The developing heart is exquisitely sensitive to its mechanical environment, and studies in model organisms such as chick or mouse indicate that mechanical loading is required for cardiac growth and morphogenesis.1–4 Similarly, the heart requires a rich coronary vascular supply for its normal growth, with the coronaries providing both nutrient exchange and paracrine growth signals.5–7 While model organism studies shed light onto general mechanisms of vertebrate development, they do not perfectly model human cardiac growth. For example, the human heart is more than a thousand times larger than that of the mouse and requires the myocardium to remain in the cell cycle much longer to achieve this mass. Similarly, the human heart beats nearly 10 times slower than the mouse heart, necessitating different systems for excitation, contraction, and relaxation, and these differences likely impart different responses to external mechanical stress. It therefore seems probable that the growth responses of immature human myocardium to mechanical load and vascular ingrowth will differ in some aspects from common laboratory models.

Early growth and maturation processes have been difficult to study in humans, due in part to difficulties in obtaining sufficient human cells. Cardiomyocytes in the postnatal human heart are essentially postmitotic,8 precluding their expansion in vitro. Fetal human tissue is difficult to obtain, and endogenous adult stem cells have not, to date, shown a robust ability to generate cardiomyocytes. In contrast, pluripotent stem cells such as human embryonic stem cells (hESCs)9 or induced pluripotent stem cells (iPSCs)10 can now be used to generate large-scale cultures of human cardiomyocytes at purities >50%.11–14 These cardiomyocytes resemble fetal human cardiomyocytes in terms of their cardiac-specific...
transcription factors and contractile proteins, and they exhibit excitation–contraction coupling and synchronous contraction in culture.\textsuperscript{15–18} This creates opportunities for studying human cardiac growth pathways.

Most in vitro studies of myocardial growth have relied on cardiomyocyte monocultures and 2-dimensional culture conditions. Monolayer growth on a rigid substrate is clearly not reproducing the heart’s native environment, however, and this has led us and other groups to explore tissue engineering. Tissue engineering can provide a more natural 3-D environment with appropriate stiffness, can improve intercellular organization, and can facilitate intercellular crosstalk, which modulates cardiomyocyte differentiation and growth. A variety of 3-D scaffolds have been used with nonhuman cardiomyocytes, including various synthetic polymers,\textsuperscript{19–21} as well as natural ones such as alginate,\textsuperscript{22} fibrin,\textsuperscript{23} fibronectin,\textsuperscript{24} and collagen.\textsuperscript{25,26} Type I collagen is attractive for cardiac tissue engineering because it is the major endogenous constituent of the heart’s extracellular matrix.\textsuperscript{27} Furthermore, collagen is self-polymerizing and can be uniformly seeded with cells as a liquid gel, molded into desirable shapes, and subjected to mechanical forces to promote cellular organization. It has been reported that rat neonatal cardiomyocytes cultured in a 3-D collagen matrix and subjected to cyclic stress are able to align in the direction of stress, express organized sarcomeres, and electrically couple by gap junctions.\textsuperscript{25,26}

In this study, we have generated 3-dimensional human cardiac tissue constructs using collagen type I and human ESC-derived and iPSC-derived cardiomyocytes to assess cardiomyocyte proliferation, maturation, and architecture under different conditions of stress. Additionally, we have examined coculture with vascular and stromal cells within the matrix as a means to further recreate cardiomyocyte and vascular organization within the cardiac construct.

Methods

Cell Culture
Undifferentiated human ESCs of the H7 line (James A. Thomson, University of Wisconsin—Madison)\textsuperscript{28–31} and IMR90-iPS cells (James A. Thomson, University of Wisconsin—Madison)\textsuperscript{32} were maintained as previously described and differentiated into cardiomyocytes in monolayer culture with activin-A and BMP4\textsuperscript{11}; for further cardiomyocyte enrichment, Percoll gradient centrifugation\textsuperscript{11} or suspension culture\textsuperscript{33} was used in some of the early experiments to enhance cardiomyocyte enrichment. Preparations averaged 53% human cardiomyocytes (hCMs) on the basis of β-myosin heavy chain (βMHC) immunostaining (Online Table I); this preparation is referred to as “cardiomyocyte” throughout the remainder of the manuscript. Rat neonatal cardiomyocytes (rNCs) were isolated\textsuperscript{34} from 1- to 3-day-old Fisher-344 rats; human umbilical vein endothelial cells (HUVEC, passage 4 to 8), human marrow stromal cells (MSCs, passage 2 to 4), and mouse embryonic fibroblasts (MEFs, passage 5 to 7) were maintained using standard culture conditions described within the expanded Methods section available in the Online Supplement at http://circres.ahajournals.org.

Cardiac Construct Generation and Mechanical Conditioning
Engineered heart tissue constructs were generated using collagen type I, basement membrane extract, medium, and cardiomyocytes\textsuperscript{25} (see Online Supplement for expanded Methods). Unless otherwise noted, each 100-µL construct contained 2 million cardiomyocytes; in bi- and triculture experiments, 2 million cardiomyocytes were mixed with 1 million HUVEC and 1 million MSCs or MEFs. The gel–cell mixture was cast in a 20-mm by 3-mm trough such that the ends of the construct impregnated into nylon mesh tabs attached to the deformable silicon floor of the well (tissue train, FlexCell International Corp., Hillsborough, NC), providing a means to transmit uniaxial tension to the construct (Online Figure I). To investigate the effects of uniaxial cyclic stress, constructs were subjected to a sinusoidal waveform setting generated with a FlexCell FX-4000T system beginning the day following construct generation (see Online Figure I). Static stress was achieved by allowing cells to contract the collagen gel against the fixed ends of the construct. For unstressed conditions, one end of the construct was cut free of the mesh tab. To measure DNA synthesis rates, we added 10 µmol/L BrdU to the medium for the last 24 hours before fixation.

Cardiac Engraftment
Animal procedures used in this study were reviewed and approved by the University of Washington Institutional Animal Care and Use Committee, and conform to federal guidelines for laboratory animal care. Male Sprague–Dawley athymic nude rats were anesthetized, their chests opened, and the pericardiums incised to expose the anterior surface of the heart. A construct that had been cultured for 1 week was sutured directly onto the epicardium.

Immunostaining, Light, Fluorescent, and Electron Microscopy, Quantitative PCR
Immunohistochemistry, light microscopy, confocal microscopy, transmission electron microscopy, and quantitative PCR were performed using standard procedures described within the expanded Methods section. Circularly polarized light microscopy was conducted for collagen matrix visualization with picrosirius red, and circular polarization was adjusted to obtain a deep uniform dark background. Images were collected with a Photometrics Coolscan camera in color mode and METAMORPH software. Illumination intensity, condenser aperture, and exposure time were maintained uniformly for all images.
Histological and Statistical Analysis

To quantify cardiomyocyte axis alignment within constructs, we analyzed 100× micrographs of slides stained for desmin (rNC constructs) or βMHC (hCM constructs) using a custom fiber orientation analysis program (developed by Dr. Michael Regnier’s laboratory, University of Washington). Briefly, this Matlab program divides a user-defined region of interest into small subimages and, by edge detection, obtains the major axis angle of each subimage. For the whole image, an average angle and the angle dispersion (standard deviation of angles of cell edges) is determined. Cellular alignment is quantified by magnitude of angle dispersion, such that low angle dispersion (low standard deviation of cell axis angles) indicates a high degree of alignment, which is graphed as the inverse of angle dispersion, expressed as a percentage of the mean (ie, the reciprocal of the standard deviation). Adult rat myocardium was used as a positive control; sections were stained for desmin, and quantification was performed only on regions with linear, longitudinal fiber orientation.

To assess cardiomyocyte proliferation, we counted in a blinded fashion slides double stained for BrdU and βMHC. Cardiomyocyte area was assessed by quantifying βMHC-positive area within each construct as described previously, normalizing to the cardiomyocyte nuclear number. To quantify vascular-like structure within the constructs, slides were analyzed in a blinded fashion for multicellular CD31+ structures scored as either cord-like structures or structures with lumens and normalized per area counted, over 4 fields per section. Unless otherwise noted, n = 3 to 6 for each experiment. Error bars represent standard error of the mean (SEM); significance was determined using single-factor analysis of variance (ANOVA) followed by Student t test with 95% or greater confidence level.

Results

Rat Neonatal Cardiomyocyte Constructs

To establish a baseline against which to compare our human constructs, we first optimized production of engineered heart tissue using primary rat neonatal cardiomyocytes (rNC). These studies determined that 1.10 to 1.25 mg/mL of type I collagen was optimal to support cardiomyocyte alignment and proliferation. Under static or cyclic stress, cardiomyocytes developed elongated, aligned structures with a high degree of alignment compared to constructs under no stress (Fig. 1A). Coculture of endothelial cells did not inhibit cardiomyocyte alignment (Fig. 1B).

Cardiomyocyte alignment developed between 1 and 4 days of static stress conditioning (Fig. 1C). Transmission electron microscopy revealed elongated cardiomyocytes within the collagen matrix, with nuclei (nuc), numerous mitochondria (m), and contractile filaments (Fig. 1D). Higher magnification revealed mitochondria among myofibrils (myof) with scattered nascent Z disks (arrow).
Collagen is required to prevent construct failure under stress and that a density of at least 2 million cells per 100 µL of construct is necessary for adequate survival of cardiomyocytes within the collagen matrix (data not shown). Constructs generated by this process have dimensions of 20 mm in length and 0.5 mm in thickness. As the cells remodel and contract the collagen gel, nylon tabs hold the construct under static tension or allow the application of controlled cyclic stress (see Online Figure I).

Cardiomyocyte constructs subjected to uniaxial static stress or cyclic stress conditioning (4 days of 1 Hz, 5% elongation) developed cell alignment not observed in 2-D cell culture (Online Figure II, A) or unstressed 3-D gels (Figure 1A). Intercellular alignment was quantified from the reciprocal of the cell axis angle dispersion, for which a low standard deviation of angles indicates a high degree of alignment (Figure 1B). We found that, in comparison to no stress (alignment value of 2.68), cardiomyocyte alignment increased by 2-fold with either cyclic or static stress conditions (alignment values of 4.86 and 5.30, respectively; \( P < 0.001 \) for each versus no stress). However, no significant difference was found between static and cyclic stress (\( P = 0.65 \)). While improved, this degree of cell alignment did not reach the level observed in longitudinal fibers within adult rat heart (alignment value of 9.08). Cardiomyocyte alignment within the collagen matrix developed between 1 day and 4 days of stress conditioning (Figure 1C); further increases in alignment were not observed between 4 and 7 days (data not shown). Addition of endothelial cells resulted in the formation of cord structures within the constructs (Online Figure IV, A), but endothelial cells did not impact cardiomyocyte alignment in either unstressed or stress-conditioned constructs (Figure 1B).

In addition to intercellular alignment within the construct, cells within this 3-D matrix also demonstrated signs of maturation, such as binucleation (Online Figure II, C) and organized sarcomeric banding (Online Figure II, D and E) perpendicular to the direction of stress, which is similar to native cardiac tissue. Some cardiomyocytes also continued to undergo DNA replication (by BrdU incorporation, Figure 1A and 1C) and nuclear division (Online Figure II, B). After several days in culture, these constructs beat spontaneously and synchronously (Online Video I). By transmission electron microscopy, rat neonatal cardiomyocytes within constructs demonstrated internal organization with active myofibrillogenesis, occasional Z lines, numerous mitochondria, and nascent intercalated disks containing desmosomes, intermediate junctions, and occasional gap junctions (Figure 1D).

Generating Human Cardiac Tissue Constructs
Cardiomyocytes derived from pluripotent human stem cells were used to make 3-D tissue constructs using conditions optimized with rNCs (Figure 2). When cultured under static stress, human tissue constructs generated from either ESC-derived cardiomyocytes or iPSC-derived cardiomyocytes began to beat synchronously and spontaneously between 1 and 4 days, indicating that these cells were capable of electromechanical coupling within the collagen matrix (Online Videos II and III, respectively). Immunostaining showed that cells throughout the construct strongly expressed the cardiac contractile proteins β-myosin heavy chain (βMHC) (Figure 2A) and α-actinin (Figure 2B and 2C), as well as cardiac troponin T (cTnT), and the cardiomyocyte transcription factor Nkx2.5 (not shown). The human cardiomyocytes underwent frequent DNA synthesis, with 15% to 45% of nuclei incorporating BrdU after an overnight pulse (Figure 2A). Furthermore, these cells demonstrated sarcomeric banding of the contractile apparatus by α-actinin immunostaining (Figure 2B). Constructs generated using cardiomyocytes differentiated from human ESC-derived cardiomyocytes stained strongly for the cardiomyocyte marker βMHC (red) and the proliferation marker BrdU (brown). High magnification (right): human cardiomyocytes were observed undergoing nuclear division within the collagen matrix. B, Constructs generated from ESC-derived cardiomyocytes subjected to static stress conditioning (bottom) or no stress conditioning (top) stained strongly for the sarcomeric protein α-actinin (green). C, Constructs generated from iPSC-derived cardiomyocytes also stained strongly for α-actinin (red). As in B, the construct edges and vector of stress conditioning are horizontal. These constructs appeared indistinguishable from the ESC-derived constructs of similar conditioning. In both cases, myofibrils appear more aligned in the static stress-conditioned constructs.
Human Cardiomyocyte Alignment With Stress

To test whether exogenous stress promotes human cardiomyocyte self-organization, we generated human cardiac constructs both with and without endothelial cells and subjected them to no stress, static stress, and 1 Hz cyclic stress conditioning for 4 days. Constructs were immunostained for βMHC (Figure 3A), and cardiomyocyte alignment was quantified as described above (Figure 3B). Unstressed human constructs did not have significantly different cell alignment than did 2-dimensional cell culture (alignment values of 1.96 versus 2.05, respectively). However, static and cyclic stress conditioning strongly increased cell alignment (4.09 for each, P<0.005 versus unstressed conditions). As with rat constructs, there were no significant differences in human cardiomyocyte alignment in static versus cyclic stress (P=1.00).

Matrix Structure Organization With Stress

We also investigated the effect of stress conditioning on the organization of the extracellular matrix within the bioengineered constructs. To analyze the collagen fiber architecture within the cardiac constructs, we combined picrosirius red staining (to determine total collagen) and polarized light (to assess the presence of organized collagen fibers).\(^{36-38}\) Circularly polarized light was utilized to avoid the orientation dependence of birefringence associated with linearly polarized light.\(^{36}\) In the absence of cells, no large collagen fibers were seen following static stress conditioning (Online Figure III, A). In human cardiac constructs, we observed large collagen fiber bundles by yellow birefringence (Figure 4A). The collagen fiber bundles were disarrayed in unstressed constructs, but fiber architecture was much more orderly following static and cyclic stress conditioning (Figure 4A). Rat neonatal cardiomyocyte constructs in the same conditions showed even more distinct collagen fiber organization (Online Figure III, A). The absence of large collagen fibers in cell-free collagen constructs indicated that these structures require cells for their synthesis and alignment within the tissue construct. Quantitative analyses (Figure 4B) showed a 2-fold increase in collagen alignment when comparing no-stress conditions (alignment value of 2.42) to static and cyclic stress conditioning (5.10 and 4.56, respectively, P<0.005).

Thus, in the context of bioengineered cardiac tissue, stress facilitates the cell-driven development of matrix architecture.
as well as self-organization of the cardiomyocytes within the construct.

Effects of Mechanical Load on Human Cardiomyocyte Proliferation and Hypertrophy

Next, we tested the hypothesis that human cardiac constructs would proliferate in response to mechanical stress. Myocardial constructs were pulsed with BrdU for 24 hours before fixation, and cardiomyocyte DNA synthesis rates were determined by measuring βMHC and BrdU double labeling (Figure 5A and 5B). Absolute BrdU incorporation rates ranged from 15% to 45%, indicating high baseline rates of proliferation. Because of run-to-run variation in baseline BrdU incorporation rates, however,39 measurements were normalized to the basal DNA synthesis rates in static stress conditions. Consistent with our hypothesis, both static and cyclic stress increased human cardiomyocyte BrdU incorporation within the ESC-derived construct by 15% to 21% over conditions of no stress (85%, 100%, and 106% for no stress, static, and cyclic, respectively; Figure 5A). The difference between no stress and cyclic stress was highly significant (*P<0.01 versus hCM only, no stress; #P<0.005 versus hCM+Endo, no stress).

Cyclic stress conditioning also increased the spontaneous beating rate of iPSC-derived constructs, from 0.71 Hz to 0.83 Hz to 0.95 Hz, respectively, for no stress, 1 day cyclic stress, 4 days cyclic stress (P<0.05 in comparison with no stress; Figure 5D), indicating changes in electromechanical structure of the cardiomyocytes within the construct in response to conditioning. To assess whether mechanical stress promoted cardiomyocyte hypertrophy, we quantified the βMHC+ area within each ESC-derived construct and divided this by total cardiomyocyte nuclear number (Figure 5C). Cardiomyocyte area within the construct markedly increased with both static and cyclic stress, more than doubling in response to cyclic stress from 62% to 136% of static stress-normalized area (*P<0.05). We assessed the effect of stress conditioning on hypertrophy in iPSC-derived cardiac constructs by quantitative RT-PCR (Figure 5E). The data showed that transcripts for βMHC, cTnT, the L-type Ca2+ channel, the ryanodine receptor, atrial and B-type natriuretic factors, and SERCA2A increased by 2- to 6-fold in response to cyclic stress conditioning. Thus, mechanical stress induces both DNA synthesis and hypertrophy in human myocardial tissue constructs.

Prevascularizing Human Cardiomyocyte Constructs

Myocardium is a highly vascular tissue, and the coronary circulation supports myocardial development through both perfusion and paracrine signaling pathways.5,6,40 We therefore explored the effects of adding a vascular cell network to our engineered human heart tissues. In constructs generated from preparations of hESC-derived cardiomyocytes, endothelial cells are occasionally detected by CD31 immunostaining, but they very rarely form any sort of multicellular structure (data not shown). However, when exogenous endothelial cells are added to cardiac constructs, both cord structures and structures containing lumens are observed (Figure 6A). The total number of endothelial structures increased markedly when support cells were added into the constructs in triculture with human cardiomyocytes and endothelial cells (Figure 6B), approximately doubling in the presence of either mouse embryonic fibroblasts (MEFs) or human marrow stromal cells (MSCs) (Figure 6C). In comparison with cardiomyocyte + endothelial cell constructs, the addition of MEFs...
increased the prevalence of endothelial cord structures by 10-fold, whereas the presence of human adult MSCs increased these structures by 8-fold (Figure 6C) \(^{10}\) (P<0.05 for MEFs, P<0.01 for MSCs). The number of luminal structures did not significantly change with either stromal cell population (P=0.76 for MEFs, P=0.56 for MSCs). Similar results were found when endothelial and stromal cells were added to neonatal rat cardiomyocyte constructs (Online Figure IV, B). Thus, in both human and rodent cardiac tissue constructs, the addition of a stromal cell population strongly augmented endothelial structure formation.

Despite formation of relatively large vascular-like structures, addition of endothelial cells had no effect on cardiomyocyte or matrix alignment in constructs, regardless of stress conditioning (Figures 3 and 4). Similarly, cardiomyocyte hypertrophy, although increased more than 2-fold with cyclic stress, did not change with endothelial cell coculture (Figure 5B). Interestingly, addition of endothelial cells increased hESC-derived cardiomyocyte DNA synthesis rates in all stress conditions (up to 19%, P=0.01), implying that endothelial-derived mitogens were stimulating cardiomyocyte proliferation (Figure 5A). The mitogenic properties of coculture with endothelial cells was also seen in human iPSC-derived cardiomyocyte constructs (Figure 5B). Cyclic stress and coculture together increased cardiomyocyte DNA synthesis by 35% (P=0.004). Thus, mitogenic pathways are induced in human cardiomyocytes by both endothelium and cyclic stress.

**Frank–Starling Relation in Bioengineered Human Cardiac Tissue**

We next studied contractile function in bioengineered human cardiac tissue that had been subjected to 3 weeks of static stress conditioning. The hESC-derived constructs were mounted between a force transducer and a post whose position was controlled by a motor, thereby varying resting tension. A representative trace and analysis is presented in Figure 7 (n=9). The tissue was subjected to a series of 4% length increases (Figure 7A) while continuously measuring force (Figure 7B). Active force transients (twitch force) were apparent starting from just above slack length and were increased in amplitude at higher magnitude stretches (Figure 7B, left and right insets, respectively). Passive force (preload) recorded 15 seconds after each stretch was normalized to

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**Figure 5. Stress and coculture modulate human cardiomyocyte proliferation and hypertrophy.** A, Cardiomyocyte DNA synthesis was measured by βMHC and BrdU double staining in hESC-derived cardiac constructs. Data are given as fold over basal rate in the hCM-only, static-stress condition. Static and cyclic stress markedly increased hESC-derived cardiomyocyte BrdU incorporation over no stress (15% and 21% increases, respectively) as did the addition of endothelial cells (19%). B, Cardiomyocyte DNA synthesis within a single experiment (n=4 per group) of iPSC-derived cardiac constructs. Coculture and cyclic stress conditioning both significantly increase iPSC-derived cardiomyocyte DNA synthesis. C, Cardiomyocyte hypertrophy within hESC-derived cardiac constructs was assessed by βMHC immunostaining, measuring stained area within each construct, and normalizing to the number of cardiomyocyte nuclei. Because of variability in input purity, data are given as fold over the hCM-only, static-stress condition. Cardiomyocyte area increased 2.2-fold in response to cyclic-stress conditioning. D, Spontaneous beating frequency with stress conditioning in iPSC-derived cardiac constructs. The following experimental conditions were used: no stress for 4 days, 1 Hz, 5% elongation cyclic stress for 1 day followed by 3 days of no stress, or 4 days under the cyclic-stress condition. Afterward, beating rate was visually assessed and a time-dependent effect due to stress conditioning was observed. E, Quantitative RT-PCR was performed on iPSC-derived cardiac constructs conditioned with no stress or cyclic stress for 4 days to determine the mRNA transcript levels of the following contractile and hypertrophy related genes: MYH7 (βMHC), TNNT2 (cTnT), NPPA (ANP), NPPB (BNP), CACNA1C (L-type calcium channel subunit 1Cα), RYR2 (sarcoplasmic calcium channel/ryanodine receptor), and ATP2A2 (SERCA2, sarcoplasmic calcium transporter). Significance was determined by single-factor analysis of variance followed by Student t test in comparison with the hCM-only, no-stress condition; error bars represent standard error.
cross-sectional area and graphed against change in length to determine the Young’s Modulus (Figure 7C). This yielded a curvilinear relationship with modest increases in tension at stretches up to 45% over slack length, and higher increases thereafter. Contractility was assessed by plotting the amplitude of active force against change in construct length (Figure 7D). This yielded a linear relationship, in which active force increased 8-fold over the first 60% of stretch over slack length and plateaued thereafter. Linear regression over the first 25% of this curve (the most physiologically relevant) yielded an \( R^2 \) value of 0.99. This active force–length relationship is analogous to Frank–Starling curves in the intact heart and indicates that our engineered tissue recapitulates a fundamental property of native cardiac muscle.

**Cardiac Engraftment**

Finally, we investigated the viability of human bioengineered cardiac tissue to engraft in the hearts of athymic rats. Constructs derived from hCM-only and human triculture constructs containing cardiomyocytes, endothelial cells, and MSCs were conditioned by static stress and engrafted onto the epicardial surfaces of uninjured athymic rat hearts (n=5 for hCM only; n=4 for Tricell; n=1 for iPSC-derived hCM only). Hearts were harvested 1 week later and studied histologically. When a subset of 3 hearts was probed for the presence of human cells by in situ hybridization for human-specific centromeric repeats, the human cardiac constructs were readily identified on the epicardial surface in each heart (data not shown). Furthermore, grafts with GFP-expressing MSCs were visible by fluorescence on the surface of the heart (Online Figure IV, D). The implanted constructs expressed the human cardiac marker \( \alpha-MHC \) in all 10 engrafted animals (Figure 8A, 8C, and 8E) as well as the cardiac transcription factor Nkx2.5 (data not shown). These human cardiomyocytes were often in close proximity to the host myocardium (Figure 8A), and there was no foreign body reaction to the implanted construct. Engrafted human ESC-derived and iPSC-derived cardiac constructs both showed sarcomeric banding, indicative of intracellular contractile organization, as well as similar cell-to-cell alignment, indicating intercellular organization (Figure 8B and 8D, respectively). Furthermore, a microvascular
network was present throughout the constructs, and these blood vessels were filled with host erythrocytes (Figure 7E and 7F). A subset of the host-perfused vessels stained for human-specific CD31 (Figure 7F) within the human triculture construct grafts (4 out of 4 grafts) but not in the hCM grafts (rare vessels in 1 out of 5 grafts, likely from “contaminating” endothelial cells) indicated that the endothelial network that had self-organized in vitro within the prevascularized construct could form bona fide blood vessels in vivo.

**Discussion**

During development, the heart responds to biomechanical cues as well as signals from resident cells and matrix to determine overall cardiac size and architecture. Cellular and mechanical factors are also important determinants of the modification that occurs when the postnatal heart has to maintain performance while adapting to physiological change such as growth, pregnancy, exercise conditioning, or injury. Although animal models have been critical in our understanding of the factors that modulate cardiomyocyte development and homeostasis, as discussed above, these have limitations due to species-intrinsic differences in cardiac size, heart rate, and so on. To our knowledge, this is the first study to examine the effects of mechanical stress and vascularization on cardiomyocyte architecture, proliferation, and maturation in human cardiac tissue. Our major findings are (1) both static and cyclic stress conditioning promote human cardiomyocyte alignment and hypertrophy within a collagen 3-D matrix, (2) mechanical stress and endothelial cell coculture both induce human cardiomyocyte proliferation, and (3) stromal cells of various types, including human MSCs, increase vascular network formation within bioengineered human cardiac tissue. Furthermore, these optimized human cardiac tissue constructs (4) can be generated from human iPSC-derived cells and (5) can generate active forces responsive to changes in construct length, analogous to Starling curves generated in the intact heart.

Multiple studies have shown that interactions between cardiomyocytes and endothelial cells are necessary for normal rodent and avian myocardial development. For instance, endothelial-specific knockout experiments using Tie2-cre mice have been used to identify a number of signaling molecules within the endothelium, including Tie2 itself, neurofibromin, and EphrinB2, which modulate myocardial development and trabecular architecture. In fact, coculture of human endothelial cells and signaling through EphB4 receptor tyrosine kinase has been determined to be critical for high-yield generation of cardiomyocytes from EphB4-null mouse ESCs, although it is not yet clear whether this effect is due to a direct influence on differentiation or on cardiomyocyte survival or proliferation. Similarly, production of neuregulin by endocardial cells is essential, as well as myocyte-specific expression of its cognate receptor ErbB4, for formation of ventricular trabeculae. It is likely, but not yet shown, that many of these endothelial–cardiomyocyte interactions directly translate to the human myocardium.

Some progress has been made toward creating in vitro models of vascularized skeletal muscle and even human cardiovascular tissue with ESC-derived cardiomyocytes. For example, Caspi et al seeded hESC-derived cardiomyocytes and vascular cells onto poly-L-lactic acid scaffolds, and they also observed that endothelial cells enhance cardiomyocyte
proliferation. Because synthetic scaffolds may not allow normal cellular remodeling and are known to elicit foreign body reactions that may limit graft–host integration, our group has been exploring scaffold-free or natural matrices for tissue engineering such as collagen type I and type III, the major components of the primate myocardial matrix. We found that scaffold-free human myocardial constructs, comprising only cardiomyocytes and the matrix they secrete, survive poorly after transplantation, but the addition of endothelial and stromal cells markedly enhances vascularization and survival. Furthermore, when implanted onto the heart, they showed no foreign body reaction at the graft–host interface.

While scaffold-free approaches show promise, the cardiomyocytes in these constructs are developmentally immature, and their assembly in rotary culture via cell–cell adhesion makes it difficult to control tissue architecture. The desire to control cardiomyocyte hypertrophy and fiber alignment prior to engraftment led us to test collagen hydrogels as vehicles for tissue engineering. Previous studies reported that cyclic stress induces cardiomyocyte alignment in rat neonatal cardiomyocytes within a collagen gel. No reports, to our knowledge, have previously demonstrated stress-induced development of alignment in human cardiomyocytes or of alignment of myocardial matrix with stress conditioning. Because isolated type I and III fibrillar collagens can polymerize into triple helical fibrils in vitro, it was long thought that the process of fiber polymerization in tissue occurred via self-assembly. However, it has more recently been established that cell surface integrins play a role in facilitating the assembly of type I and type III collagen fibers in MEF cultures, and that this assembly is independent of collagen synthesis. The data presented here suggest that cell-directed organization of collagen matrix occurs in cardiomyocyte cultures as well, and that stress increases the ability of cells to organize these fibers and align them with vectors of external force.

It has been postulated that mechanical stress modulates architecture of the developing, mature, and injured myocardium. In vitro, the establishment of cardiomyocyte cellular organization due to stress has been studied thus far in rat neonatal cardiomyocytes. Optimal sarcomere length in rNCs is reestablished during sudden longitudinal static stress and involves PKC phosphorylation, whereas detection of transverse static stress is accomplished through FAK and ERK1/2 phosphorylation. Sensing directionality of mechano-
ical stress has been hypothesized to be a mechanism by which cardiomycytes may add sarcomeres in series in response to diastolic stretch or in parallel due to increased systolic stress, leading to reestablishment of homeostasis and preservation of cardiac performance under changing physiological conditions. Here we demonstrated that human cardiomycytes within unstressed constructs show no more alignment than do cells in 2-dimensional cell culture. Conversely, static and cyclic stress conditioning both promoted cell and matrix alignment much closer to that measured in native cardiac muscle (Figures 1, 3, and 4). And although cyclic stress conditioning promoted more cardiomycyte hypertrophy within the construct, we were surprised that it conferred no additional benefit to cardiomycyte alignment over static stress conditioning. It is possible that further increased exogenous cyclic stress—greater amplitude, faster rate, or longer culture conditions than used here—may further improve alignment. However, our pilot studies showed that conditions of 10% elongation caused mechanical failure in some constructs, most often at the points of attachment to the nylon tabs, and conditions of 5% cyclic stress up to 7 days did not show an increase in alignment over 7-day static stress (data not shown). Another possible explanation for comparable alignment in static and cyclic stress conditions is the spontaneous contractions observed in both situations. The cardiomycytes may induce their own cyclic stress conditioning under "static" external loading conditions.

One of the only other reports on human myocardial tissue engineering demonstrated an increase in both endothelial structure formation and proliferation due to coculture with stromal cells. This study found a similar significant increase in vascular structure formation; however, we observed that the presence of stromal cells actually decreases endothelial cell proliferation within the human constructs (Online Figure IV, C). Several differences exist between the 2 studies, including the scaffold material (poly l-lactic acid versus collagen), the metric used for measurement of proliferation (Ki67 versus BrdU incorporation), and the stromal cell type, ie, we investigated both MEFs and human MSCs. The latter are clinically more relevant because of their human origin, smooth muscle differentiation capabilities, and putative cardiac function benefit on injection following injury. We found similar results with both stromal cell types. Furthermore, the time point of the proliferation measurement in the study by Caspi et al is not clearly delineated, although other analyses within that study have endpoints from 1 hour to 7 days. In our study, we measured proliferation after 5 days in culture with a 1-day pulse of BrdU. At 5 days, significant vascular structure development was evident. Endothelial cells within the cardiac construct may be involved in a high degree of proliferation followed by apoptosis as part of normal vascular pruning, and the presence of stromal cells may play a role stabilizing the nascent endothelial cell structures.

In summary, we developed a collagen-based, bioengineered human cardiac tissue construct in a self-organizing coculture with endothelial and stromal cells and demonstrated the development of cardiomycyte alignment, proliferation, and hypertrophy due to mechanical stress and coculture. Furthermore, we determined that these constructs engraft into the myocardium with cardiac and endothelial contributions of human origin, and ascertained that engrafted constructs are perfused through connections to host vasculature within a week. These cardiac constructs may provide additional engraftment benefit over cell injection therapies for infarct repair because of positioning of a cardiac repair construct over and across an infarct scar rather than within it. This differential placement may increase therapeutic performance due to (1) separation of the graft from the inflammatory infarct environment and (2) positioning that may promote electric coupling with intact myocardium on both sides of the infarct. These studies validate mechanical conditioning and vascular and stromal coculture as practical and constructive methods of affecting human cardiomycyte organization, replication, and maturation in bioengineered human cardiac tissue.

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Growth of Engineered Human Myocardium

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Novelty and Significance

What Is Known?

- Cardiomyocytes can be cultured in monolayer or 3-dimensional matrices of synthetic or natural origin; interactions with endothelial cells are necessary for normal myocardial development.
- Human cardiomyocytes can be generated from human embryonic stem cells or reprogrammed “induced pluripotent stem cells” derived from differentiated tissues.
- Rat neonatal cardiomyocytes cultured in 3-dimensional collagen matrix respond to mechanical strain with organization and hypertrophy.

What New Information Does This Article Contribute?

- In a 3-dimensional bioengineered cardiac tissue generated with a type I collagen scaffold, human cardiomyocyte proliferation and vascular structure formation is promoted in vitro by coculture with vascular and stromal cell types.
- Mechanical stress conditioning promotes human cardiomyocyte proliferation, intercellular organization, matrix/scaffold organization, and cellular hypertrophy.
- This bioengineered human cardiac muscle is spontaneously contractile, responds appropriately to stretch with increased force of contraction, and can engraft onto the heart, where it is perfused through anastomosis of engineered human vascular networks to the host coronary circulation.

The regulation of heart growth by mechanical stresses and vascularization is poorly understood, yet both these factors are necessary for the heart to reach its proper size, shape, and architecture. We therefore investigated the effects of human vascular cells and mechanical stress conditioning of human myocardium derived in vitro using tissue engineering approaches. We found that vascular cells increased proliferation of cardiomyocytes in 3-dimensional bioengineered tissue generated using a type I collagen scaffold. Mechanical stress conditioning induced intercellular organization, matrix organization, cardiomyocyte proliferation, and hypertrophy. Furthermore, we discerned that addition of a third, stromal cell type allowed more complex vascular structures to form within the human bioengineered cardiac tissue. Moreover, this engineered human tissue was able to contract spontaneously and synchronously and respond with increased force of contraction when stretched, demonstrating force/length relationships analogous to Starling curves generated in the intact heart. Finally, we determined that these bioengineered cardiac tissue constructs can be engrafted onto the heart in vivo and are quickly perfused by host coronary circulation through connection to the pre-existing human vascular network. This work has implications for both models of human cardiac development as well as human therapeutics using vascularized human cardiac tissue.
Growth of Engineered Human Myocardium With Mechanical Loading and Vascular Coculture
Nathaniel L. Tulloch, Veronica Muskheli, Maria V. Razumova, F. Steven Korte, Michael Regnier, Kip D. Hauch, Lil Pabon, Hans Reinecke and Charles E. Murry

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Expanded Methods

Cell Culture
Undifferentiated human ESCs of the H7 line (James A. Thomson, U. Wisconsin-Madison) were maintained as described previously. IMR90-iPS cells (James A. Thomson, U. Wisconsin-Madison) were maintained similarly. Pluripotent cells underwent directed differentiation into cardiomyocytes in monolayer culture with activin-A and BMP4 as described previously. Briefly, human ESCs were grown to confluence in MEF-conditioned medium supplemented with 4 ng/mL basic FGF (Peprotech) on Matrigel (BD Biosciences). At confluence, the cells were switched into a RPMI medium and B27 supplement (Invitrogen) and 100 ng/mL activin-A (R&D) was added for 1 day followed by 10 ng/mL BMP4 (R&D) for 4 days. Cells were cultured for an additional 15 days, after which they were spontaneously contracting and expressing cardiac markers. For further cardiomyocyte enrichment, Percoll gradient centrifugation or suspension culture (where cardiomyocytes self-aggregate and non-myocytes die off) was used. Percoll was followed by at least 1 day each of recovery in suspension culture and replating on gelatin, both in Human Embryoid Body medium (80% Knockout-DMEM, Invitrogen; 20% fetal bovine serum, HyClone; 1 mmol/L L-glutamine, Gibco; 0.1 mmol/L β-mercaptoethanol; 1% nonessential amino acid stock (Gibco); penicillin G, 100 U/mL, Cellgro; and streptomycin, 100 mg/mL, Cellgro) before cells were used for construct generation. Preparations averaged 52% cardiomyocytes based on β-myosin heavy chain (βMHC) immunostaining of the input population at the time of construct generation. This preparation is referred to as “cardiomyocytes” through the remainder of the manuscript.

Rat cardiomyocytes were isolated from 1-3 day old Fisher-344 neonates (Charles River) as previously described and maintained on gelatin for a period of no more than 4 days before use in Rat NeoCardio medium (DMEM; Invitrogen, and M199; Sigma, in a 4:1 ratio supplemented with 10% horse serum, 5% fetal bovine serum, penicillin G; 100 U/mL, and streptomycin; 100 mg/mL). Human umbilical vein endothelial cells (HUVEC, Lonza) of passage 4-8 were maintained on gelatin in EBM2 medium (Lonza). Human marrow stromal cells (MSCs) of passage 2-4 were maintained on gelatin (Sigma) in MSCGM (Lonza), and mouse embryonic fibroblasts (MEFs, Lonza) of passage 5-7 were maintained in DMEM (Invitrogen) with 10% fetal bovine serum, penicillin G (100 U/mL), and streptomycin (100 mg/mL). For the construct preparation, cells were enzymatically dispersed using TrypLE (Invitrogen).

Generation of Cardiac Constructs
Engineered heart tissue constructs were generated using collagen type I (final concentration 1.25 mg/mL, neutralized with NaOH; Gibco), 11% mouse basement membrane extract (Geltrex, Invitrogen), and 57% Human Embryoid Body Medium or, when appropriate, Rat NeoCardio medium, mixed together on ice with cells gently added. Unless otherwise noted, each 100 µL suspension contained 2 million cardiomyocytes; in bi- and tri-culture experiments, 2 million cardiomyocytes were mixed with 1 million HUVEC and 1 million MSCs or MEFs.

A FlexCell system was used for tissue engineering and mechanical conditioning. The gel-cell mixture was pipetted into a trough of 20 mm by 3 mm formed in tissue train plates mounted over trough loader posts on a loading station set in a Bioflex baseplate under vacuum (Flexcell International Corp.). Each end of the construct was impregnated into a nylon mesh tab attached to the deformable silicon floor of each well, providing a means to transmit tension to the construct. After one hour at room temperature, the constructs were placed in medium (for
human and rNC constructs, RPMI medium with B27 supplement and Rat NeoCardio medium, respectively).

To investigate the effects of cyclic stress conditioning, constructs in tissue train plates were placed onto an Arctangle loading station connected to an FX-4000T system (Flexcell International Corp) on the day following construct generation. Uniaxial cyclic strain was applied at 1 Hz, 5% elongation for 4 days using a square sine waveform setting. Static stress was achieved by allowing cells to contract the collagen gel against the fixed ends of the construct. For culture without stress conditioning, one end of the construct was cut free of the mesh tab. For electron microscopic analysis, constructs were conditioned in static stress or no stress for 7 days. For α-actinin immunohistochemistry of iPSC-derived constructs, static stress conditioning or no stress conditioning was applied for 14 days, and conditioning for active and passive force measurements took place for 21 days before analysis. All other experiments were conducted with 4 days of conditioning. To measure DNA synthesis rates, 10 μmol/L BrdU was added to the medium for the last 24 hours before fixation.

Cardiac Engraftment
Animal procedures used in this study were reviewed and approved by the University of Washington Institutional Animal Care and Use Committee, and conform to federal guidelines for laboratory animal care. Male Sprague Dawley athymic nude rats (Charles River, n=10) were anesthetized with 5% inhaled isofluorane (Terrell) with an O₂ flow rate of 1.5-2 liters/minute, intubated, and mechanically ventilated with 2.5% isofluorane. After intubation, each animal received a subcutaneous injection of buprenorphine hydrochloride (0.05-0.1 mg/kg, Hospira). The chest was opened and the pericardium incised to expose the anterior surface of the heart. Following 1 week of in vitro culture, one construct was sutured directly onto the epicardium (n=5 for ESC-derived hCM-only, n=4 for ESC derived tri-culture, n=1 for iPSC-derived hCM-only). One week following engraftment, animals were anesthetized with isofluorane and sacrificed with a 1.5 ml intraperitoneal injection of Beuthanasia-D (390mg/mL pentobarbital sodium, 50mg/mL phenytoin sodium, Schering-Plough Animal Health) and the hearts were removed for fixation overnight in Methyl Carnoy’s fixative or 4% paraformaldehyde, followed by paraffin embedding and sectioning.

Mechanical Measurements
Constructs were carefully dissected away from the nylon mesh tab and placed in 30°C Tyrode’s buffer (containing, in mmol/L: CaCl₂ 1.8, MgCl₂ 1.0, KCl 5.4, NaCl 140, HEPES 10, NaH₂PO₄ 0.33, glucose 5.5; pH 7.4). Constructs were further dissected into 1-2 mm long sections and then suspended on stainless steel hooks attached to a force transducer (Aurora Scientific, model 400A) and a length controller (Aurora Scientific, model 308B). The dimensions of the constructs were 1.67 ± 0.13 mm by 0.57 ± 0.03 mm (L x W) Initial length (Lₒ) of the preparation was standardized by stretching constructs to just-above-slack-length similar to as previously described. Force of spontaneously contracting constructs (1.3 ± 0.1 Hz) was then continuously monitored as preparation length was changed by adjusting the position of the length controller arm. Length was increased in small increments with 20 sec between length changes in order to monitor several contractions at each length. Force and length signals were digitally recorded and analyzed using custom LabView software. Passive tension and the amplitude of spontaneous isometric twitch force were measured on 3 or more transients at 15 sec after each length step. Force was normalized to cross-sectional area of the preparation, calculated by measuring the diameter at non-strained length and assuming circular geometry. All measurements were acquired at 30°C.
**Quantitative RT-PCR**
RNA was isolated from each construct using the RNeasy Fibrous Tissue Kit (Qiagen). 1ug of RNA was used to generate cDNA using random hexamers (Promega) and Superscript II Reverse Transcriptase (Invitrogen). For each set of cDNA, one sample was duplicated in parallel without reverse transcriptase as a check for genomic contamination. cDNA was diluted 1:10 and 5µL were used per 20µL reaction, along with 0.2µM primers and 2x SensiMix SYBR reagent (Bioline), including 3mM MgCl₂. Samples were run in triplicate in a 96-well plate format with no template controls for each primer set on a 7900HT Fast Real Time PCR System (Applied Biosystems) with the following reaction design: Step 1: 95°C, 15min; Step 2: 95°C 15sec, 60°C annealing 30sec, 72°C elongation 30sec (40 cycles); Step 3: 72°C cleanup 5min; followed by a melting curve (Step 4) of 95°C 15sec, 60°C 15sec, and a 2% ramp rate to 95°C 15sec to check for single product formation by melting curve peaks. Primers specificity was additionally verified by gel electrophoresis for amplicon size; primer specifications are listed in Table S2. qPCR was performed as described above with the following exceptions— for SERCA2: 62°C annealing temperature; for RYR2: 0.6µM primer concentration and 62°C annealing temperature; for NPPB: 0.1µM primers, 4mM MgCl₂, and 58°C annealing temperature. For qPCR analysis, Cts were determined automatically using SDS 2.2.1 software (Applied Biosystems), and mRNA expression was normalized to levels of HPRT transcripts.

**Immunostaining**
For immunohistochemistry, constructs were fixed for 30 minutes in cold methanol or 4% paraformaldehyde and embedded in paraffin. 5 µm sections were cut, and primary antibody staining was performed overnight, followed by one hour of secondary antibody incubation. For light microscopy, biotinylated secondaries were used followed by a thirty-minute incubation in the enzyme-based ABC reagent (Vector Labs); the binding was visualized by DAB (Sigma) when horseradish peroxidase (Vector Labs) was used and by Vector Red (Vector Labs) when alkaline phosphatase (Vector Labs) was used, all followed by hematoxylin nuclear counterstain. For immunofluorescence, Alexa fluorophore-conjugated secondary antibodies were employed; Hoechst (Sigma) counterstain was used to visualize the nuclei. The following primary antibodies were used with methanol-fixed constructs and tissue: mouse monoclonal anti-β-myosin heavy chain clone A4.951 (ATCC,1:10 dilution of hybridoma supernatant), mouse monoclonal anti-desmin (Dako, 1:5), mouse monoclonal anti-human CD31 (Dako, 1:15), mouse monoclonal anti-cardiac troponin T (Developmental Studies Hybridoma Bank, 1:1000), mouse monoclonal anti-cardiac troponin I (AbCam, 1,800), and mouse monoclonal anti-BrdU-POD (Roche, 1:40). Mouse monoclonal anti-α-actinin (1:800, Sigma) was used with paraformaldehyde-fixed constructs and tissue with proteinase K digest (Roche). Light microscopy secondary antibodies used were biotinylated goat anti-mouse IgG (Jackson Labs, 1:500) and biotinylated horse anti-goat IgG (Jackson Labs, 1:500). Immunofluorescent secondaries included Alexa 488- or 594-conjugated goat anti-mouse or horse anti-goat (Invitrogen, 1:100). For collagen matrix visualization, slides were stained with Sirius Red (1%, Sigma) and counterstained with FastGreen (1%, Sigma) made up together in saturated picric acid (1.3% in water, Sigma). Permount (Fisher) and Vectashield (Vector Labs) media were used to mount #1 glass coverslips (Corning) onto light microscopy and immunofluorescent slides correspondingly.

**Transmission Electron Microscopy**
Entire constructs were fixed in half-strength Karnovsky’s fixative (2.0% paraformaldehyde, 2.5% glutaraldehyde, 0.1mol/L cacodylate buffer, 3mmol/L CaCl₂, pH 7.3) overnight. They were washed in 0.1mol/L cacodylate buffer for 1h and then either stored in 0.1mol/L cacodylate buffer or immediately post-fixed in 1.0% OsO₄, rinsed and dehydrated through a graded series of alcohols and propylene oxide. Before embedding into Eponate resin (Ted Pella), each construct...
was subdivided into three longitudinal segments. In this study, only middle segments were visualized. Sections for light and transmission electron microscopy were cut using a Reichert Ultracut E microtome. Sections were mounted on 0.25% formvar coated rhodium/copper grids and stained with uranyl acetate and lead citrate.

Microscopy and Image Preparation
Light micrographs were taken at room temperature using Nikon Eclipse 80i microscope fitted with dry 10x- and 60x-Nikon objective with lenses of 0.30 and 0.95 NA correspondingly. The images were captured by Olympus Qcolor 3MB camera operated by Qcapture Pro software. Circularly polarized light microscopy was conducted as described previously\cite{12} at room temperature using a Nikon E800 microscope with a 10x/0.45 objective. Images were collected with a Photometrics Coolsnap camera in color mode (Roper Scientific) and METAMORPH software (Molecular Devices). Circular polarization was used and adjusted to obtain a deep uniform dark background. Illumination intensity, condenser aperture and exposure time were maintained uniformly for all images.

All immunofluorescent images were collected by a Nikon A1 Confocal System attached to a Nikon Ti-E inverted microscope platform and using water-immersion Nikon 60x CFI Plan Apo objective lens with 1.2 NA. Image acquisition was performed at room temperature using Nikon NIS Elements 3.1 software to capture 12-bit raw files that were then rescaled to 16-bit images for further processing. All images were collected as a single scan with the pinhole adjusted to 1 Airy unit at 1024x1024 pixel density. For electron microscopy, samples were examined using a JEM 1200EX II transmission electron microscope (JEOL Ltd, Tokyo). Operating conditions for the instrument included an accelerating voltage of 80kV, a 300u condenser aperture, a 50u objective aperture and a spot size setting of 3. Images were collected with Olympus Morada digital camera using iTEM image acquisition and analysis software. For figure preparation, images were exported into Photoshop 7.0 (Adobe). If necessary, brightness and contrast were adjusted for the entire image and the image was cropped.

Histological and Statistical Analysis
To quantify cardiomyocyte axis alignment within constructs, we analyzed 100x micrographs of slides stained for desmin (rNC constructs) or βMHC (hCM constructs) using a custom fiber orientation analysis program (developed by Dr. Michael Regnier's lab, University of Washington). Briefly, this Matlab program divides a user-defined region of interest into small subimages and, by edge detection, obtains the major axis angle of each subimage. For the whole image, an average angle and the angle dispersion (standard deviation of angles of cell edges) is determined. Cellular alignment is quantified by magnitude of angle dispersion, such that low angle dispersion (low standard deviation of cell axis angles) indicates a high degree of alignment, which is graphed as the inverse of angle dispersion, expressed as a percentage of the mean (i.e. the reciprocal of the coefficient of variance). Adult rat cardiac tissue was used as a positive control for cardiomyocyte alignment: sections were stained for desmin and quantification was performed only on regions with linear, longitudinal fiber orientation without fibers in cross-section, large vascular structures, or intersecting fiber planes.

To assess cardiomyocyte proliferation, slides double stained for BrdU and βMHC were counted in a blinded fashion, with 500 cardiomyocyte nuclei counted per section as either BrdU positive or negative. Cardiomyocyte area within the construct was assessed by quantifying βMHC positive area within each construct as described previously.\cite{2,13} The number of red pixels in 100x micrographs were counted in Photoshop and expressed as a percentage of total pixel area. This area was then normalized by the number of cardiomyocyte nuclei per micrograph. To quantify vascular structure within the constructs, slides were analyzed in a blinded fashion.
for multicellular CD31 positive formations that were scored as either cord structures or structures with lumens and normalized per area counted, over 4 fields per section.

N=3-6 for each experiment unless otherwise noted. Error bars represent standard error of the mean (SEM), significance was determined using single factor ANOVA followed by Student’s t-test with 95% or greater confidence level.
Online Supplement References


Online Videos

Online Video I. Contractility of engineered rat cardiac tissue construct. Primary rat neonatal cardiomyocytes were used to generate cardiac constructs a density of 2 million cells per 100µL construct and conditioned with static stress for 4 days. At this time, constructs demonstrated spontaneous and synchronous contractions along its long axis at room temperature.

Online Video II. Contractility of engineered human tri-culture myocardial tissue. Human ESC-derived cardiomyocytes (2 million) were used in conjunction with human endothelial cells (1 million) and human MSCs (1 million) to generate 100µL human tri-culture constructs, which were exposed to static stress for 4 days. Similar to rat neonatal constructs, they displayed spontaneous and synchronous contractions along the full construct length at room temperature.

Online Video III. Contractility of engineered human iPSC-derived myocardial tissue. Human IMR90-iPS cells were differentiated into cardiomyocytes and used to generate cardiac constructs at a density of 2 million cells per 100µL construct and conditioned under static stress. Similar to rat neonatal and human ESC-derived constructs, human iPS cell-derived constructs demonstrated spontaneous, synchronous contractility visible under low magnification and by eye. After 2 weeks, contractions were recorded at room temperature with one end of the construct cut free of the tab to demonstrate the extent of length shortening.
Online Table I

<table>
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<th>Immunostain</th>
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<th>Cardio Ave (%) ± SEM</th>
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<td>βMHC</td>
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<td>36.3 ± 32.0%</td>
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<td>Cytokeratin</td>
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<td>3.8 ± 3.1%</td>
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<tr>
<td>α-fetoprotein</td>
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<td>6.2 ± 6.2%</td>
</tr>
<tr>
<td>β3-tubulin</td>
<td>0.0%</td>
<td>0.1 ± 0.1%</td>
</tr>
</tbody>
</table>

Online Table I. Characterization of differentiated cells. The hESC-derived input cardiomyocyte population was stained and quantified using lineage markers for cardiovascular and support cell types (βMHC, CD31, SMA) as well as for endodermal, ectodermal, and pluripotent cell types (α-fetoprotein, β3-tubulin, and Oct4, respectively). Undifferentiated hESCs (n=1) were analyzed as well.
### Online Table II

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<th>Gene</th>
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<th>Size</th>
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Online Table II: Characteristics of primers used for quantitative RT-PCR.
Online Figure I. Cardiac Construct Manufacture and Conditioning. A) Constructs under low magnification in 6-well plate. B) Closeup view of construct end enmeshed in nylon tab with 20x objective. C) 10x objective view of length of contractile cardiac construct. D) Program of cyclic stress conditioning: 5% elongation, 1Hz, square sine wave generated with the FX-4000T system (Flexcell International Corp).
Online Figure II. Alignment and protein expression within rat neonatal constructs. A) Desmin and BrdU staining (red and brown, respectively). Cardiomyocytes cultured under normal 2-D culture conditions did not align with each other, whereas those in the 3-D collagen matrix under static stress conditioning became aligned in parallel with the direction of stress. B) Cardiomyocytes within the construct express cardiac troponin I and can undergo nuclear division. C, D, and E) Desmin, α-actinin, and cardiac troponin T staining, respectively. Cardiomyocytes within the construct demonstrate internal alignment and signs of maturation including sarcomeres and binucleated cardiomyocytes, similar to native cardiac tissue.
Online Figure III. Collagen alignment in rat neonatal cardiac constructs. A) Staining was performed using picrosirius red for assessment of collagen fiber bundle organization by birefringence under circularly polarized light. Large bundle collagen fibers fluoresced yellow in rNC constructs in contrast to cell-free collagen constructs, demonstrating an absence of large collagen fibers in the starting material. These fibers appeared disarrayed in the unstressed condition but closely aligned with conditioning, indicating that that collagen fiber architecture was also greatly improved by static and cyclic stress.
Online Figure IV. Vascularization and implantation of rat and human cardiac constructs. A) CD31 immunohistochemistry (brown). Constructs generated with rat cardiomyocytes and human endothelial cells contain endothelial structures dispersed throughout. Right, closeup of endothelial structures. B) In rat neonatal cardiac constructs under static strain, endothelial structure formation was increased by the addition of human MSCs. *, p<0.05 for cord structures compared to rNC+Endo. C) Endothelial proliferation was measured in statically strained human cardiac constructs pulsed with BrdU on day 4 and fixed on day 5. Slides were double stained for BrdU and CD31 and the ratio of BrdU-positive to BrdU-negative endothelial cells was quantified. Addition of a 1:1 ratio of MSCs to Endos (+1MSC) or half has many MSCs (0.5MSC) decreased endothelial proliferation rate between day 4 and day 5 (n=3 for hCM only, hCM+Endos, hCM+Endos+1MSC, n=2 for hCM+Endos+0.5MSC). D) Human triculture cardiac constructs were generated with MSCs expressing GFP, conditioned with static strain for 7 days, and grafted into the myocardium of an athymic rat. After an additional seven days, the construct is brightly visible on the epicardial surface under fluorescent light.
Online Figure V. Contractility of Human and Rat Cardiac Constructs with Tri-culture. A) Human ESC-derived constructs generated with or without 1 million human endothelial cells, 1 million human MSCs (+1MSC), or 0.5 million MSCs (+.5MSC) were conditioned with static strain for 5 days before beating rate was assessed visually (n=2). B) rNC constructs generated with or without 1 million human endothelial cells (rNC+E) and 1 million human MSCs (+1MSC) were conditioned with static strain for 3 days before spontaneous beating rate was assessed visually (# = p < 0.005).
Online Figure VI. BrdU labeling of proliferating ESC-derived and iPSC-derived human cardiac constructs. A) hCM constructs derived from hESCs with or without endothelial cells were placed under conditions of no stress, static stress or cyclic stress and immunostained for βMHC (red) and BrdU (brown). Cardiomyocytes undergoing DNA synthesis were double-labeled with brown nuclei and red cytoplasm. Levels of DNA synthesis in cardiomyocytes were quantified in Figure 5A. B) hCM constructs derived from iPSCs with or without endothelial cell were placed under conditions of no stress or cyclic stress and immunostained for βMHC (red) and BrdU (brown). Representative double-labeled cells within the construct are shown. Levels of DNA synthesis were quantified in Figure 5B. N=4.