our cellular knowledge is specific to the 2D, tissue culture polystyrene context, as different responses have been achieved in more physiological environments[14–15]. By allowing independent tuning of variables, control over conditions, and good throughput, the *in vitro* 3D culture environment enables prospective studies of specific biological questions that would be challenging to achieve in animals. Additionally, some studies have indicated that even simplistic 2D cultures with human cells produce more predictive results than studies performed in rodents[16], and there are diseases for which appropriate animal models have yet to be identified. These limitations in both 2D and animal platforms outline a distinct gap which 3D engineered tissues seem ideally suited to fill.

Our ability to understand disease mechanisms and develop therapies relies heavily upon our ability to develop appropriate experimental models. In combination with the delivery of physiological risk factors, 3D tissue-engineered platforms can help us identify conditions that support both the onset of pathology and its progression. These environments can be manipulated and probed to gain mechanistic insight on the specific signaling pathways involved in pathogenesis, thereby revealing potential treatment targets. Tissue-engineered disease platforms can be employed to test potential disease treatments, acting as an extra measure of *in vitro* drug screening that may significantly reduce the use of animals in subsequent *in vivo* studies. Finally, disease tissue engineering is not completely divorced from traditional healthy tissue engineering, as investigating the physical and biochemical factors that promote disease pathogenesis also serves to identify factors that promote maintenance of a healthy tissue environment, and this information may be applied to inform the design of healthy engineered tissues.

This review article will focus on progress made toward the development of tissue-engineered models of cardiovascular diseases, addressing pathologies found in vessels, cardiac muscle, and the aortic heart valve. This is a burgeoning area of investigation and distinct from the pursuit of generating healthy engineered tissues to perform cardiotoxicity screening of drugs[17]. In theory, any healthy engineered tissue may become a disease model if combined with the appropriate stimuli, but this review will concentrate solely on instances where mimicking the disease environment was the specified goal.

Cardiac

Cardiovascular diseases affect nearly half of all American adults and are responsible for 18 million deaths per year worldwide, or 1 out of every 3 deaths in the U.S.[18]. These diseases span both congenital and acquired conditions, but almost half of the deaths from cardiovascular disease are attributed to complications resulting from a myocardial infarction (MI)[18]. While the immediate post-MI survival rate has been increasing in recent years[19],

patients that survive the initial acute MI injury remain at elevated risk of eventual heart failure due to the tissue death and remodeling that occur in response to ischemic injury[19–20]. Meanwhile, it is the heart's own healing and compensatory mechanisms that cause further dysfunction, as attempts to normalize the mechanical stresses to account for the loss of functional contractile tissue can ultimately lead to cardiac fibrosis and pathological hypertrophy[21]. Post-MI treatment regimens generally consist of blood pressure-lowering and anti-platelet drugs[22], which do not directly address the mechanistic cascades causing hypertrophy or fibrosis. Ischemic damage to the heart can also occur in a chronic fashion, not associated with an acute cardiac event; such is the case of chronic ischemic heart disease due to atherosclerosis[23]. Similarly, pathological cardiac hypertrophy can result from non-acute events, such as systemic hypertension[24]. In both of these conditions, pharmacological treatment tends to focus upon modifying risk factors, such as cholesterol levels and blood pressure, as well as lifestyle modification (e.g., smoking cessation, exercise)[22, 25].

The prevalence and severity of these various types of myocardial dysfunction has motivated a wealth of studies aimed at creating healthy tissue-engineered myocardium to regenerate or repair this lost function. While we leave a thorough discussion of healthy cardiac tissue engineering to other reviews[26–29], it is worth noting the foundational work in this area that ultimately set the stage for the pursuit of disease-inspired cardiac models. In particular, the engineered heart tissue (EHT) approach described by Eschenhagen et al. in 1997[30] has become a widely used platform for 3D myocardial culture. EHTs have evolved to take many forms[31] and have been an enabling technology with respect to the creation of cardiac disease models. While the vast majority of cardiac tissue engineering research remains focused on creating healthy tissues, the application of these approaches to specifically study the pathology of cardiac dysfunction or treatment of diseased tissues is an area of steady growth. Such disease-mimetic platforms are poised to address ongoing concerns about the predictive ability of current *in vitro* and animal models[17] across all of the pathologies mentioned above. Additionally, our mechanistic knowledge of these pathologies remains extremely limited. Advancements in the area of cell source have dramatically altered the landscape of this research field[32] and made it easier for researchers to apply 3D tissue engineering techniques, which often require large numbers of cells. In the sections below, we highlight recent work aimed at creating tissue-engineered disease-mimetic platforms for studying both disease pathophysiology and potential treatments (Figure 1).

Disease-specific cells

The majority of work in cardiac disease modeling has been in the form of creating disease-specific cardiomyocytes[33–36]. For decades, the logistics of culturing cardiomyocytes *in vitro* were challenging and often prohibitive in terms of throughput. Due to their limited proliferation capacity, cardiomyocytes had to be obtained as primary cultures, without the ability to sub-culture. Neonatal rodents still serve as the most common source of *in vitro* cardiomyocytes, but their applicability to modeling human cells is questionable[37]. The ability to generate cardiomyocytes from human pluripotent stem cells (hPSCs)[38], followed by dramatic improvements in differentiation efficiency[39], have been transformative events in cardiomyocyte culture, and the culture of hPSC-CMs in 3D hydrogels (e.g., EHTs) is increasingly used to make healthy myocardial cultures *in vitro*[31]. The maturity of hPSC-

CMs does remain a concern and can significantly impact their use as predictive models[40]. But, given the current trajectory, the use of hPSC-derived cardiomyocytes (hPSC-CMs) may soon surpass the use of animal-derived primary cells for *in vitro* studies.

The advent of induced pluripotent stem cell (iPSC) techniques has also enabled the generation of plentiful diseased CM cultures from patients with congenital cardiac conditions (e.g., arrhythmias, cardiomyopathies)[41]. Further revolutionizing the *in vitro* usage of diseased cardiomyocytes was the development of genome editing techniques, which have expanded the ability of researchers to create disease-specific cardiomyocytes from any cell type, modeling both congenital and acquired conditions[42]. Diseased hPSC-CMs produced via both patient-specific and gene-edited approaches are even sold commercially (Cellular Dynamics, Inc., Madison, WI). In most cases, these diseased hPSC-CMs have been used in a traditional (2D) context, although an increasing number of studies have embedded these cells in collagen I and/or fibrin, an approach that is commonly used to enable quantification of contractile function, but is not necessarily modeled upon mimicking the native extracellular environment. Because much of the work with disease-specific hPSC-CMs has not yet involved the creation of 3D disease-inspired environments, we leave more thorough coverage of these cells to other reviews[35, 42]. However, they are positioned to become a critical element in the generation of future 3D tissue-engineered disease models, so we will highlight some of the key advances in this area (Table 1).

Congenital arrhythmias, including Long QT Syndromes (LQTS), comprise the bulk of current diseased hPSC-CM research[35, 43–44]. LQTS hPSC-CMs in standard 2D culture exhibit prolonged action potential duration and responsiveness to current and potential anti-arrhythmic drugs, indicating that these cells are capable of recapitulating the disease phenotype and predicting response to pharmacological treatments[45–46]. In the case of cardiomyopathies, gene-edited hPSC-CMs have primarily been used to characterize the genetic and molecular contributors to disease pathogenesis[47] in traditional *in vitro* culture environments, with a focus on genes related to contraction. EHTs have been formed using hPSC-CMs to yield 3D constructs intended to improve iPSC maturation and the physiological relevance of disease models[48–49]. For example, both patient-specific and gene-edited iPSC-CMs were cultured in EHTs to examine the pathogenicity of different titin gene mutations in dilated cardiomyopathy (DCM), as well as elucidate the downstream pathways by which these mutations ultimately caused decreased force generation[49]. In this case, 2D iPSC-CM cultures failed to recapitulate the disease phenotype observed in patients, while the EHT constructs revealed mechanistic information about the pathogenesis of DCM. While the aforementioned studies have generally been confined to monogenic cardiac diseases, recent work employed the novel Biowire platform[50] to investigate the differences between hypertensive individuals who develop left ventricular hypertrophy (LVH) and those who do not, an issue which is likely polygenic in nature. iPSC-CMs were derived from hypertensive individuals with or without LVH, cultured in combination with cardiac fibroblasts in collagen/matrigel EHTs, and subjected to regimes of electrical stimulation for up to 8 months[51]. This platform enabled monitoring of contractile function and yielded distinct gene expression profiles across the patient groups, which may help in understanding the pathogenesis of this dysfunction. Finally, the construction of EHTs using patient-derived iPSC-CMs has also fueled commercial developments in cardiac disease modeling.

InvivoSciences (Madison, WI) generates EHTs using patient-specific iPSC-CMs[52] in order to define personalized approaches to treating various cardiac diseases as well as to perform rare disease drug discovery.

The generation of a diseased cardiac phenotype does not necessarily need to come from the use of cells with genetic mutations. Manipulating the composition of the cell population in a co-culture model can also provide a cell-driven approach to yield a disease-mimetic culture platform[53–54]. For example, the Biowire platform[50] was recently used as the basis to generate not only healthy and fibrotic engineered myocardium, but also an integrated model of adjacent healthy and fibrotic tissue, to mimic the post-infarct border zone and scar[53]. This 3D culture employed healthy iPSC-CMs and cardiac fibroblasts embedded in a mixture of fibrin and matrigel, with fibrosis mimicked by increasing the ratio of fibroblasts to cardiomyocytes. These tissues exhibited a physiologically relevant fibrotic phenotype and may be used to test both the efficacy and timing of anti-fibrotic therapies[53].

Disease-inspired physical cues

Several types of biomaterials have been used in the pursuit of creating healthy tissue-engineered myocardium[55–56]. In general, these scaffolds have been in the form of hydrogels, which are attractive for myocardial applications for several reasons, including mechanical properties with broad similarities to native cardiac tissue, allowing functional contraction of encapsulated cardiomyocytes. However, scaffolds used in the context of disease-inspired cardiac tissue engineering have not branched out to the same extent, and the majority of 3D disease platforms use collagen I, fibrin, Matrigel, or a mixture of these materials[55]. These materials have been well-established for encapsulation of cardiomyocytes and supporting cell types, but are not necessarily aimed at recapitulating native healthy or disease-specific cardiac ECM composition. Rather, the disease-mimicking aspect of these systems has generally come in the form of using disease-specific cells (discussed above), or application of external stressors: exogenous biomolecules associated with disease pathogenesis (e.g., transforming growth factor beta 1, TGF-β1), hypoxic conditions, or pathophysiological mechanical stresses.

During a myocardial infarction, vessel occlusion limits blood flow to the heart, leading to hypoxia, metabolic waste accumulation, and nutrient deficiency[57]. While reperfusion of the occluded vessel is necessary to reduce acute ischemic injury and minimize infarct size, it can also cause additional injury, termed ischemia reperfusion injury (IRI)[58]. Current preclinical models of IRI have not successfully translated to the clinic[59], leading to the recent emergence of engineered cardiac tissues to test potential therapeutic strategies to protect the heart from IRI damage[60]. In one of the first examples of this application, collagen I-based EHTs were constructed using neonatal rat cardiomyocytes and transiently cultured in hypoxic conditions in the presence or absence of potential cytoprotective agents cyclosporine A and acetylcholine[61]. The application of hypoxic conditions initiated a physiologically relevant ischemic cellular response, and treatment of these constructs with cytoprotective agents was able to successfully inhibit hypoxia-induced damage, thereby indicating potential for use of EHTs for studying the cardiac response to stressors, as well as for screening pharmacological agents that may help treat myocardial injuries. However, the

cytoprotective agents in this study were administered prior to the onset of hypoxia, which would not be relevant to the clinical setting. Subsequent studies using neonatal rat cardiomyocytes in EHTs further refined the IRI-mimicking conditions and found moderate cardioprotective effects offered by a nitric oxide donor or B-type natriuretic peptide[62]. A recent study was the first to use human cells in a tissue-engineered environment to study IRI[63]. This work encapsulated hPSC-derived CMs in collagen I/fibrin scaffolds and further improved upon the physiological relevance of the IRI-mimicking conditions by subjecting the engineered constructs to both hypoxia and a small volume of ischemia-mimicking buffer solution to reproduce the metabolic waste accumulation, nutrient deprivation, hyperkalemia, low pH, and high lactate concentration found during ischemia. Using this novel system, the authors investigated both IRI pathophysiology (e,g., distinguishing between ischemic and reperfusion injury) and possible treatments, with potential cardioprotective agents added at the time of reperfusion[63]. Importantly, this work also addressed how hPSC-CM maturity affected the ischemic response and employed a strategy to further hPSC-CM maturation. Together, these studies have established a strong platform for evaluation of potential strategies to inhibit IRI.

Pathological hypertrophy occurs in response to volume or pressure overload in the heart[64]. Although it can be intended to compensate for altered wall stresses post-injury, it is always a maladaptive process in the adult myocardium, leading to extensive cardiac remodeling and heart dysfunction or failure[64–65]. Despite many years of considerable effort to elucidate the mechanisms of pathological hypertrophy, there is still much to be learned about its pathogenesis, which is critical to develop effective treatments for this condition. Limitations of traditional *in vitro* cell cultures and animal models are often named as major factors that have impeded progress on this front[66]. In combination with primary or hPSC-derived cardiomyocytes, fibrin-based EHTs have been used as a platform for applying both biochemical and mechanical hypertrophic stimuli[67–68]. Humoral factors such as endothelin-1 and phenylephrine are commonly used to stimulate hypertrophic behaviors, but do not recapitulate the chronically increased hemodynamic load that triggers hypertrophy. To examine the latter, novel methods have been applied to mimic increased afterload in these 3D cultures. EHTs are typically strips of tissue anchored to a flexible post on either end, where contraction of the EHT results in post deflection[69]. Afterload in EHTs containing rat cardiomyocytes was increased by 12-fold by fitting metal braces to these posts, without alteration to the beating rate. Using this approach, the authors found that a hypertrophic gene program was enacted by this application of pathological afterload, with significant increases in cardiomyocyte size, glycolysis, and fibrotic ECM remodeling[67]. This platform of enhanced afterload laid the foundation for multiple subsequent studies that have uncovered mechanistic features of pathological hypertrophy, such as differential regulation of multiple microRNAs[70] and characterization of DNA methylation signatures[71]. In the EHT platform, miR-21–5p was strongly upregulated by hypertrophic afterload, consistent with its elevation in human heart failure[72]; additionally, antagonism of this microRNA attenuated the afterload-induced hypertrophic response in EHTs[70]. In another demonstration of the potential for the hypertrophic EHT platform to discover new treatment targets, the DNA methyltransferase inhibitor RG108 was found to attenuate some hypertrophic features in the EHTs[71], which also held true in a follow-up study in rats with pressure overload-induced

hypertrophy[73]. These studies have been performed using animal CM sources, but others have employed a similar system to apply increased afterload to fibrin EHTs containing hPSC-CMs[68]. Although performed for the purpose of using afterload to enhance the maturity of hPSC-CMs, the authors observed that application of high levels of afterload (~9 μN/μm) significantly upregulated markers of pathological hypertrophy and fibrosis.

Efforts made by the heart to ameliorate the biological and mechanical insufficiency of the post-MI environment include not only hypertrophy of resident cardiomyocytes but also extensive remodeling of the extracellular matrix (ECM) by cardiac fibroblasts[74–75]. Cardiac fibroblasts differentiate into a myofibroblast phenotype and produce excessive amounts of ECM proteins such as collagen I and fibronectin[76]. While these changes can stabilize heart tissue integrity in the short-term, the increase in tissue stiffness ultimately leads to impaired ability to contract and increased risk of arrhythmia[77]. A few recent efforts have employed tissue engineering techniques to intentionally create fibrotic cardiac tissues[78–81]. A proof of concept for this approach was provided by encapsulating neonatal rat cardiac fibroblasts and cardiomyocytes within scaffolds of methacrylated gelatin (GelMA) and then treating with a known pro-fibrotic agent, TGF-β1[78]. The use of GelMA enabled mechanical tuning of the 3D environment to best match the *in vivo* setting and promote normal, quiescent cardiac fibroblast behavior as a healthy baseline. Addition of TGF-β1 stimulated a fibrotic response, as measured by both phenotypic indicators as well as altered functional activity (e.g., asynchronous beating), demonstrating the ability of this approach to serve as a platform for modeling cardiac fibrosis. Mechanical cues have been introduced into these tissue-engineered systems by constructing microdevices that applied cyclic compressive strain to GelMA[79] or fibrin[82] hydrogels containing cardiac fibroblasts, resulting in increasing fibrotic activity in a strain-dependent manner. The value of taking a tissue engineering-based approach to study cardiac fibrosis was also illustrated by work in which collagen density and fibroblast number were independently varied in order to decipher their relative influences on contraction, revealing that fibroblast number affected contractile tissue function more than collagen density[54]. Recent studies have begun to construct cardiac fibrosis platforms using entirely human-derived cells[80–81] and demonstrated similar successes in mimicking fibrotic outcomes upon treatment with pro-fibrotic stimuli. Together, these advancements have provided an important foundation for constructing tissue-engineered models of cardiac fibrosis that will permit elucidation of its pathogenesis and testing of potential therapeutics.

Future Opportunities

As described in the preceding sections, many types of disease-specific cardiomyocytes have been generated, as have several approaches to provide disease-specific physical environments. However, relatively little research has been done at the intersection of these areas (i.e., placing disease-specific cells in disease-specific extracellular environments). Furthermore, the diseased ECM has been highly underappreciated in the context of mimicking cardiac diseases[83], and most of the aforementioned approaches have not attempted to replicate disease on the ECM level. Increased ECM deposition is a hallmark of hypertrophy, cardiomyopathy, and heart failure[84–86], and merging diseased hPSC-CMs with disease-mimicking 3D scaffolds has significant applicability to the study of these conditions. For example, ECM alterations are thought to be a driving force in the pathogenesis of

cardiomyopathies[87], but these potentially influential features are omitted when cardiomyopathies are studied using a model that is based solely on disease-specific cells. Recreating a dysfunctional ECM environment is even important in pathologies that are typically only cell-focused (e.g., arrhythmias). Although arrhythmias are not known to cause changes to the ECM environment, the occurrence of ECM changes – due to other cardiac events or simply the fibrosis that happens with natural aging[83] – can strongly influence the arrhythmia and its drug responsiveness[88]. The design and implementation of pathological scaffold environments, combined with innovations in delivering pathological stimuli such as hypoxia[89], could greatly advance the ability of these environments to faithfully mimic native disease conditions.

Vascular

Myocardial infarctions are generally caused by coronary artery disease (CAD), or atherosclerosis of the vessels supplying the heart. CAD is the most common type of cardiovascular disease and is the leading cause of death worldwide[18]. Yet, the onset and progression of ischemic vascular diseases tend to go undetected until symptoms indicate an advanced stage of disease[90]. The pursuit of tissue-engineered vascular grafts is one of the earliest examples of tissue engineering, when Weinberg and Bell described seeding endothelial cells, smooth muscle cells, and fibroblasts within a collagen I matrix in 1986[91]. As reviewed elsewhere[92], numerous innovative advancements in vascular tissue engineering have followed, ultimately yielding multiple different approaches that have reached clinical tests in humans[93–95].

While promising strides have been made to construct tissue engineered blood vessels for the purposes of vascular grafting[96–98], engineered models of vascular diseases could also prove useful for earlier detection of disease as well as the development of preventative drugs and therapies. Significant challenges face the development of these models, given the complexity of native vessel composition, architecture, and pathogenesis of disease. The native arterial wall is concentrically composed of endothelial cells (ECs), smooth muscle cells, and fibroblasts, each residing within a different ECM environment. Endothelial cells in the healthy blood vessel intima provide a selectively permeable layer that does not allow infiltration of lipoproteins into the subendothelial space. However, hypercholesterolemia or other pathological stimuli can disrupt the integrity of this barrier, allowing the infiltration and accumulation of low density lipoproteins (LDL)[99]. Following LDL entrapment, monocytes infiltrate the vessel wall through the compromised endothelial layer, uptake LDL, and become foam cells[100]. The inflammatory state that is then assumed ultimately culminates in the characteristic lesion and plaque formation seen in atherosclerosis. As described in subsequent sections, progress toward fabricating disease-inspired models of atherosclerosis has focused on cellular-based methods to produce diseased EC phenotypes as well as the construction of complex scaffold environments to replicate key risk factors and features of disease onset (Figure 2).

Disease-specific cells

Endothelial cells, smooth muscle cells, and fibroblasts that comprise the vessel structure are each uniquely impacted throughout the progression of atherosclerosis and other vascular pathologies. EC dysfunction has long been considered an initiating event in atherosclerosis, in which there is a reduction in EC-produced nitric oxide and increase in inflammatory cytokine production by ECs[101]. EC dysfunction also affects processes such as angiogenesis, which is crucial to restoring myocardial function in response to ischemic conditions. Sourcing and culturing ECs has not been limited by the same challenges described above for cardiomyocytes, and *in vitro* experiments have often utilized human umbilical vein endothelial cells (HUVECs) as the cell source. However, efforts are increasingly moving towards the use of iPSC-derived ECs or direct reprogramming of somatic cells (e.g., fibroblasts) into ECs as an alternative[102–104]. Similar to the case of cardiomyocytes, these derivation approaches have opened up the possibility of generating disease-specific EC cultures, either by starting with cells from diseased individuals, or by the application of gene editing techniques to introduce targeted mutations. For example, using CRISPR/Cas9 gene editing, iPSC-ECs deficient in hypoxia inducible factor-1 alpha (HIF-1α) were recently generated[105]; HIF-1α plays a crucial role in regulating the response to ischemia but is impaired with advanced age. *HIF1A* knockout iPSC-ECs expressed key EC characteristics and, in normoxic conditions, exhibited few differences compared to wild type ECs. However, their response to ischemic conditions was significantly altered, as these diseased iPSC-ECs demonstrated decreased viability and angiogenic potential, both in traditional 2D conditions and in a GelMA-based 3D environment[105]. This study provides an important foundation for generating tissue-engineered models of EC dysfunction.

Overall, the creation of disease-specific ECs is in a relatively nascent stage in comparison to the extensive work done thus far with cardiomyocytes, but iPSC-ECs have been derived to represent other conditions associated with high risk of cardiovascular disease, such as familial pulmonary arterial hypertension[106], Fabry disease[107], or diet-induced obesity[108]. Smooth muscle cells (SMCs) are in a similar position[109], with a few cardiovascular disease-specific phenotypes being generated thus far (e.g., elastin mutation in supravalvular aortic stenosis[110] or fibrillin-1 mutation to mimic Marfan syndrome[111]), but the integration of these cells within the context of 3D disease modeling has not been explored.

Disease-inspired physical cues

The effect of altered shear stresses and atherogenic stimuli on endothelial dysfunction has been investigated for several decades using traditional 2D environments, providing many insights on atherosclerosis and thrombosis mechanisms[112–114]. The recent development and refinement of microfluidic cell culture techniques has enabled further advancements in disease-inspired vascular platforms[115]. Many of these investigations remain focused on what is functionally a 2D culture setup (i.e., ECs lining a channel)[116–117]. These systems may be used to introduce tunable channel constrictions that mimic atherosclerotic plaques of varying severity, thereby allowing an examination of EC dysfunction and inflammatory cell adhesion in response to disease-mimicking hemodynamic alterations. However, few of these current vascular platforms represent the vessel as a 3D, multi-layered structure, which is important for studying inflammatory cell infiltration and plaque formation. Additionally, in the human

body, the vessels that are most affected by atherosclerosis are not micro in size, raising concerns about whether a microfluidic approach is best suited to investigate atherosclerosis-mimicking flow profiles.

Thus, more traditional methods of fabricating tissue-engineered blood vessels (TEBVs) have been employed to create platforms that mimic elements of atherosclerosis. Early work in this area described the production of fibrin-based scaffolds containing smooth muscle cells and an EC lining to study LDL accumulation in normal and hypercholesterolemic conditions[118]. LDL deposition was dose-dependent and associated with increased monocyte infiltration and foam cell formation, illustrating the ability of a tissue-engineered vessel to replicate key features of atherosclerosis. However, these studies were not done in the presence of fluid flow, which has been viewed as a significant limitation. Thus, more recent iterations have focused upon TEBVs cultured in the presence of flow. For example, TEBVs made from smooth muscle cells embedded in tubular polymeric scaffolds[119] or collagen I hydrogels[120] and an EC-populated lumen were used to study lipoprotein accumulation[119] as well as inflammatory cell adhesion and transendothelial migration[119–120] under physiological fluid flow. These platforms were able to mimic early inflammatory events in atherogenesis. Additionally, the EC dysfunction induced by the inflammatory factor tumor necrosis factor-alpha was reduced by administration of anti-inflammatory drugs, suggesting the potential for this platform to be used to evaluate potential therapeutics[120].

Future Opportunities

Advances in both microfluidics and TEBV creation have yielded 3D culture platforms with great promise for studying vascular disease onset, progression, and treatment. Work in these areas has already progressed to mimicking multiple complex features of the *in vivo* environment, including delivery of shear stresses, presence of systemic risk factors (e.g., hypercholesterolemia), and presence of inflammatory cell types. The tunability of these existing platforms can be further exploited in the future to mimic key features of aging or common comorbidities. Advanced age is the strongest risk factor for atherosclerosis[121] and is well known to be associated with significant arterial stiffening and ECM remodeling[122]. Current *in vitro* models of atherosclerosis do not account for these changes, but evidence that monocyte behavior is stiffness-dependent[123–124] suggests that age-related stiffening may impact disease onset and progression. There is also room to expand these models to mimic different stages of atherosclerosis, as existing studies have focused upon only the earliest events in disease onset. These efforts would require tailoring scaffold features to mimic the ECM and biophysical alterations that occur with progression of atherosclerosis and plaque development[125]. Until recently, no models had been described to mimic the advanced atherosclerotic plaque itself. However, new work describes an innovative approach to mimic late-stage atherosclerotic lesions in a spheroid culture model[126], creating a future opportunity to combine tissue-engineered atherosclerosis models with advances in organoid technology to produce plaque-containing vessels.

Aortic Heart Valve

Non-rheumatic calcific aortic valve disease (CAVD) leads to the fibrotic thickening and calcification of the aortic heart valve, and is the most common heart valve disorder in developed countries[127–128]. CAVD affects more than 25% of individuals over the age of 65, and the number of individuals with CAVD is expected to triple by the year 2050[129]. However, much of our knowledge of this disease is derived from end-point analyses of diseased valves, and the only treatment option remains surgical valve replacement[130–131]. Because total valve replacement is the sole treatment for CAVD, numerous advances have been made toward creating healthy tissue-engineered valves. As reviewed elsewhere[132], pioneering work in this area has been performed by multiple individuals, with Shinoka and Mayer describing large animal implantation of a tissue-engineered valve in 1995[133], followed by further work in sheep by Hoerstrup[134] and in humans by Dohmen et al.[135] in the early 2000s. Preclinical studies of tissue engineered heart valves have been promising[136], and these foundational studies have laid the groundwork for the ongoing development of novel valve scaffolds and cell sources. However, there is also a strong desire to develop strategies that target CAVD progression prior to the disease advancing to the stage that requires valve replacement surgery.

Despite sharing numerous risk factors and pathological similarities with atherosclerosis, therapies and lifestyle changes that are efficacious in slowing atherosclerosis have not been met with the same success in CAVD[137–139], illustrating that CAVD has a unique pathogenesis. Our incomplete understanding of CAVD etiology ultimately acts as a major limiting factor in developing CAVD treatments. Thus, the development of 3-D *in vitro* models of valvular calcification is being investigated in order to both characterize the cellular and molecular events that lead to the initiation and progression of CAVD. Such *in vitro* engineered diseased models could aid in the development of therapeutics to slow or reverse CAVD progression, but would need to account for changes in valve cell phenotypes and leaflet architecture at different stages of disease, as we discuss below (Figure 3).

Disease-specific cells

Aortic valve leaflets are lined with valvular endothelial cells while the most abundant cell type within the leaflets are valvular interstitial cells (VICs). In healthy valves, VICs exhibit a quiescent, fibroblastic phenotype. Following injury, quiescent VICs become activated to a myofibroblastic phenotype and begin to proliferate and remodel their surrounding ECM[140]. While VIC activation is necessary to maintain valve homeostasis, when myofibroblastic VICs fail to apoptose, the valve progressively transitions to a diseased state characterized by an aberrant ECM[140] and formation of calcified nodules via dystrophic mechanisms[141]. To a lesser extent, heterotopic ossification also contributes to calcification as VICs may transdifferentiate into an osteoblast like phenotype[141]. In contrast to the other cardiovascular tissues discussed in this review, VICs have not yet been directly derived from hPSCs. Because VICs obtained from porcine valves are viewed by many as an adequate mimic for human VICs, the need to identify alternative cell sources has not been as pressing as for other cell types. Additionally, human valves that are explanted at the time of valve replacement surgery have been able to act as a relatively accessible source of diseased

VICs[142–143]. However, iPSC sources offer greater potential for manipulation and improved consistency, and recent efforts have achieved the generation of VICs from mesenchymal stem cells that had been derived from iPSCs[144]. It is also postulated that new efforts describing the derivation of second heart field progenitors from iPSCs may be used to generate VICs[145].

Rather, efforts to mimic disease-specific cell phenotypes have generally relied on exogenous administration of biochemical stimuli, such as TGF-β1 for myofibroblastic VIC activation[146–147] and mineralization media for generating osteoblastic VICs (obVICs)[148–149]. Interestingly, it is actually the generation of healthy VICs (qVICs) that has been more challenging. Even when creating disease-inspired environments, the ability to support a healthy phenotype is essential for generation of appropriate baseline or control conditions. But, when VICs are cultured on standard tissue culture polystyrene, they rapidly assume a myofibroblastic phenotype[150–151], which is problematic when expanding a cell population for use in an experiment. Only recently have researchers been able to prevent myofibroblastic differentiation or reverse this phenotype to a quiescent state in human[152] and porcine[151] VICs with the use of a fibroblastic media formulation in 2D cultures. Others have found that qVICs can also be generated by adjusting the elastic modulus of the culture substrate[153–154], which provides an alternative method for qVIC expansion in addition to informing the construction of 3D scaffolds.

Disease-inspired physical cues

The ECM of the native aortic valve leaflet exhibits a trilayered arrangement of collagen, glycosaminoglycans (GAGs), and elastin. This ECM composition and arrangement impacts both the mechanical and biochemical behavior of the valve[155]. In particular, the fibrosa layer on the aortic outflow side of the valve mainly consists of collagen and a small amount of elastin, allowing it to withstand much of the mechanical stress as the leaflets repeatedly coapt. In contrast, the ventricularis layer on the inflow side is mainly composed of elastin and a small amount of collagen, accommodating for shear stress due to blood flow. The spongiosa layer, between the fibrosa and ventricularis, is composed of GAGs and proteoglycans (PGs) that act to dampen pressure differences between the fibrosa and ventricularis. During CAVD pathogenesis, the valvular ECM undergoes progressive alterations[156]. In early-stage CAVD, the valve becomes highly enriched in GAGs and PGs[157], leading to early leaflet thickening and stiffening. As CAVD progresses, there is elastin fragmentation and increased deposition of disorganized collagen I, particularly in the spongiosa[158]. Several 2D studies have illustrated that ECM composition and substrate stiffness can modulate VIC differentiation to a myofibroblastic phenotype[159–162], thereby setting the stage to create 3D tissue-engineered models of CAVD.

One of the first detectable hallmarks of CAVD is thickening of valve leaflets due to increased deposition of PGs and GAGs[157]. Motivated by the field's poor understanding of CAVD pathogenesis, particularly during early stages of the disease, our group recently created tissue-engineered models of early CAVD[163]. Scaffolds were constructed using GelMA as the base material, to mimic the collagen foundation of the normal valve structure, and copolymerized with either healthy or diseased amounts of methacrylated hyaluronic acid

(HA) or chondroitin sulfate (CS) to mimic disease-associated GAG enrichment. Using this early CAVD platform, we demonstrated that increased GAGs decreased inflammatory cytokine production by VICs, indicating that this ECM alteration was not directly responsible for the development of an inflammatory environment that is seen in early CAVD. Rather, the function of the GAG enrichment varied by GAG type, with CS indirectly stimulating inflammatory activity. Specifically, CS enrichment enabled binding and retention of LDL and oxidized LDL (oxLDL) to the matrix; oxLDL, in turn, stimulated the production of numerous inflammatory cytokines by VICs[163]. Meanwhile, HA enrichment was not linked to inflammatory activity, but did significantly increase the production of angiogenic factors, another hallmark of CAVD. Moreover, the oxLDL retained by the CS stimulated further production of CS and HA, thereby creating a positive feedback loop. This *in vitro* disease-inspired culture platform allowed us to recreate and decipher a complex, multi-step mechanism in CAVD pathogenesis[163]. Additionally, it illustrated the benefits of using tissue-engineered systems to investigate disease etiology, such as being able to separately study individual ECM components to discover differential functions of each one in promoting disease progression.

The creation of diseased ECM conditions has also been pursued using a top-down tissue engineering approach, wherein the ECM composition of native valve leaflets was altered to produce pathological changes[164–165]. Motivated by observations that collagen I and HA promote quiescent (i.e., 'healthy') behavior by VICs, these studies examined the effects of collagen I or HA degradation on VIC behavior in *ex vivo* leaflet organ cultures. Mild enzyme treatments yielded alterations in collagen I or HA without affecting other ECM components and showed that disruption of either of these ECM components was sufficient to promote VIC differentiation to a myofibroblastic phenotype and significant tissue mineralization[164–165]. This top-down tissue engineering approach offers a unique complement to the traditional bottom-up engineered tissues; although it is not as precisely controlled as bottom-up engineering, it retains the complexity of the intact, native tissue and can thus be used to validate the physiological relevance of findings from more reductionist systems.

Others have exploited this ECM-based control of diseased VIC phenotype to construct systems that investigate disease pathogenesis. Consistent with the aforementioned findings that VIC interactions with HA are necessary to maintain a healthy phenotype, recent work found that VICs in GelMA-HA hybrid scaffolds did not undergo the spontaneous myofibroblastic differentiation seen in GelMA-only scaffolds[166]. These scaffolds provided a platform for examining how soluble factors (e.g., TGF-β1 or osteogenic medium) enacted development of disease features and mineralization and postulated a sequence by which VICs can become osteoblastic VICs[167]. A different approach to gaining mechanistic insight is the use of peptide-modified polyethylene glycol (PEG)-based hydrogels, which allow the investigation of how individual ECM-derived peptides influence VIC behavior. Modification of PEG hydrogels with peptides derived from different valve ECM proteins revealed that key markers of VIC fibrosis (e.g., myofibroblastic differentiation, ECM production) could be differentially modulated by individual peptides[168], thus providing a platform for studying how specific cell-ligand interactions may be regulating disease pathogenesis. A similar

PEG-based platform has also been implemented to study how exogenously added agents may be used to attenuate or halt fibrotic events in VIC-populated scaffolds[169].

Finally, the mechanical stiffening that occurs in CAVD has also been replicated in 3D tissue-engineered platforms to create disease-inspired conditions. Interestingly, these studies have not yielded the results that were anticipated. Despite numerous 2D studies demonstrating increased myofibroblastic differentiation on stiffer substrates[154, 161–162, 170] and stiffening being associated with native disease progression, VICs encapsulated in modified HA scaffolds[171] as well as dynamically-stiffened PEG-based hydrogels[172] exhibited decreased myofibroblastic differentiation in stiffer scaffolds. The source of this discordance remains unclear, but provides an interesting mechanobiological question to probe using these tailored microenvironments.

Future Opportunities

The pursuit to decipher CAVD pathogenesis has yielded great advancements in the design of ECM-inspired scaffold environments. It is believed that CAVD will be most treatable in its earliest stages, which is why initial development of disease-inspired environments have focused on early events[163]. However, there is still a need to elucidate subsequent steps in CAVD pathogenesis, necessitating the construction of tissue-engineered platforms that mimic the biochemical and biophysical stimuli that are characteristic of more advanced stages of the disease. Additionally, the majority of tissue-engineered CAVD platforms have focused upon examining how the disease-mimicking microenvironment impacts VIC differentiation to a myofibroblastic phenotype. However, VIC differentiation is just one part of CAVD, and CAVD is not solely driven by VICs. Infiltration of inflammatory cells such as macrophages and T lymphocytes also occurs fairly early in CAVD[157, 173], and these cells are thought to significantly contribute to CAVD progression[174–175]. The pathological ECM environment can indirectly[163] and directly[176] influence inflammatory activity, motivating future work to merge disease-inspired CAVD platforms with inflammatory cell co-culture; many of the biomaterials approaches described by researchers developing tissue-engineered tumor microenvironments may prove useful in this endeavor[176]. Additionally, evaluating immune cell behavior in these systems may aid in understanding ECM remodeling and angiogenesis throughout CAVD progression, as macrophages also contribute to these processes[177–178].

Conclusions and Recommendations

Let's Redefine "Tissue Engineering"

Other recent reviews have covered advances made toward generating tissue-engineered disease models for many other tissue types, ranging from bone to tumors[179–180]. Herein, we present highlights from the cardiovascular disease modeling, an emerging field that is poised to have dramatic impact, with over 90 million people in the U.S. alone affected by the cardiovascular diseases noted above. Yet, as noted at the start of this review, the textbook definition of "tissue engineering" still focuses on the creation of healthy constructs; it is time to revisit this definition and expand to include disease-inspired tissue engineering. The current definition fails to accurately capture the diverse ways in which the application of

tissue engineering techniques can advance both our mechanistic knowledge and discovery of therapeutic treatments for a disease.

Sex as a Biological Variable

Most researchers are now familiar with sex as a biological variable within the NIH-mandated framework of justifying the sex of vertebrate animals used in experiments. However, cellular-scale sex is often overlooked as a potentially influential factor, despite calls to account for cellular-scale sex in *in vitro* cultures[181–182]. Many cardiovascular diseases exhibit strong sexual dimorphism[182–185], and sex-separated cultures of several types of cardiovascular cells have illustrated that cellular sex can have a powerful influence on disease pathogenesis and responsiveness to extracellular stimuli[186–189]. Ideally, our tissue-engineered disease models will take into account these cellular-scale sex differences, thereby offering a potentially powerful platform for investigating sources of sexual dimorphism in cardiovascular pathologies and perhaps even the development of sex-specific treatments.

Integration Across the Cardiovascular System

Although the myocardium, aortic valves, and vessels all fall under the umbrella of cardiovascular tissues, these areas tend to be quite siloed from each other. There is surprisingly little overlap across the researchers creating tissue-engineered disease models of the myocardium, aortic valves, and vessels, with individual labs generally specializing in one of the three. However, while each of these tissues experiences a unique suite of pathologies, there are also numerous commonalities across these tissues that could be better shared and exploited to advance disease model creation. For example, heart valve researchers have been investigating myofibroblastic differentiation in ECM-inspired 3D environments for over a decade – experience that could be harnessed to inform the relatively nascent field of tissue engineering cardiac fibrosis. In a similar vein, atherosclerosis shares many common elements with aortic valve stenosis[190], but there is relatively little crosstalk across these fields to examine how the more developed mechanistic knowledge of atherosclerosis may be applied to model CAVD. Greater advancements and innovation in tissue-engineered cardiovascular disease modeling will grow from increased communication across researchers specializing in the different cardiovascular tissues.

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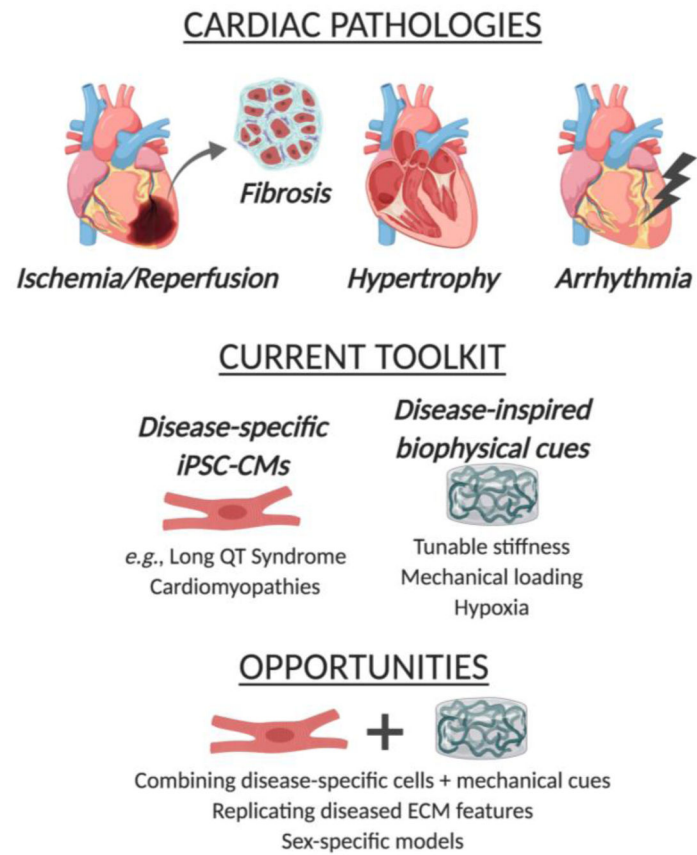
Figure 1.
Overview of disease-inspired tissue engineering approaches to mimic cardiac pathologies
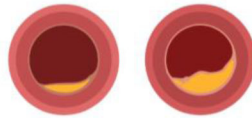
Figure 2.
Overview of disease-inspired tissue engineering approaches to mimic atherosclerosis
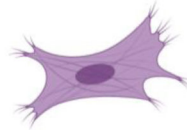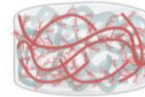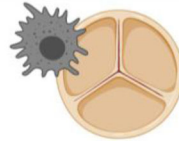
Figure 3.
Overview of disease-inspired tissue engineering approaches to mimic calcific aortic valve disease

Table 1:

Summary of approaches used to mimic cardiac diseases

CARDIAC DISORDER	Disease-specific cells	Disease-inspired physical cues
Dilated cardiomyopathy	hPSC-CMs [49]	--
Left ventricular hypertrophy	hPSC-CMs [51]	Increased afterload [67–73]
Ischemia-reperfusion injury	--	Hypoxia [61–63]
Fibrosis	CM/fibroblast ratio [53–54]	Cyclic strain [79,82]
		Biomolecules [78–81]
		ECM density [54]