Published in final edited form as:

Trends Cardiovasc Med. 2013 November ; 23(8): 282–286. doi:10.1016/j.tcm.2013.04.001.

Trends in Cardiovascular Engineering: Organizing the Human Heart

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Abstract

The regulation of heart growth through the interaction of cell types, matrix molecules, and mechanical cues is poorly understood, yet is necessary for the heart to reach its proper size and function. Using mechanical load and vascular cell co-culture in combination with a tissue engineering approach, we have recently been able to generate organized human myocardium *in vitro* and to modulate cardiomyocyte alignment, proliferation, and hypertrophy within the engineered tissue construct; further, we measured contractile function and the force-length dependence of the engineered tissue as a whole. The goal of these studies has been to characterize *in vitro* models of human cardiac development and to work towards human therapeutics using organized, vascularized, contractile human cardiac tissue. This review will touch on the current state of knowledge in this field, give an overview of the results of our own recent findings, and present areas of active investigation and new directions for future research.

Introduction

Generating engineered cardiovascular tissue has been an important goal for the field of cardiac biology for 3 distinct reasons: it presents a way to replicate and understand the normal human myocardial development, it is required for the development of human models of cardiomyopathies and other cardiovascular diseases, and finally, it has alluring potential for the creation of cell and tissue-based therapies for patients with cardiovascular disease.

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There are no potential conflicts of interest.

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Cardiac tissue engineering has its roots in both scaffold-based (Birla et al, 2007; Caspi et al, 2007; Dar et al, 2002; Fink et al, 2000; Liao et al, 2011; Madden et al, 2010; Marsano et al, 2010; Schaaf et al, 2011; Zimmermann et al, 2002a; Zimmermann et al, 2006; Zimmermann et al, 2002b) and scaffold-free approaches (Kreutziger et al, 2011; Stevens et al, 2009), often using readily available rat neonatal cardiomyocytes (Birla et al, 2007; Fink et al, 2000; Zimmermann et al, 2002a; Zimmermann et al, 2006; Zimmermann et al, 2002b) or mouse embryonic stem cell (ESC)-derived cardiomyocytes (Badie & Bursac, 2009; Liao et al, 2011). A large body of work has been created using collagen gel-based scaffolding (Eschenhagen et al, 2002; Fink et al, 2000; Naito et al, 2006; Zimmermann et al, 2002a; Zimmermann et al, 2006; Zimmermann et al, 2002b), although studies have been conducted as well with hydrogels composed of alginate (Dar et al, 2002), fibrin (Birla et al, 2007; Liao et al, 2011; Schaaf et al, 2011), or polyethylene glycol (PEG) (Benton et al, 2009; Benton et al, 2008; Shapira-Schweitzer & Seliktar, 2007). Pioneering studies by Eschenhagen and Zimmermann demonstrated that— at least in the rat neonatal cardiomyocyte-based system— stress conditioning can provide cues for alignment and maturation (Zimmermann et al, 2002b), generating force-producing functional tissue (Naito et al, 2006; Zimmermann et al, 2002a; Zimmermann et al, 2002b). These investigators also demonstrated the importance of stress conditioning in scaling engineered tissue constructs into the range necessary for *in vivo* repair of the infarcted rat heart (Eschenhagen et al, 2002; Zimmermann et al, 2002a; Zimmermann et al, 2006).

Interestingly, highly purified cardiomyocytes do not perform nearly as well on a number of parameters as those in mixed cultures of naturally associating cell types, such as neonatal cardiomyocytes and cardiac fibroblasts (Naito et al, 2006; Radisic et al, 2008). Such cross-talk has been observed by other groups, such as Caspi and Levenberg's, who generated tissues from cardiomyocytes derived from human ESCs (hESCs), human umbilical vein endothelial cells (HUVEC), and mouse embryonic fibroblasts (MEFs) as a stromal cell type to generate a hybrid tri-cell tissue (Caspi et al, 2007). They found that endothelial cell viability and proliferation were increased by the presence of the fibroblasts and that nascent endothelial structures were stabilized in the tri-cell mixture, compared to the bi-cell mixture of endothelial cells and cardiomyocytes. Others have found that using vascular explant-derived tubular networks, developed over topographic substrates and engineered to scaffold rat neonatal-derived cardiac tissue, were able to improve cardiac cell-cell junction, increase sarcomere organization, and lower excitation thresholds, compared to similar cardiac constructs generated without prevascularization (Chiu et al, 2012). Our own group demonstrated that scaffold-free engineered heart tissue containing a tri-cell mixture (cardiomyocytes, endothelial cells and stromal cells) self-assembled vessel-like networks, which became bona fide blood vessels after transplantation (Stevens et al, 2009). After transplantation into hindlimb muscle, these “pre-vascularized” constructs had more than 10-fold enhanced survival compared to constructs made from cardiomyocytes-only. More recent studies have demonstrated the feasibility of transplanting rat neonatal-derived cardiac constructs, scaffolded *in vivo* upon a syngeneic arterio-venous loop, to ectopic or cardiac sites in the same animal, while preserving contractile function of the engineered tissue (Tee et al, 2012).

The techniques available for differentiation of human cardiomyocytes from hESCs or their adult cell-derived counterparts, human induced pluripotent stem cells (hiPSCs; Takahashi et al, 2007), have markedly improved over the years (Laflamme et al, 2007; Mummery et al, 2007; Murry & Keller, 2008; Yang et al, 2008; Zhu et al, 2011). Early studies used serum to induce differentiation in cellular spheroids termed embryoid bodies, typically yielding <1% cardiomyocytes (Caspi et al, 2007). Since then a number of protocols have been published by our group (Laflamme et al, 2007) and others (Yang et al, 2008) that use chemically defined factors and give purities well over 50% cardiomyocytes without purification steps,

and protocols also have been developed for differentiation of endothelial cells as well (Lu et al, 2007; Nourse et al, 2009). Two robust protocols, in particular, have been used to generate large numbers of human cardiomyocytes from human embryonic stem cells (Laflamme et al, 2007; Yang et al, 2008) and, more recently, from hiPSCs (Figure 1A).

Current Results with Engineered Human Myocardium

We recently generated all human tri-cell based myocardium, in a collagen scaffold, using both hESC-derived and hiPSC-derived cardiomyocytes, along with human endothelial cells and utilizing human MSCs as a preferred stromal cell type (Tulloch et al, 2011). The human engineered heart tissues were cast as trabecula-like cylinders and subjected to mechanical stress by attaching the ends to fixed posts (static stress) or subjected to a 5% stretch at a rate of 60/min (1 Hz cyclic stress). Control constructs had only one end fixed to a post and developed no tension during contraction. Engineered heart tissues were grown for up to 2 weeks, and we quantified changes in cellular alignment, proliferation, and hypertrophy within the bioengineered tissue. We found that stress conditioning and the presence of human endothelium increased cardiomyocyte proliferation, that stress conditioning increased hypertrophy and cell alignment, and that the presence of vascular cells and structures did not interfere with cardiomyocyte alignment. Furthermore, we found both static stress conditioning and exogenously provided 1 Hz cyclic stress conditioning were similar in their ability to promote alignment and hypertrophy over no stress conditions, likely because the cardiomyocytes themselves within the engineered tissue are synchronously contracting, providing, in the “static stress” condition, their own endogenously produced cyclic stress as an organizing cue.

The addition of stromal cells markedly increased the number of vascular structures formed within the tissue. Interestingly, the species origin of the stromal cell was relatively unimportant, with similar results from both human mesenchymal stem cells (MSCs) and mouse embryonic fibroblasts (MEFs) on the extent of human endothelial-derived vascular structure. This nascent vasculature appeared histologically as networks of cord structures and structures with lumens throughout the bioengineered tissue *in vitro*, but the functionality of these networks for hemodynamic perfusion remained unknown from *in vitro* experiments. We therefore implanted both human ES cell-derived and iPS cell-derived cardiovascular tissue into the myocardium of immuno-compromised athymic rats and assayed for success of engraftment. After as little as one week we found healthy human myocardium engrafted in the host tissue (Figure 1B). These myocardial grafts contained vessels of both human and rodent origin. Furthermore, the presence of rat erythrocytes in the human vessels indicated anastomosis of the graft vessels to the host coronary circulation (Figure 1C).

In addition to the vascular function of this engineered tissue, we examined synchronous contraction and force production of the tissue *in vitro*. Spontaneous and synchronous contraction was observed in stress-conditioned and unconditioned tissue, however, the observed beating rate in tissue conditioned with a 1 Hz, 5% elongation strain for 4 days was very close to 1 Hz, whereas beating rate of tissue maintained in no stress conditions for the same length of time was significantly lower. We examined the potential of this engineered human cardiac tissue to produce force and to respond to increased stretch with increased contractility. We determined that the active twitch force was measureable and that the amplitude of twitch height markedly increased over stepwise increases in length starting from slack length. This force-length relationship is analogous to Starling curves generated in the intact heart (Figure 1, D – F).

Future Directions

These studies indicate that we are at a point where we can now begin examining structure-function relationships of human myocardium *in vitro*. This helps us in our pursuit of understanding the biological cues required for the developing human heart, such as mechanical conditioning effects on cell alignment and hypertrophy, or neighboring cell-type interactions important for cardiomyocyte proliferation. Furthermore, the ability to create these tissues using human iPSC-derived myocardium now allows us to begin to explore important unanswered questions of human myocardial biology and disease, such as the pathogenesis of human hypertrophic and dilated cardiomyopathies. These diseases affect large numbers of people, often present in childhood, and tend to become more severe with age, commonly resulting in sudden death in young adulthood or congestive heart failure similar to post-myocardial infarction disease (Grünig et al, 1998; Manolio et al, 1992; Maron et al, 1995; Sadoul et al, 1999). Both hypertrophic and dilated cardiomyopathies are now known to have genetic determinants, typically presenting with autosomal dominant inheritance (Paul et al, 2009).

Although the genetic basis for many cardiomyopathies has been known for more than two decades, the mechanistic link between contractile gene mutation and disease phenotype such as myocardial disarray, fibrosis, or cardiac hypertrophy has not been determined. Additionally, different mutations in the same contractile proteins can cause either hypertrophic or dilated cardiomyopathy (Paul et al, 2009). The technique of inducing pluripotency in differentiated, adult human cells now theoretically could allow us to reprogram cells from these patients back to an undifferentiated, pluripotent state, then to use our current cardiac differentiation protocols to derive mutant genotype-specific cardiomyocytes. Using these cardiomyocytes, we can apply our current tissue engineering techniques to generate heart tissue that perfectly reflects the genetics of the patient. These cardiomyopathic tissues can be compared functionally and structurally to genetically normal engineered human tissue, assessing contraction, relaxation and calcium handling, for example. This sort of hiPSC-based approach has now been performed on a single-cell phenotyping basis for the cardiomyocyte-based diseases long-QT syndrome (Moretti et al, 2010) and Leopard syndrome (Carvajal-Vergara et al, 2010) but has yet to be utilized for assessment of the origin of multicellular and tissue-based phenotypes such as myocardial disarray and fibrosis found in familial hypertrophic cardiomyopathy. It should be noted that engineered human heart tissues also conveniently lend themselves to drug screening, as has been already demonstrated on a boutique basis with a number of known arrhythmia producing compounds (Schaaf et al, 2011).

Finally, the goal of producing functional engineered myocardium for developmental and therapeutic purposes would be greatly enhanced by an ability to generate the multiple differentiated cell types found in mature myocardium from the same genetic background. This could hypothetically be done by subjecting a single line of pluripotent cells to several specific differentiation protocols, for instance by differentiating vascular endothelium using endothelial specific differentiation protocol (Nourse et al, 2009) and cardiomyocytes using a cardiogenic protocol (Laflamme et al, 2007), and then mixing the resulting cell types, in a method analogous to our previous study (Tulloch et al, 2011). However, the discovery and description of a tri-potential cardiovascular progenitor population (Yang et al, 2008), existing part-way between the pluripotent state and the differentiated cardiac and vascular fates, provides an even more attractive route to differentiated human cardiovascular tissues. These KDR and PDGFR⁺-expressing progenitor cells could theoretically be used to generate 3-dimensional tissues containing myocardium with genotype-specific vasculature, both for induction of cardiomyocyte proliferation, and for “plug and play” readiness

necessary for therapeutic grafting purposes. These tissues would be particularly useful in the study of a mutation that affects multiple cell types, e.g. a signaling pathway.

Given our current success in generating human cardiomyocytes and engineered heart tissue with roughly fetal stage maturity, an important next goal is maturing these types of tissues to more adult-like states. Human cardiomyocytes in engineered tissue, even when subjected to weeks of stress conditioning (Tulloch et al, 2011) or implanted into myocardium *in vivo* for months (Laflamme et al, 2007; Stevens et al, 2009), remain relatively immature, with only some modest increases in hypertrophic or maturation parameters. We should continue to define new methods for maturing human cardiac tissue, using not only mechanical conditioning but also assessing the potential of electrical conditioning as well as cytokine or small molecule inducement of maturation. The ability to replicate adult cardiac structure and function will teach us important lessons about normal development and provide additional tools for modeling disease and developing new treatments.

Acknowledgments

We thank Sarah Dupras and Jennifer Deem for surgical assistance and engraftment, Veronica Muskeli for fluorescent staining and confocal imaging, and Maria Razumova and F. Steven Korte for assistance with active force measurement in contractile human tissue constructs. This work was supported by National Institute of Health Grants R01HL64387, R01HL084642, P01HL094374, U01HL100405, and P01GM81619 (to CEM), T32GM726638, and T32HL007312 (to NLT) and the Poncin Scholarship (to NLT).

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Figure 1. Bioengineered cardiovascular progenitor tissue constructs mature into cardiovascular tissue

A, Undifferentiated human iPS cells of the IMR90 line were differentiated into cardiomyocytes using activin A and BMP4 and stained for the sarcomeric protein alpha-actinin (green) and the cardiomyocyte transcription factor Nkx2.5 (red). **B**, hiPSC-derived cardiac tissue graft and human fetal cardiac tissue, both stained for alpha-actinin. **C**, Engrafted cardiovascular tissue construct with human endothelium (CD31, green) perfused with host red blood cells (Ter119, red). **D**, Contractility of human cardiac tissue construct generated with cardiomyocytes, endothelial cells, and stromal cells is increased with stepwise increases in preparation length. **E**, Passive force (baseline) recorded 15 seconds after each acute stretch increased with increasing length. **F**, Active force twitch height increased with increasing length, similar to Starling curves generated from native myocardium ($R^2 = 0.99$ for initial 20% length increase).