Engineered early embryonic cardiac tissue retains proliferative and contractile properties of developing embryonic myocardium

Kimimasa Tobita, Li J. Liu, Andzej M. Janczewski, Joseph P. Tinney, Jill M. Nonemaker, Serena Augustine, Donna B. Stolz, Sanjeev G. Shroff, and Bradley B. Keller

1Cardiovascular Development Research Program, Children’s Hospital of Pittsburgh of University of Pittsburgh Medical Center, and Department of Pediatrics, University of Pittsburgh School of Medicine; and Departments of 2Bioengineering and of 3Physiology and Cell Biology, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania

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Tobita, Kimimasa, Li J. Liu, Andzej M. Janczewski, Joseph P. Tinney, Jill M. Nonemaker, Serena Augustine, Donna B. Stolz, Sanjeev G. Shroff, and Bradley B. Keller. Engineered early embryonic cardiac tissue retains proliferative and contractile properties of developing embryonic myocardium. Am J Physiol Heart Circ Physiol 291: H1829–H1837, 2006. —Embryonic myocardium has a high rate of cell proliferation and regulates cellular proliferation, contractile function, and myocardial architecture in response to changes in external mechanical loads. However, the small and complex three-dimensional (3D) structure of the embryonic myocardium limits our ability to directly investigate detailed relationships between mechanical load, contractile function, and cardiomyocyte proliferation. We developed a novel 3D engineered early embryonic cardiac tissue (EEECT) from early embryonic ventricular cells to test the hypothesis that EEECT retains the proliferative and contractile properties of embryonic myocardium. We combined freshly isolated White Leghorn chicken embryonic ventricular cells at Hamburger-Hamilton (HH) stage 31 (day 7 of a 46-stage, 21-day incubation period), collagen type I, and matrix factors to construct cylindrical-shaped EEECTs. We studied tissue architecture, cell proliferation patterns, and contractile function. We then generated engineered fetal cardiac tissue (EFCT) from HH stage 40 (day 14) fetal ventricular cells for direct comparison with EEECT. Tissue architecture was similar in EEECT and EFCT. EEECT maintained high cell proliferation patterns by culture day 12, whereas EFCT decreased cell proliferation rate by culture day 9 (P < 0.05). EEECT increased active contractile force from culture day 7 to day 12. The culture day 12 EEECT contractile response to the β-adrenergic stimulation was less than culture day 9 EFCT (P < 0.05). Cyclic mechanical stretch stimulation induced myocardial hyperplasia in EEECT. Results indicate that EEECT retains the proliferative and contractile properties of developing embryonic myocardium and shows potential as a robust in vitro model of developing embryonic myocardium.

cardiomyocyte; morphogenesis; growth and development; hyperplasia; tissue engineering

THE EMBRYONIC HEART is the first functioning organ, and through a rapid process of cell proliferation, death, and differentiation, the heart transforms from a single straight tube into a complex four-chamber heart (6). Primary cardiac morphogenesis occurs between weeks 4 and 8 of human pregnancy (21), between days 8 and 15 in the mouse embryo (34), and between days 2 and 10 of a 21-day gestation in the chick embryo (13, 34). At the onset of the heart beat, the embryonic myocardium is composed of an inner single cell endocardial layer, a middle layer of thick acellular extracellular matrix (cardiac jelly), and an outer thin myocardial layer (31). The three-layered, smooth-walled myocardium then rapidly transforms into a three-dimensional (3D) complex porous trabecular myocardium with a thin outer compact myocardium. The trabecular and compact myocardium increase in mass and then condense to form a solid mature myocardium with transmural variation in myofiber angle (31, 38). The developing myocardium increases myocardial function via cardiomyocyte (CM) proliferation and differentiation (1, 3, 4, 36, 40, 44). CM ultrastructure and myofiber architecture mature in parallel with chamber morphogenesis (3, 31). The cardiac conduction system and coronary vascular system differentiate later in morphogenesis (1, 12). Embryonic CMs have the highest rate of DNA synthesis during the period of cardiac morphogenesis, and CM proliferation rate then decreases during development (4, 16, 32, 36).

Mechanical load is one of the major exogenous (epigenetic) factors that regulate cardiac morphogenesis (12, 23, 30, 39). In neonatal and mature myocardium, altered mechanical load triggers various molecular signaling pathways, resulting in cardiac hypertrophy or atrophy (25, 26, 41). In contrast, the developing embryo/fetal myocardium responds to altered mechanical load by regulating cellular proliferation (hyperplasia or hypoplasia) and differentiation (3, 11, 21, 22, 27, 28, 32, 35, 38, 40). Numerous studies have investigated the relationships between external mechanical load, myocardial mechanical properties, CM differentiation, proliferation, death, and molecular mechanisms in the developing myocardium (3, 12, 20, 22–24, 28–30, 38–40, 43). Previous studies of detailed biomechanical measurements of in vivo embryonic myocardium used various assumptions, such as cylindrical or ellipsoidal chamber geometry, or porous compressible vs. solid incompressible myocardium (20, 28, 39, 40, 43). However, it is difficult to validate these assumptions directly within the in vivo embryonic myocardium that has nonuniform myofiber architecture and heterogeneous cellular proliferation and differentiation (24, 29, 31, 38). Therefore, we have pursued the development of an in vitro 3D tissue that would maintain the properties of developing embryonic myocardium but aid our investigation of load-sensitive myocardial function and differentiation.

To date, most in vitro model systems used to investigate embryonic CM maturation and function have used two-dimen-
sional (2D) culture system where CM phenotype and behavior often differ from “normal” in vivo biology (5, 8, 27). In 2D culture, embryonic CMs lose their in vivo rod-shaped phenotype and develop a flattened, stellate-shape phenotype (27). Embryonic CMs often do not attach efficiently, proliferate poorly, and fail to initiate spontaneous contraction in 2D culture (8). The use of a 2D planar-aligned collagen substrate induces cultured embryonic CMs to display a more in vivo-like phenotype (27). However, cell-cell and cell-extracellular matrix interactions remain restricted by the planar geometry. Finally, 2D culture systems cannot apply “realistic” biomechanical loads to myocardial tissue in culture, restricting the ability to determine the material properties of the tissue. Thus traditional 2D culture techniques do not provide a system in which embryonic CMs can proliferate and differentiate while maintaining correct morphology and an active mechanical environment.

Recently, several research groups have described the development of 3D in vitro CM culture systems (2, 7, 8, 45). The in vitro phenotype and molecular regulation of CMs in 3D culture more closely resemble native in vivo myocardium (8, 45), perhaps because of the fact that 3D culture systems expose CMs to biomechanical and electrical stimulation resembling native myocardium. These 3D culture systems have used CMs isolated from fetal hearts after the completion of primary cardiac morphogenesis or from neonatal hearts. It is important to note that primary cardiac morphogenesis (embryo) is followed by further myocardial growth and maturation (fetus) and that the myocardium continues to mature in the early postnatal period. In contrast to larger mammals where embryonic and fetal periods of gestation are clearly defined, there is a tendency to refer to all stages of development as embryonic in the chick and in rodents (for example, embryonic day 19.5 in the mouse embryo is close to the end of gestation). The biochemical and biophysical properties of “embryonic” CMs during the primary morphogenesis period differ markedly from those of “postmorphogenesis fetal” and “neonatal” CMs (3, 11, 22, 33, 36). Therefore, it is important to use immature embryonic cardiac cells in 3D tissue culture system to define the dynamic relationships between external mechanical loads and myocardial proliferation, differentiation, and function in developing embryo/fetal myocardium.

In the present study, we developed a novel 3D engineered early embryonic cardiac tissue (EEECT) to test the hypothesis that embryonic cardiac cells, obtained during the primary cardiac morphogenesis period, form myocardial tissue within in vitro 3D tissue culture, maintaining cell proliferative capacity and contractile function similar to native developing embryo/fetal myocardium.

MATERIALS AND METHODS

Cell sources. We incubated fertile White Leghorn chicken eggs (Utah State University, Logan, UT) in a forced-draft, constant-humidity incubator until Hamburger-Hamilton (HH) stage 31 (day 7 of a 46-stage, 21-day incubation period) or HH stage 40 (day 14) (13). HH stage 31 represents the period of cardiac morphogenesis at which the embryonic ventricle has differentiated from a single primitive ventricular chamber into right and left ventricular (LV) chambers and the coronary vascular system has not yet differentiated (1, 30). The developmental stages correspond with 6-wk-old human embryo. HH stage 40 represents the postcardiac morphogenesis period and corresponds with 12-wk-old human fetus (13, 34). Our research protocols conform to the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH Publication No. 85-23, Revised 1985), and the animal protocol was approved by the Animal Research and Care Committee of Children’s Hospital of Pittsburgh.

Construction of 3D EEECT and engineered fetal cardiac tissue. We used 15 embryonic ventricles of HH stages 29–31 to construct each individual EEECT and three fetal ventricles of HH stage 40 to construct individual engineered fetal cardiac tissue (EFCT). Excised embryonic/fetal ventricles were enzymatically digested by using 2 mg/ml of collagenase type II followed by 0.05% trypsin-EDTA solution (Invitrogen, Carlsbad, CA), and the isolated cells were preplated for 1 h to reduce noncardiac cell population, such as large debris or red blood cells. We then cultured the isolated cells on a gyratory shaker (60–70 rotations/min) for 24 h to reaggregate viable CMs (42). Approximately 3.0 × 10^6 cells/ml CMs were mixed with acid-soluble rat-tail collagen type I (Sigma, St. Louis, MO) and matrix factors (Matrigel, BD Science, Franklin Lakes, NJ) as previously described by Zimmermann et al. (45). We made cell/matrix mixture as follows. 1) Isolated cells were suspended within a culture medium (Modified Dulbecco’s Essential Medium, Invitrogen) containing 20% FBS (Invitrogen). 2) Acid-soluble collagen type I solution (pH 3) was neutralized with alkali buffer (0.2 M NaHCO3, 0.2 M HEPES, 0.1 M NaOH) on ice. 3) Matrigel (17% of total volume, BD Sciences) was added to the neutralized collagen solution. 4) Cell suspension and matrix solution were mixed. The final concentration of collagen type I was 0.67 mg/ml. Cylindrical-shaped EEECT and EFCT were constructed by using a collagen type I-coated silicone membrane culture plate (Tissue Train, Flexcell International, Hillsborough, NC) and FX-4000TT system (Flexcell International) (10). Briefly, the center of the silicone membrane of a Tissue Train culture plate was deformed by vacuum pressure to form a 20-mm-length × 2-mm-width trough using a cylindrical loading post (Tissue Train and FX-4000TT) (10). Approximately 200 μl of cell/matrix mixture was poured into the trough and was incubated for 120 min in a standard CO2 incubator (37°C, 5% CO2) to form a cylindrical-shaped construct. Both ends of the construct were held by anchors attached to the Tissue Train culture plate. When the tissue was formed, the culture plate was filled with a growth medium containing 10% FBS and 1% chick embryo extract (SLI, Horsted Keynes, UK). The vacuum pressure was then gradually released, and the construct was floated within the growth medium (10). The culture medium was changed every other day.

To study the impact of mechanical stretch, we applied uniaxial cyclic mechanical stretch into the longitudinal axis of the tissues for 48 h at culture day 10 in EEECT (0.5 Hz, 8% elongation from the original length) and at culture day 7 in EFCT (0.5 Hz, 4% elongation) using the Flexcell FX-4000TT system.

Confocal microscopy. Each tissue was fixed with 4% paraformaldehyde-PBS for 15 min. The sample was embedded in a 13% polyacrylamide gel oriented in the longitudinal or transverse direction, and 150-μm-thickness serial sections were made by using a standard vibrating microtome (Vibratome-1000, Vibratome.com, St. Louis, MO) (38). Sections were permeabilized with 0.1% Triton X-100 for 15 min and stained for α-actinin with a mouse-monoclonal α-sarcoglycan primary antibody (EA53, Sigma) and Alexa Fluor 488 (Molecular Probes, Eugene, OR) secondary antibody, and for β-tubulin with a mouse-monoclonal β-tubulin primary antibody (TU-06, Abcam, Cambridge, MA) and Alexa Fluor 594 secondary antibody (Molecular Probes). CMs were identified by positive α-actinin staining, and non-CMs were identified by negative α-actinin staining and positive β-tubulin staining. We reconstructed 3D projection images from stacks of z-axis optical scans by using a standard laser confocal microscope system (FV500, Olympus, Tokyo) and Scion Image software (Scion) (38).
Transmission electron microscopy. Each tissue was fixed with 2% glutaraldehyde containing 10−5 M verapamil, 10−5 M EDTA, and adjusted to pH 7.4 and then postfixed with aqueous 1% osmium tetroxide (20, 30). The fixed sample was dehydrated and embedded with epon. The 50-μm ultrathin sections were cut and stained with 2% uranyl acetate and lead. The prepared sections were viewed with a JEOL 1210 TEM system (JEOL USA, Peabody, MA).

Cell proliferation assays: bromodeoxyuridine, cell density, and total protein/DNA ratios. Each tissue was incubated with 60 μg/ml bromodeoxyuridine (BrdU, Sigma) for 16 h before fixation (29). Standard 7-μm-thickness frozen sections were stained for BrdU with a mouse monoclonal anti-BrdU antibody (Molecular Probes) and 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) (Vector Laboratories, Burlingame, CA). Ten to 20 sections from each tissue were randomly chosen in each sample, and the cell density within the outer layer of the construct was calculated by counting DAPI-positive nuclei. Cell proliferation rate was calculated from double-stained sections as ([BrdU-positive nuclei]/[DAPI-positive nuclei]) × 100 (%). Whole cell lysates were prepared from EEECT for the measurement of total protein-to-DNA ratio. DNA content (μg) was quantified by the enhancement of fluorescence of Hoechst dye 33258. Total protein content (μg) was determined by Bio-Rad protein microassay on the basis of the differential color change of a dye in response to increasing concentration of protein (Bio-Rad Laboratories, Hercules, CA) (3).

SDS-PAGE and Western blot analysis for sarcomeric α-actinin. Whole cell lysates were prepared from each tissue and separated by SDS-PAGE (10% separating gel, Bio-Rad Laboratories). Immuno-blotting was carried out by using routine protocols. Each lane contained 20 μg of total protein prepared from a single tissue. Mouse monoclonal α-sarcemeric actinin antibody (EA53, Sigma) and mouse monoclonal β-actin antibody (Abcam) were visualized with IR-Dye 800 donkey anti-mouse secondary antibody (Rockland Immunochemicals, Gilbertsville, PA) by using an infrared Western blot imaging system (Odyssey, LI-COR Biosciences Lincoln). All Western blot experiments were performed in triplicate to ensure that experimental observations were reproducible. Immunoblots were quantified by using densitometry, and α-sarcemeric actinin-to-β-actin expression ratio was calculated (Scion Image, Scion).

Contractile force measurements. We excised a central 10-to-15-mm-length tissue segment and transferred the specimen to a dissection chamber filled with cold (25°C) Tyrode solution containing (in mM) 119.8 NaCl, 5.4 KCl, 2.5 CaCl2, 1.05 MgCl2, 22.6 NaHCO3, 0.42 NaH2PO4, 0.05 Na2EDTA, 0.28 ascorbic acid, 5.0 glucose, and 30.23-butanedione monoxime (BDM), gassed with 95% O2-5% CO2 (pH 7.4). One end of the engineered tissue segment was gently attached to a force transducer (model 401A or 403A, Aurora Scientific, Ontario, Canada) and the other end to a high-speed length motor. EEECT was electrically stimulated at a rate of 1 Hz (scale bar, 1 mm).

1 The online version of this article contains three supplemental movies. Supplemental movie 1 shows EEECT mounted on a force transducer and a length motor. EEECT was electrically stimulated at a rate of 1 Hz (scale bar, 1 mm).

2 Supplemental movie 2, available with the online version of this article, shows spontaneous contraction of culture day 6 EEECT.

Table 1. Impact of cyclic mechanical stretch on culture day 12 EEECT and day 9 EFCT

<table>
<thead>
<tr>
<th>CSA, mm²</th>
<th>Engineer Tissue</th>
<th>Nonstretch</th>
<th>Stretch</th>
</tr>
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<tbody>
<tr>
<td>EEECT</td>
<td>1.23 ± 0.20 (n = 8)</td>
<td>0.79 ± 0.10* (n = 6)</td>
<td></td>
</tr>
<tr>
<td>EFCT</td>
<td>1.80 ± 0.09* (n = 4)</td>
<td>1.47 ± 0.13* (n = 6)</td>
<td></td>
</tr>
<tr>
<td>Passive stress, mN/mm²</td>
<td>EEECT</td>
<td>3.93 ± 0.98 (n = 8)</td>
<td>2.25 ± 0.72 (n = 5)</td>
</tr>
<tr>
<td></td>
<td>EFCT</td>
<td>23.6 ± 1.45* (n = 5)</td>
<td>30.4 ± 3.76* (n = 6)</td>
</tr>
<tr>
<td>BrdU-positive ratio</td>
<td>EEECT</td>
<td>42.7 ± 3.9 (n = 4)</td>
<td>53.3 ± 3.0* (n = 4)</td>
</tr>
<tr>
<td>Total protein/DNA ratio</td>
<td>EEECT</td>
<td>4.2 ± 0.6* (n = 9)</td>
<td>4.2 ± 0.5 (n = 8)</td>
</tr>
<tr>
<td>α-Actinin/β-actin ratio</td>
<td>EEECT</td>
<td>0.73 ± 0.05 (n = 6)</td>
<td>0.72 ± 0.03 (n = 5)</td>
</tr>
<tr>
<td></td>
<td>EFCT</td>
<td>1.00 ± 0.04* (n = 5)</td>
<td>1.00 ± 0.03* (n = 5)</td>
</tr>
</tbody>
</table>

Data are means ± SE; n, no. of samples. CSA, cross-sectional area; α-actinin/β-actin, sarcomeric α-actinin-to-β-actin expression ratio; EFCT, engineered fetal cardiac tissue; BrdU, bromodeoxyuridine. *P < 0.05 vs. nonstretch within group. †P < 0.05 vs. engineered early embryonic cardiac tissue (EEECT).

Microscopy system (model KPD-50, Hitachi), and the cross-sectional area (CSA, mm²) was calculated using circular geometry. We measured (1) active force at Lmax, (2) passive force at Lmax, (3) effects of 2.5, 5.0, and 10.0 mM extracellular [Ca2+]o on active force at Lmax, and (4) effects of β-adrenergic stimulation with isoproterenol (1 μM) on active force at Lmax. Force was normalized by each specimen’s adjusted CSA to yield stress values [active and passive stress (σ) at Lmax, mN/mm²]. Slack length (Ls), which was the length when the tissue segment was excised, was used to calculate the strain (ε) corresponding to a given length.

Statistical analysis. Data are expressed as averages ± SE. One-factor ANOVA was performed to compare the active stress of native LV trabeculae and nonstretched EEECT and EFCT. Two-factor ANOVA was performed to compare the contractile response to extracellular calcium and isoproterenol on active stress at Lmax. Individual comparisons were performed by using a Tukey test. Statistical significance was defined by a value of P < 0.05. All calculations were performed by using SigmaStat (Systat Software, Point Richmond, CA).

RESULTS

Myofiber architecture and cellular proliferation patterns. During culture the tissues condensed (reducing the external diameter), and spontaneous CM contraction was observed after 4–5 days of initial culture in both EEECT and EFCT. The contraction patterns of EEECT and EFCT gradually changed from asynchronous to synchronous by culture day 6 (see supplemental movie 2). The CSA of culture day 9 EFCT was larger than day 12 EEECT (Table 1). CMs that were randomly distributed at initial tissue construction gradually formed a myocardial tissue with the majority of CMs oriented in parallel to the longitudinal axis of the construct (Fig. 1A). The highest density of CMs was observed in a layer around the outer border (thickness of 100–150 μm, Fig. 1B). Tissue structure and myofiber architecture were similar in both EEECT and EFCT. In the outer high-density-CM layer, the myofibers oriented to the construct longitudinal axis. This myofiber architecture of
EEECT was similar to the native fetal chick LV trabeculae where myofibers aligned with the longitudinal axis (Fig. 1. D–F, and supplemental movie 3). Figure 2 shows that CMs within EEECT contained organized sarcomeres oriented along the longitudinal cell axis. A-bands, I-bands, and Z-lines were clearly recognized (5). Other CM organelles, such as mitochondria and desmosomes, were also present. Very few CMs showed apoptotic or necrotic changes.

BrdU-positive ratios of EEECT increased from culture day 7 to culture days 9 and 12 [23.6 ± 3.1% (SE) at culture day 7 (n = 4), 36 ± 2.2% at day 9 (n = 7), and 42.6 ± 3.8% at day 12 (n = 4), respectively] and then decreased to 8.2 ± 0.7% (n = 4) at culture day 21 (P < 0.05 vs. culture day 7, Fig. 3). In contrast, the BrdU-positive ratio of EFCT decreased from 36.6 ± 1.0% at culture day 7 (n = 5) to 4.2 ± 0.6% at culture day 9 (P < 0.05, n = 9, Fig. 3).

Mechanical properties and contractile responses to the increase in external calcium ion and β-adrenergic stimulation. Figure 4 shows representative active stress tracings of culture day 7 EEECT (Fig. 4A), culture day 12 EEECT (Fig. 4B), and culture day 9 EFCT (Fig. 4C). The active stress of culture day 7 EEECT (n = 7) was similar to native embryonic day 14 LV trabeculae (n = 8, Fig. 5A). The culture day 7 EEECT corresponds to a cumulative age of 14 incubation days of native myocardium. The active stress increased at culture day 12 EEECT (n = 4, Figs. 4A and 5A). The active stress of culture day 9 EFCT (n = 5) was larger than culture day 12 EEECT (Figs. 4A and 5A). Passive stress of day 9 EFCT was larger than day 12 EEECT (Table 1). Increasing extracellular calcium enhanced active stress in a dose-dependent manner, and the inotropic response was similar in both EEECT (n = 6, 50% increased from baseline) and EFCT (n = 5, 70% increased from baseline, Fig. 6A). Physiologically maximum effective concentration of isoproterenol (1 μM) increased active stress of EFCT ~100% from baseline, while EEECT increased only 15% (Fig. 6B) (22). Passive stress did not change after extra-
cellular calcium or β-adrenergic stimulation. The inotropic response to maximal isoproterenol stimulation of EEECT was less than the response to 10 mM extracellular calcium ion, indicating that EEECT contractile properties are similar to immature developing embryo/fetal myocardium (Fig. 6, A and B) (11, 22, 33, 35).

Impact of cyclic mechanical stretch stimulation. Cyclic mechanical stretch reduced the CSA of both tissues (Fig. 1, B and C, and Table 1). The CM-to-total CSA ratio of EEECT was increased after stretch stimulation [67.6 ± 1.7% in stretched EEECT (n = 5) vs. 53.2 ± 1.7% in nonstretched EEECT (n = 5), P < 0.05]. Active stress increased in both EEECT and EFCT (P < 0.05, Fig. 5B). Mechanical stretch did not change EEECT passive stress, while the mechanical stretch increased EFCT passive stress (Table 1). Contractile responses to the extracellular calcium or β-adrenergic stimulation were unchanged after stretch stimulation (Fig. 6, A and B). Cell proliferation rate of EEECT increased after cyclic stretch stimulation while that of EFCT did not change (P < 0.05, Table 1). Total protein-to-DNA ratios were unchanged by mechanical stretch in EEECT and EFCT. Cyclic mechanical stretch did not change the actinin-to-actin expression ratio in both EEECT and EFCT (Fig. 7 and Table 1). These results indicate that EEECT increased contractile properties by CM hyperplasia, not hypertrophy (3).

DISCUSSION

Our present study demonstrates that an in vitro 3D EEECT derived from early chick embryonic ventricle during the period of primary morphogenesis retains the proliferative and contractile properties of developing embryo/fetal myocardium. Specifically, we found that 1) EEECT myofibril architecture resembled that of native embryonic trabeculae, 2) high cellular proliferative activity was maintained during culture (33, 36, 44), 3) EEECT contractile properties were more sensitive to changes in extracellular calcium than to β-adrenergic stimulation (11, 22), and 4) mechanical stretch induced EEECT CM hyperplasia (3, 4). All of these features are characteristic of developing embryonic myocardium.

EEECT architecture. We generated cylindrical EEECTs by mixing cells in suspension with a liquid extracellular matrix to allow spontaneous cell-matrix interactions and tissue remodeling (45). A cylinder is the preferred simple geometric construct...
negligible bending effects) for evaluating the relationship between tissue geometry/architecture and mechanical properties. Both embryonic and fetal CMs proliferated within the cylindrical constructs and remodeled the surrounding matrix to form a myocardial tissue containing a 100- to 150-μm-thick concentric ring of high-density CMs on the EEECT or EFCT outer circumference with myofibers oriented parallel to the longitudinal axis (Fig. 1). We noted that this aligned myofiber architecture was not seen in preliminary experiments with spherically shaped EEECTs (unpublished data). The CMs distributed mainly laterally with myofibers oriented in the circumferential direction in the ring-shaped construct described by Zimmermann et al. (45). Our results and those of Zimmermann et al. suggest that spontaneous tissue remodeling is closely related to tissue geometry. Other factors, such as fluid diffusion, fluid viscosity influenced by nutrients, growth factors, and material properties of scaffold, are also likely to influence tissue formation, growth, and remodeling (15). Zimmermann et al. (45) described that a collagen concentration of 0.8 mg/ml does not interfere with fluid diffusion within engineered heart tissues. Native embryonic myocardium acquires oxygen and nutrients by direct diffusion until the coronary vascular system starts to function at developmental stages close to completion of cardiac morphogenesis (1). It remains unknown how the embryonic myocardium changes myocardial structure while optimizing contractile function before oxygen and nutrient delivery via coronary vascular flow. Thus EEECT may provide new insights by allowing the investigation of the critical relationships among nutrient availability, tissue geometry, and subsequent myocardial architecture and mechanical properties in the developing embryonic myocardium.

In the present study we applied rotation culture to form CM aggregate before tissue construction. In our preliminary study we found that CM aggregate formation significantly increased CM volume fraction (CM volume/total cell volume within EEECT, %) within outer high-cell-density regions at culture day 7 EEECT (n = 4) vs. 63.4 ± 4% in preplating group (n = 4), P < 0.05. Watzka et al. (42) showed that CM aggregate formation using neonate mouse heart cells with 3D floating and rotation culture methods improves cell viability, reduces cell apoptosis rate, and increases CM population. They described that extracellular ma-
trix laminin and fibronectin are deposited between the cells and that the viable CM attach to each other by tight junctions (42). Their results suggest that the spontaneous production of new extracellular matrix by CMs also plays an important role in CM survival, myocardial tissue formation, and proper contractile function. Thus CM aggregate formation may protect embryonic CMs from apoptosis triggered by cell isolation and help to reconstitute engineered cardiac tissue by preserving effective CM-CM connections in vitro.

Both the cell-to-matrix ratio and matrix composition are likely to have a significant impact on optimizing myocardial tissue geometry, cell-cell and cell-matrix interactions, and composite mechanical properties. Small changes in the cell-to-matrix ratio impact both tissue formation and mechanical properties. In preliminary experiments we used a liquid matrix and determined the optimal cell-to-matrix ratio for EEECT construction. We found that a collagen concentration <0.45 mg/ml did not support the formation of viable embryonic CM constructs, while higher collagen concentrations (>0.8 mg/ml) interfered with cell outgrowth (expansion), cell proliferation, and spontaneous contractile function. In the present study we report the optimal collagen concentration to be 0.60–0.7 mg/ml. This collagen concentration supported embryonic CM proliferation and spontaneous remodeling. The specific cell-to-matrix ratio and composition are likely to have a significant impact on optimizing myocardial tissue geometry, cell-cell and cell-matrix interactions, and composite mechanical properties.

**Cellular proliferation in EEECT.** Numerous studies have shown that the developing embryo/fetal myocardium has high cell proliferation activity and that this high cell proliferation rate declines shortly after birth in mammalian and gradually decreases in avian myocardium (14, 16, 17, 36). CMs shift from an embryonic/fetal proliferative (hyperplastic) phenotype to a mature cell-growth (hypertrophic) phenotype during this transition process (36). In the present study, we found that the BrdU-positive ratio of EEECT was maintained at a high level for 12 culture days. The BrdU-positive ratio of culture day 12 EEECT was similar to culture day 7 EFCT. The culture day 12 EEECT and the culture day 7 EFCT correspond to a cumulative age of 19 incubation days (late fetus) of a 21-day gestation and 21 incubation days (hatching), respectively. We note that BrdU-positive ratio of culture day 21 EEECT and culture day 9 EFCT, which are corresponding to age of posthatch 7 days and 2 days, respectively, significantly decreased from culture day 7 (Fig. 3). Evans et al. (8) recently developed a collagen-based, tubular-shaped engineered cardiac construct by using gestational day 15 rat embryonic CMs, noting that CMs within this construct proliferated until culture day 6 and then decreased significantly in later culture days. The timing of decrease in cell proliferation rate corresponded to the time of birth for native rat myocardium (cell isolation at embryo day 15 plus 6 days), similar to our results.

In contrast, Eschenhagen et al. (7) described a cylinder-shaped engineered cardiac construct by using incubation days 9–11 chicken embryonic cardiac cells (HH stages 35–37). They noted increased cellular proliferation rate for only the initial 2 days in culture followed by a decline in proliferation rate after 5 days in culture. Li et al. (18) described an engineered cardiac construct by using a biodegradable gelatin mesh and embryo day 19 rat cardiac cells. The cell number/construct continuously increased up to 30 days of culture. Many factors, such as cell density, various growth factors, and matrix composition, influence cell proliferation activity both in vivo and in vitro, and the results of these studies suggest that the extracellular matrix plays an important role in CM survival, myocardial tissue formation, and proper contractile function.
vitro. In the present study, initial cell density was similar to previous studies (7, 9, 45), and the cell density at tissue construction was set as the same in both EEECT and EFCT. We used the same standard tissue culture growth medium that was also similar to previous studies (7, 9, 45). The mechanism by which our EEECT maintained longer high cellular proliferative activity, while not yet proven, is likely related to the earlier developmental stage of cells isolated to form EEECT. The changes in cell proliferation patterns correspond to those in native myocardium. Further studies are necessary to investigate the factors that regulate high cell proliferation patterns and the transition of CM phenotype from hyperplasia to hypertrophy in EEECT.

**Contractile properties and response to cyclic mechanical stretch in EEECT.** We showed that active stress of EEECT increased with duration of culture, and the positive inotropic response to increased external calcium ion was greater than the response to β-adrenergic stimulation at culture day 12 (cumulative age of 19 incubation days of native myocardium). In contrast to EEECT, the EFCT at culture day 9 (posthatch 2 days in native myocardium) responded to β-adrenergic stimulation similarly to the neonate and mature myocardium (11, 22). Our results indicate that the contractile properties of EEECT are likely related to the earlier developmental stage of cells (11, 22, 33, 35).

Fink et al. (9) showed that engineered cardiac tissue from postmorpheogenesis and neonatal CMs responds to the cyclic mechanical stretch stimulation by cardiac hypertrophy rather than hyperplasia. In the present study, we found that cyclic mechanical stretch induced further CM proliferation within EEECT. In addition, cyclic mechanical stretch increased EEECT active stress, while mechanical stretch did not change the inotropic response to the extracellular calcium ion and β-adrenergic stimulation. Previous studies in native embryonic myocardium showed that increased external mechanical loads increased both contractile force and end-diastolic myocardial strain and triggered cellular hyperplasia within vivo early embryonic ventricular myocardium (3, 40). Miller et al. (19) showed that embryonic chick heart cells at HH stage 31 respond to cyclic mechanical stretch by cellular proliferation rather than hypertrophy in the 2D tissue culture system (19). Those studies in embryonic myocardium support our results that CMs within EEECT respond to cyclic mechanical stretch similar to native embryonic myocardium. None of the previously described engineered cardiac constructs display contractile properties similar to immature embryonic myocardium (2, 7–9, 45).

In the present study, we used HH stage 31 embryonic ventricular cells to construct EEECT. At HH stage 31 the embryonic chick right and left ventricles are in transition from secondary trabecular ventricles to tertil trabecular (mature type) ventricles. This developmental stage is relatively late in the period of primary morphogenesis (12, 13, 31). We found that embryonic cardiac cells from this period in morphogenesis (HH stage 31) maintain their proliferative capacity for longer duration than cells isolated in the late fetal period, raising the possibility that cells harvested earlier during cardiac morphogenesis could proliferate even longer in culture.

The mechanical stretch protocols of EEECT and EFCT were the minimal stretch stimulation required to increase active stress using our active culture system. Mechanical stretch applied to EEECT before culture day 7 induced permanent tissue elongation shortly after the stretch stimulation with no subsequent stretch effect after tissue elongation. We found that cyclic stretch magnitude <5% did not trigger additional cellular hyperplasia or increased active stress vs. nonstretched EEECT. In contrast to the EEECT, both of the active stress and cell proliferation rate of EFCT were decreased when we applied the same stretch protocol of EEECTs (8% stretch). The EFCTs were twisted after the stretch stimulation, and non-CMs within EFCT tended to align to the perpendicular direction of the stretch direction (data not shown) (37). We showed that culture day 9 EFCT passive stress at $L_{\text{max}}$ was larger than day 12 EEECT, suggesting that EFCT is stiffer. Mechanical stretch stimulation at 8% did not change EEECT passive stress, while 4% stretch stimulation increased EFCT passive stress. The higher passive stress of EFCT vs. EEECT could be due to intrinsic differences in the material properties of cells isolated at different developmental stages (fetal cells could be stiffer than embryonic cells) and/or due to differences in cell-cell and cell-matrix interactions. Differences in passive stress may influence the stretch threshold values that influence active force production and/or cell proliferation rates of EEECT and EFCT. Further studies are necessary to investigate how embryonic CMs and non-CMs within EEECT regulate biochemical signaling, material properties, and tissue architecture within a mechanically active environment.

It is important to note that native embryonic myocardium is continuously exposed to 15–20% cyclic mechanical stretch during diastole from the onset of heartbeat (39, 40). Stretch frequency (heart rate) and velocity also increase during cardiac morphogenesis (3). Obviously further studies are necessary to define the mechanisms by which stretch magnitude and frequency and envelope regulate the maturation of EEECT growth, architecture, and mechanical properties.

In conclusion, our results demonstrated that the EEECT provides a robust in vitro model of developing embryonic myocardium. We expect that EEECT will greatly facilitate the investigation of myocardial growth, differentiation, maturation, and adaptation to external biomechanical loads within a realistic and reproducible 3D in vitro environment.

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