Engineered Fetal Cardiac Graft Preserves Its Cardiomyocyte Proliferation Within Postinfarcted Myocardium and Sustains Cardiac Function

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The goal of cellular cardiomyoplasty is to replace damaged myocardium by healthy myocardium achieved by host myocardial regeneration and/or transplantation of donor cardiomyocytes (CMs). In the case of CM transplantation, studies suggest that immature CMs may be the optimal cell type to survive and functionally integrate into damaged myocardium. In the present study, we tested the hypothesis that active proliferation of immature CMs contributes graft survival and functional recovery of recipient myocardium. We constructed engineered cardiac tissue from gestational day 14 rat fetal cardiac cells (EFCT) or day 3 neonatal cardiac cells (ENCT). Culture day 7 EFCTs or ENCTs were implanted onto the postinfarct adult left ventricle (LV). CM proliferation rate of EFCT was significantly higher than that of ENCT at 3 days and 8 weeks after the graft implantation, whereas CM apoptosis rate remained the same in both groups. Echocardiogram showed that ENCT implantation sustained LV contraction, whereas EFCT implantation significantly increased the LV contraction at 8 weeks versus sham group ($p < 0.05$, analysis of variance). These results suggest that active CM proliferation may play a critical role in immature donor CM survival and the functional recovery of damaged recipient myocardium.

Introduction

EMBRYONIC/FETAL CARDIOMYOCYTES (CMs) possess high proliferative activity during development, which declines shortly after birth, and terminally differentiated mature CMs have limited proliferative activity.^{1,2} This limited proliferative activity of mature CMs is the major barrier to myocardial regeneration after injury in higher vertebrates. Each of the therapeutic options for injured myocardium, including medical management, cardiac transplantation, mechanical circulatory left ventricular assist devices, and other experimental surgical approaches, has limitations.^{3,4}

Cellular cardiomyoplasty is considered to be a promising therapeutic option for cardiac repair.⁵⁻⁹ Currently, a wide range of donor cell types are under investigation. Initial results from trials in human patients using autologous bone marrow cells have shown that improvement in cardiac function is not satisfactory and remuscularization mediated by donor cells within damaged myocardium is unlikely to be

the basis for functional recovery.⁷ Recently, emerging tissue engineering approaches, such as synthetic biomaterial cardiac patch,¹⁰ hydrogel,^{11,12} or cell sheet,¹³⁻²⁰ offer another pathway to achieve damaged myocardium recovery and may overcome the insufficiency of current treatments.²¹

Studies suggest that a three-dimensional (3D) tissue culture environment is necessary for efficient CM survival, functioning myocardial tissue formation *in vitro*, functional integration, and sustained cardiac recovery.²²⁻²⁸ Using neonate CMs, pioneering studies from Eschenhagen and Zimmermann validated an engineered heart tissue paradigm for cardiac graft implantation onto postinfarct left ventricle (LV) myocardium.^{22,23,26} Their results were reported for 4 weeks after graft implantation, and graft survival required the use of immunosuppression.²³

In contrast to neonatal CMs, studies suggest that fetal CMs display a high level of cell survival and sustained cardiac function when transplanted into injured myocardium.²⁹⁻³⁶ We have developed a 3D engineered cardiac tissue from

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embryonic chick cardiac cells, termed engineered early embryonic cardiac tissue (EEECT). Chick EEECT maintains high cellular proliferation activity and contractile properties that mimic the native developing fetal myocardium.³⁷ In response to cyclic mechanical stretch stimulation, chick EEECT increases CM proliferation, rather than the CM hypertrophic response noted in adult and neonate CMs.^{37,38} Therefore, in the current study, we tested the hypothesis that the proliferating fetal CMs within engineered cardiac tissue graft maintain CM proliferative activity *in vivo*, survive as a donor myocardial tissue, and contribute to the cardiac functional recovery of injured recipient myocardium in a syngeneic rat model.

Materials and Methods

Experimental animals

Gestational day 14 Lewis rat fetal hearts (engineered fetal cardiac tissue [EFCT]), neonatal day 3 Lewis rat hearts (engineered neonatal cardiac tissue [ENCT]), and 12-week-old adult female Lewis rats (tissue graft recipients) weighing 200 to 250 g were used (Harlan Sprague Dawley Inc.). To track the fate of implanted CMs within EFCT, we used gestation day 14 EGFP(+)-[(SD-TgN(acro/act-EGFP)4Osb] transgenic rat fetal hearts identified by using a fluorescent light (Dark Reader Stop Lamp; Clare Chemical Research) during heart excision from the fetuses. We used 12-week-old adult female rnu/rnu nude rats (200 to 250 g body weight; Harlan Sprague Dawley Inc.) as recipients of EGFP(+)-EFCTs. The EGFP-transgenic rats were originally generated by Dr. Masaru Okabe (University of Osaka, Osaka, Japan)³⁹ and the EGFP transgenic rat colonies were maintained within the animal facility of the Rangos Research Center Animal Facility, Children's Hospital of Pittsburgh of UPMC.¹⁰ All experimental protocols followed the National Institutes of Health guidelines for animal care and were approved by the University of Pittsburgh's Institutional Animal Care and Use Committee.

Construction of rat EFCT and ENCT

For EFCT construction, pregnant mothers were anesthetized using 3% isoflurane inhalation with 100% oxygen, and hysterectomy was performed. Immediately after hysterectomy, the mother was euthanized by induced asystole under 5% isoflurane anesthesia. The excised uteri were transferred to a sterilized Petri dish filled with cold (25°C) phosphate-buffered saline and 1% antibiotic-antimycotic solution containing 100 units/mL of penicillin, 100 units/mL of streptomycin, and 0.25 µg/mL of amphotericin-B (Invitrogen), and the fetuses were excised by hysterotomy, and then fetal hearts were harvested. Great vessels and atrium were removed from each fetal heart, and ventricular tissue was collected and pooled. For ENCT construction, neonatal day 3 rat pups were euthanized by cervical truncation under 5% inhaled isoflurane with 100% oxygen and the ventricular tissue was excised and pooled. Pooled fetal or neonatal ventricles were then enzymatically digested by 2 mg/mL of collagenase type II followed by 0.05% trypsin-EDTA solution (Invitrogen). Isolated cells were preplated for 1 h and then cultured on a gyratory shaker (50 to 55 rotations/min) for 24 h to reaggregate viable CMs.³⁷ Approximately 5.0×10^5

of cardiac cells/construct (350 cells/cell aggregate) were mixed with acid-soluble rat-tail collagen type I (Sigma) and matrix factors (Matrigel; BD Science).³⁷ Cell/matrix mixture was performed as follows. (1) Cells were suspended within a culture medium (high glucose-modified Dulbecco's essential medium; Invitrogen) containing 20% fetal bovine serum (Invitrogen). (2) Acid-soluble collagen type I solution (pH 3) was neutralized with alkali buffer (0.2 M NaHCO₃, 0.2 M HEPES, and 0.1 M NaOH) on ice. (3) Matrigel (15% 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 EFCT or ENCT were constructed using a collagen type I-coated silicone membrane culture plate (Bioflex culture plate; Flexcell International) and FX-4000TT system (Flexcell International). Briefly, the center of the silicone membrane of a Tissue Train culture plate was deformed by vacuum pressure to form a 20-mm-length $\times$ 2-mm-width trough using a cylindrical loading post (FX-4000TT). Approximately 200 µL of cell/matrix mixture was poured into the trough and incubated for 120 min in a standard CO₂ incubator (37°C, 5% CO₂) to form a cylindrical construct. Both ends of the construct were held by anchors attached to the Tissue Train culture plate (BioFlex; Flexcell International). When the tissue was formed, the culture plate was filled with a growth medium containing 10% fetal bovine serum (Invitrogen) and 1% antibiotic-antimycotic solution (Invitrogen). Constructed engineered tissues were cultured for 7 days and the culture medium was changed every other day.

Contractile force measurement

Contractile force was measured on culture day 7 EFCT ($n = 7$) and ENCT ($n = 8$) as previously described.³⁷ In brief, each engineered cardiac construct was excised from anchors of BioFlex culture plate and transferred to a cold (25°C) calcium free Ringer solution containing (in mM): 135.0 NaCl, 4.0 KCl, 10.0 Trizma-HCl, 8.3 Trizma-base, 11.0 glucose, and gassed with 95% O₂/5% CO₂ (pH 7.4). One end of the EFCT or ENCT was attached to a rigid stainless steel bar connected to force transducer (model 403A; Aurora Scientific) and the other end to a rigid stainless steel bar mounted on a micro-manipulator using 10-0 mono-filament nylon sutures (Fig. 1A). The perfusion chamber containing the construct was then filled with warmed buffer (37°C, 2 mL chamber volume) perfused at 1 mL/min with 2 mM [Ca²⁺] Ringer solution. We performed measurement of force-length relations followed by 1 µM isoproterenol (ISP) treatment from the same construct. After 10 min preconditioning of the construct at a slack length (by adjusting construct length as the same length as that in a Bioflex culture plate) under perfusion of oxygenated, 2 mM [Ca²⁺] ion containing warmed ringer solution (baseline ringer solution), the tissue was electrically stimulated at 1 Hz, 4 ms, 50 to 70 V using a field stimulator for 5 min. We then measured active contractile force at the slack length (0%, L₀) and 15% elongation (L_{0.15}) from L₀. We repeated force measurement at L₀ and L_{0.15} three times and averaged in each construct. We note that the weakening of force was not found throughout the test. Measured force at given length were averaged in each construct. The construct was then maintained at L_{0.15} and was perfused for 5 to 10 min with baseline ringer solution and then treated with

FIG. 1. External shape of constructed EFCT, EFCT implantation onto postinfarcted LV surface, and tissue structure of EFCT and ENCT. **(A)** Culture day 7 cylindrical EFCT (black arrow) is suspended within a mechanical testing chamber and is field stimulated to measure contractile force. Minor scale indicates 1 mm. **(B)** Photograph of EFCT graft implantation onto the postinfarcted myocardium. Four EFCTs (indicated by *) were implanted into LV circumferential direction to cover the infarct myocardium. Scale bar indicates 5 mm. White arrows indicate LV circumferential direction. **(C)** Confocal microscope image of α -sarcomeric actinin and DAPI staining of culture day 7 EFCT tissue. CMs oriented into the longitudinal axis (white arrows) of the EFCT forming myocardial tissue-like structure. **(D)** Confocal microscope image of α -sarcomeric actinin and DAPI staining of culture day 7 ENCT tissue. Myocardial tissue architecture of

ENCT is similar to EFCT (**C**). Scale indicates 20 μ m. CM, cardiomyocyte; EFCT, engineered fetal cardiac tissue; ENCT, engineered neonatal cardiac tissue; LV, left ventricle. Color images available online at www.liebertonline.com/ten.

1 μ M ISP. The contractile force was monitored for 5 min during ISP treatment and the largest contractile force was measured as the contractile response of ISP treatment at 5 min after ISP treatment. After the force measurement, ISP was washed out by baseline ringer solution to confirm force level was returned to the baseline. We repeated ISP treatment for three times and averaged in each construct.³⁷

Chronic left ventricular infarction model

EFCT or ENCT recipient adult (Lewis or nude) rats were anesthetized using 3.0% isoflurane inhalation with 100% oxygen gas followed by endotracheal intubation and connection to a rodent volume controlled mechanical ventilator (Model 683; Harvard Apparatus). The heart was exposed through a left thoracotomy, monitoring electrocardiogram (THM 1000; VisualSonics). The proximal left anterior descending coronary artery was ligated with 7-0 polypropylene. Myocardial ischemia was confirmed by regional cyanosis and changes in electrocardiogram (ST-segment elevation). The incision was closed in layers with 4-0 silk continuous sutures.¹¹ A total of 45 rats underwent left coronary artery ligation, 7 animals died within 24 h of permanent coronary artery ligation, and 38 animals survived. These surviving rats were recipient candidates for cardiac grafts. The survival

rate at 2 weeks after permanent left coronary artery ligation was 84.4%.

EFCT or ENCT implantation

Two weeks after permanent coronary artery ligation (preimplantation), animals were anesthetized with isoflurane, and the infarction size and cardiac function were assessed by transthoracic echocardiography. LV infarction size was estimated in a standard LV short-axis view by the percentage of scar area (akinetic or dyskinetic regions) to LV free wall area.¹¹ We excluded 4 Lewis rats because of insufficient LV infarction, and 34 rats with infarcts >25% of the LV free wall were included in the present study. We performed CM proliferation (histone H3) and apoptosis (caspase-3) assays at 3 days after EFCT graft implantation ($n=6$) and ENCT implantation ($n=6$). For the longitudinal echocardiography and histological assessment, eight EFCT-implanted, six ENCT-implanted, and eight sham-operated animals were studied at 8 weeks after graft implantation. We used three nude rats with infarcts to allow EGFP(+)-EFCT cell tracking and to evaluate EFCT-derived cell fate. The LV anterior wall was exposed through left thoracotomy again. Using 7-0 polypropylene with peripheral sutures, the anterior infarcted myocardium was covered with four

engineered tissue constructs into the LV circumferential direction (Fig. 1B). For the sham-operated group, a thoracotomy was performed 2 weeks after coronary ligation; however, no EFCT implantation was performed. EGFP(+) transgenic rat EFCTs were implanted onto postinfarcted LV myocardium in nude rats.

Histological assessment

Culture day 7 EFCTs or ENCTs were fixed with 4% paraformaldehyde/phosphate-buffered saline for 15 min. For BrdU staining each tissue was incubated with 60 μ g/mL bromodeoxyuridine (BrdU; Sigma) for 16 h before fixation. Fixed EFCTs or ENCTs were embedded in a 13% polyacrylamide gel oriented in the longitudinal direction of the construct, and 150 μ m thickness serial sections were made using a standard vibrating microtome (Vibratome-1000; Vibratome.com).³⁷ At 3 days, 2 weeks, or 8 weeks after the EFCT or ENCT implantation, the rats were exposed under 3.0% isoflurane inhalation with 100% oxygen gas, and the heart was arrested by apical injection of 2 mL of a hypothermic arresting solution (28 mM NaCl, 100 mM KCl, 36 mM NaHCO₃, 2.0 mM MgCl₂, 1.4 mM Na₂SO₄, 11 mM dextrose, 30 mM butanedione monoxime, and 10,000 U/L of heparin). The embedded frozen LV tissues were serially sectioned at 8 μ m in the LV transverse direction using a standard cryo-microtome (MICROM HM505E; Pacific Southwest Lab Equipment Inc.).

Sections were permeabilized with 0.1% Triton X-100. The DNA synthetic-phase nuclei were identified by double staining with a secondary antibody (Alexa Fluor 594; Invitrogen)-conjugated anti-BrdU antibody (Invitrogen) and 4',6-diamidino-2-phenylindole (DAPI) (Vector Labs). Mitotic phase CMs were identified by triple staining with anti- α -sarcomeric actinin (EA53; Sigma), anti-phospho-S10 Histone H3 (Upstate cell signaling solutions) with Alexa Fluor secondary antibodies, and DAPI (Vecta-Shield; Vector Labs).^{38,40} To identify cellular apoptosis, sections were triple-stained with anti- α -sarcomeric actinin (EA53), anti-active caspase-3 (Abcam), and DAPI. Presence of vascular cells was investigated by using a triple staining of sections with anti-von Willebrand factor (vWF; Abcam), anti- α -smooth muscle actin (SMA; Abcam), and DAPI (Vecta-Shield) in culture day 7 EFCT, a double staining of sections with vWF (Abcam) or SMA (Abcam) and DAPI (Vecta-Shield) for GFP-labeled EFCT-implanted myocardium at 2 weeks after implantation, and a triple staining of sections with anti- α -sarcomeric actin (Abcam), vWF (Abcam), and DAPI (Vecta-Shield) for EFCT-implanted myocardium at 8 weeks implantation, respectively.

Identification of CM proliferation or CM apoptosis

We reconstructed 3D composite images from stacks of z-axis optical scans of each stained sample using a standard laser confocal microscope system (FV1000; Olympus) and Scion Image software (Scion Corp).³⁸ The images were observed at different rotation angles to identify proliferating/apoptotic CMs by co-localization of phospho-histone H3 or active caspase-3-positive nuclei staining and α -sarcomeric actinin staining. To quantify total cellular and CM proliferation activities, 10–20 regions from multiple representative sections from each EFCT or ENCT tissue, or noninfarct areas of myocardium (>200 μ m apart from graft tissue implanta-

tion site or infarction border in each section, which expressed α -sarcomeric actinin similar to the normal, noninfarcted myocardium) were randomly chosen for analysis at 400 $\times$ or 600 $\times$ magnification. Total cellular proliferation (both CM and non-CM fractions), and CM proliferation or apoptosis ratios were calculated as follows: [Total cellular proliferation] = [BrdU (+) nuclei]/[DAPI (+) nuclei] (%) and [CM proliferation or CM apoptosis] = [Phospho-histone H3 or Caspase-3 (+) nuclei within α -sarcomeric actinin (+) cells]/[DAPI (+) nuclei] (%), respectively. To measure CM ratio of EFCT and ENCT at before and 8 weeks after graft implantation, we randomly captured at least 20 fields at 400 $\times$ magnification of α -sarcomeric actinin/DAPI sections in each tissue sample and the CM ratio was calculated as follows: [α -sarcomeric actinin-positive nuclei number]/[total nuclei number] $\times$ 100 (%).

Capillary density measurement of EFCT and ENCT-implanted myocardium

We measured capillary density in EFCT ($n=8$), ENCT ($n=6$), and sham ($n=8$) myocardial sections at 8 weeks after graft implantation by counting vWF-positive cells. Using a confocal microscope, 20 nonoverlapped image fields at a 400 $\times$ magnification were randomly selected in each section and vWF-positive cells were counted. Data were normalized as vWF(+) counts/mm².

Assessment of in vivo recipient cardiac function

LV function was measured by noninvasive echocardiography (Acuson Sequoia and 13-MHz 15L8 probe) at preimplantation (2 weeks after left coronary artery ligation), 4 weeks, and 8 weeks (10 weeks after coronary ligation) after graft implantation. End-diastolic area (EDA) and end-systolic area (ESA) of the LV cavity were measured by endocardial planimetry, and % LV fractional area change (FAC) was measured as [(LVEDA–LVESA)/LVEDA] $\times$ 100%. Echocardiography was performed using isoflurane anesthesia (1.5% with 100% oxygen) via nose cone.

Statistical analysis

Data are expressed as mean $\pm$ standard error. Student's *t*-test was performed to compare the data of maximum active contractile force of EFCT and ENCT, and capillary density measurement of EFCT and ENCT donor cardiac grafts. Paired Student's *t*-test was performed to compare the data of *in vitro* active contractile force and contractile response to the ISP stimulation. One-factor analysis of variance (ANOVA) with Turley *post-hoc* test was performed to compare capillary density among EFCT, ENCT, and sham-operated myocardium. Two-factor ANOVA with a Tukey *post-hoc* test was performed to compare the BrdU, phospho-histone H3, or active caspase-3-positive cells. Two-factor repeated ANOVA with a Tukey test was performed to compare the echocardiogram data. Statistical significance was defined by a value of $p < 0.05$. All calculations were performed using SigmaStat (Systat Software Inc.).

Results

In vitro EFCT morphology and contractile force

Both EFCT and ENCT displayed spontaneous tissue contraction from culture day 4. CM orientation and striation

patterns within engineered constructs assessed by α -sarcomeric actinin staining at culture day 7 were similar to each other (Fig. 1C, D). Culture day 7 EFCT and ENCT generated active contractile force in response to cyclic field electric stimulation and displayed a positive Frank-Starling response to increased tissue longitudinal length ($p < 0.05$, Fig. 2A). Although the active contractile force of ENCT at $L_{0.15}$ was much higher than that of EFCT (2.60 ± 0.31 mN [$n = 7$] vs. 1.45 ± 0.39 mN [$n = 8$], $p = 0.001$), physiologically maximum ISP stimulation ($1 \mu\text{M}$) in the presence of 2 mM calcium ions did not increase contractile force in both tissues, suggesting that fetal and neonatal CM phenotypes within the engineered cardiac graft remain immature (Fig. 2B, Table 1).³⁷

In vitro culture day 7 EFCT and ENCT total cellular and CM proliferation

Total cellular proliferation ratio of culture day 7 EFCT assessed by BrdU (DNA synthetic phase) was significantly higher than that of culture day 7 ENCT ($23.3\% \pm 3.0\%$ [$n = 7$] vs. $1.69\% \pm 0.4\%$ [$n = 8$], $p = 0.001$). CM proliferation ratio of EFCT assessed by triple staining of phospho-histone H3 (mitotic phase), α -sarcomeric actinin, and DAPI was also significantly higher than that of ENCT ($16.0\% \pm 3.7\%$ vs. $6.8\% \pm 0.7\%$, $p = 0.018$).

Cardiac graft survival after implantation onto postinfarcted adult LV myocardium

There were no early or late postoperative deaths related to tissue graft implantation in any experimental groups. Two weeks after EGFP-EFCT implantation onto the postinfarcted, nude rat myocardial surface, EGFP-positive cells within EFCT did not migrate out from the implanted EFCT and maintained EFCT myocardial tissue integrity (Fig. 3A–C). At 8 weeks after Lewis rat graft implantation, the implanted EFCT or ENCT grafts were still recognizable in the site at the original implanted area and the grafts were seen to be merged with host LV surface by visual inspection (Fig. 3D). The implanted grafts were covered with thin connective tissue and no significant adhesions with the chest wall were found. Hematoxylin–eosin staining of microtome sections revealed that implanted EFCTs and ENCTs were recognizable as eosin-positive staining muscle-like tissues at the im-

planted sites (epicardial surface) of infarcted myocardium in all hearts, which was not observed in the sham-operated LVs (Fig. 3E, upper panel). Typical ring-shaped postcapillary vessel was also identified within the implanted tissue graft. Implanted grafts were positive to α -sarcomeric actinin, and the high magnification images showed that CMs within implanted EFCT grafts preserved a typical striated sarcomere structure (Fig. 3E, middle and lower panels). Although we did not quantify implanted myocardial tissue survival from histological sections beyond measures of cell death and proliferation, EFCT myocardial tissue appeared to be preserved better than that of ENCT at 8 weeks. The CM ratio identified by α -sarcomeric actinin-positive nuclei of culture day 7 EFCT ($n = 7$) or ENCT ($n = 8$) was similar ($60.9\% \pm 3.8\%$ in EFCT vs. $60.4\% \pm 6.6\%$ in ENCT, $p = 1.0$, ANOVA), whereas the CM ratio of EFCT at 8 weeks after implantation was higher than that of ENCT ($33.8\% \pm 7.2\%$ in EFCT [$n = 7$] vs. 13.4 ± 2.7 in ENCT [$n = 7$], $p = 0.039$). We screened all graft-implanted LVs for evidence of tumor-like tissue formations by hematoxylin–eosin staining and found no evidence for tumor formation in EFCT- and ENCT-implanted LVs.

Capillary vessel formation within implanted grafts

The vWF-positive cells were recognized well within EFCT or ENCT-implanted LV myocardium (Fig. 4A–C). The capillary density of EFCT (133 ± 5 counts/ mm^2) or ENCT (115 ± 10 counts/ mm^2)-implanted LV myocardium was significantly higher than that of infarction controls (68 ± 5 counts/ mm^2 , $p < 0.05$, ANOVA, Fig. 4D), whereas the capillary densities within donor EFCT (183 ± 16 counts/ mm^2) or ENCT (176 ± 10 counts/ mm^2) grafts were similar ($p = 0.35$). To investigate whether vascular network was derived from donor EFCT graft or recipient myocardium, we assessed vWF and SMA expressions in GFP-labeled EFCT graft implanted onto nude rat LV myocardium at 2 weeks after implantation. The majority of vWF or SMA-positive cells were negative to GFP (Fig. 5, white arrow heads) and only few GFP (+) cells were positive to vWF or SMA (Fig. 5E, F) suggesting that vessel formation is mainly derived from the host myocardium at 2 weeks after graft implantation. We did not recognize typical vessel network formations in culture day 7 EFCT before implantation (data not shown).

FIG. 2. Culture day 7 EFCT and ENCT contractile force. **(A)** Representative contractile force waveform tracings at slack length (L_0) and 15% stretched length ($L_{0.15}$). EFCTs were field stimulated by an electric pacing stimulator at 1 Hz. EFCT increased contractile force at L_{max} . **(B)** Comparison of maximum active force at $L_{0.15}$ between EFCT and ENCT. ENCT active force was significantly higher than EFCT at culture day 7.

TABLE 1. *IN VITRO* CONTRACTILE RESPONSE OF ENGINEERED FETAL CARDIAC TISSUE AND ENGINEERED NEONATAL CARDIAC TISSUE TO β -ADRENERGIC STIMULATION

	Baseline (Pre)	1 μ M-ISP	% of Change versus Pre	p
EFCT	1.45 $\pm$ 0.39	1.67 $\pm$ 0.43	13.8 $\pm$ 8.5	0.37
ENCT	2.60 $\pm$ 0.31 ^a	2.99 $\pm$ 0.59 ^a	15.2 $\pm$ 10.0	0.31

Data are mean $\pm$ standard error. Baseline active force at L_{0.15}, ^a*p* < 0.05 versus ENCT. Five minutes after 1 μ M isoproterenol (ISP) stimulation did not increase active contractile force in both EFCT and ENCT. Contractile force was measured under presence of 2 mM [Ca²⁺] containing Ringer solution.
EFCT, engineered fetal cardiac tissue; ENCT, engineered neonatal cardiac tissue.

In vivo graft CM proliferation and apoptosis activities

We assessed phospho-histone H3 and active caspase-3-positive CM ratios within implanted EFCT, ENCT, and noninfarcted host myocardium at 3 days and 8 weeks after

graft implantation (Fig. 6). Phospho-histone H3 (histone-H3)-positive CM ratios of EFCT, ENCT, or noninfarcted LV myocardium remained at the same levels, respectively (EFCT: 16.7% $\pm$ 8.0% at 3 days after implantation, and 21.9% $\pm$ 4.1% at 8 weeks [*p* = 0.39], ENCT: 1.0% $\pm$ 1.0% at 3

FIG. 3. Histological assessments of implanted EFCT fate within postinfarcted LV myocardium. **(A–C)** Representative fluorescent histological sections of 2 weeks after EGFP transgenic EFCT implantation onto postinfarcted nude rat LV myocardium. Cells within EFCT stayed within the implanted site and preserved the graft myocardial tissue structure *in vivo*. Green color indicates GFP signal, red color indicates α -sarcomeric actinin, and blue color indicates nuclei (DAPI). Scale indicates 50 μ m. **(D)** Implanted EFCTs at 8 weeks after implantation were well merged onto host infarcted myocardial surface and thin connective tissue covered EFCT implantation site. Yellow dot curves indicate implanted EFCTs and black dotted line indicates histological section of **(E)**. Scale indicates 10 mm. **(E)** Implanted EFCT were recognized at epicardial implantation sites in all samples at 8 weeks after graft implantation. Typical round postcapillary vessel formation was found within implanted EFCT (inset). Immunohistochemistry revealed that implanted EFCT preserved myocardial tissue and the α -sarcomeric actinin staining showed typical striated muscle patterns, indicating that EFCT maintained its myocardial tissue structure. Scale bars indicate 50 μ m (**E**, inset), 500 μ m (hematoxylin-eosin staining, upper panel), 250 μ m (middle panel), and 20 μ m (lower panel), respectively. Color images available online at www.liebertonline.com/ten.

FIG. 4. Capillary vessel formation of EFCT graft-implanted postinfarct myocardium at 8 weeks after graft implantation. **(A)** Triple staining of α -sarcomeric actinin (green), von Willibrand factor (vWF, red), and DAPI in EFCT graft-implanted myocardium at low magnification; scale indicates 200 μ m. **(B, C)** Higher magnification images; scale indicates 100 μ m. Capillary formation (vWF-positive nuclei, white arrow head) was detected though the postinfarcted myocardium and vessels also penetrated into EFCT graft. **(D)** Capillary density assessed by vWF-positive cell counting within postinfarcted myocardium indicates that both EFCT and ENCT increased capillary density significantly higher than sham-operated myocardium ($p < 0.05$), whereas these between EFCT and ENCT was not statistically different. Color images available online at www.liebertonline.com/ten.

days, and $1.1\% \pm 2.0\%$ at 8 weeks [$p = 0.97$], noninfarcted LV: $3.5\% \pm 1.1\%$ at 3 days, $0.8\% \pm 1.0\%$ at 8 weeks [$p = 0.11$]). The EFCT histon-H3-positive CM ratio tended to be higher than that of ENCT or host noninfarcted myocardium at 3 days ($p = 0.11$), and the CM-positive ratio at 8 weeks after tissue implantation was significantly higher in EFCT ($p < 0.001$, Fig. 6A). Active caspase-3-positive CM ratios of EFCT, ENCT, or noninfarcted LV myocardium also remained at the same relative levels at 3 days and 8 weeks after graft implantation (EFCT: $2.3\% \pm 1.5\%$ at 3 days after implantation, and $3.0\% \pm 1.0\%$ at 8 weeks [$p = 0.71$], ENCT: $2.0\% \pm 1.0\%$ at 3 days, and $1.0\% \pm 1.0\%$ at 8 weeks [$p = 1.0$], noninfarcted LV: $2.0\% \pm 1.7\%$ at 3 days, $0.6\% \pm 0.4\%$ at 8 weeks [$p = 0.45$]) (Fig. 6B). We noted that the caspase-3-positive CM ratio of EFCT was substantially lower than histone H3-positive ratio at both 3 days and 8 weeks after tissue implantation ($p < 0.001$ vs. CM H3-positive ratio each time point).

Cardiac function of EFCT and ENCT-implanted postinfarction LV

At 2 weeks after permanent coronary artery ligation (preimplantation state, 0 week) LV EDA increased to approximately twice the precoronary artery ligation EDA ($0.34 \pm 0.02 \text{ cm}^2$ precoronary ligation; $0.68 \pm 0.03 \text{ cm}^2$ at 2 weeks after ligation EDA, $n = 34$, $p < 0.001$, paired t -test) and

LV FAC decreased to less than half of precoronary artery ligation FAC ($61.0\% \pm 1.4\%$ precoronary ligation; $26\% \pm 2.6\%$ 2 weeks after ligation, $p < 0.001$), indicating permanent coronary artery-induced postmyocardial infarction heart failure (LV dilatation and impaired LV contraction). We noted no statistical differences in LV EDA ($p = 0.80$, ANOVA) or LV FAC ($p = 0.93$) among experimental groups before graft implantation at 2 weeks postinfarction (0 week). Longitudinal echocardiography after graft implantation showed that the sham-operated LVs further increased EDA at 8 weeks ($p < 0.001$, 8 vs. 0 week), whereas LV EDA did not increase after EFCT or ENCT implantation indicating that implanted EFCTs or ENCT prevented further LV dilatation (Fig. 7A). LV FAC decreased in the sham-operated LV by 8 weeks ($p = 0.030$, 8 vs. 0 week), whereas ENCT-implanted LV preserved contractile function and EFCT implantation significantly increased FAC at 8 weeks ($p = 0.04$, 8 vs. 0 week, Fig. 7B). At 8 weeks after graft implantation, LV FAC in EFCT was significantly higher than sham-operated LVs ($p = 0.009$), whereas the LV FAC of ENCT tended to higher than sham-operated LV ($p = 0.07$ vs. sham).

Discussion

In the current study we found that EFCT maintained active CM proliferation *in vitro* (preimplantation) and that this

FIG. 5. Vascular network formation in GFP-labeled EFCT graft at 2 weeks after implantation. (A–C) α -smooth muscle actin (SMA) staining of GFP-labeled EFCT-implanted postinfarcted nude rat myocardium at 2 weeks after implantation. Some SMA-positive cells formed ring-shaped vessels (while white arrow heads). There were few vascular-like cells that exhibit SMA and GFP colocalization (white arrow). (D–F) vWF staining of GFP-labeled EFCT graft. The majority of vWF-positive cells with typical ring- or capillary-like formation did not colocalize with GFP (white arrow heads), suggesting that vascular network was mainly recruited by host myocardium. Color images available online at www.liebertonline.com/ten.

proliferative activity was maintained for 8 weeks *in vivo* after EFCT implantation onto postinfarcted adult rat myocardium. The implanted EFCT graft survived, preserved cardiac tissue structure, and improved the contractile function of the postinfarct LV. Our previous study of chick EECT showed that EECT maintains a high cellular proliferation activity.^{37,38} In the current study, we constructed EFCT from gestational day 14 fetal rat cardiac cells to investigate the efficacy of an EFCT postinfarction cardiac repair paradigm. Culture day 7 rat EFCT, which developmentally corresponds to gestational day 21 rat fetal hearts, displayed active CM

proliferation activity whereas culture day 7 ENCT, which developmentally corresponds to postnatal day 10, displayed a relatively low CM proliferation rate similar to postnatal myocardium.¹ We used the same method of tissue construction for these two types of cardiac tissue. Therefore, our data suggest that the differences in *in vitro* CM proliferation activity between EFCT and ENCT parallel intrinsic differences in fetal and neonatal CM properties.^{1,40,41}

Eschenhagen *et al.* first reported the generation of engineered heart tissue (EHT) from postorganogenesis chick embryonic or neonatal rat cardiac cells using a 3D liquid

FIG. 7. Echocardiographic assessment of EFCT and ENCT-implanted post-infarcted myocardium. Data are expressed as % change in both LV EDA and FAC $\pm$ standard error. The value of 100% EDA indicates EDA at cardiac graft implantation. While sham-operated LV cavity area increased by 8 weeks after graft implantation (A, $*p < 0.05$, ANOVA), EFCT and ENCT-implanted LV did not increase LV cavity area. LV contraction of sham-operated LV decreased

by 8 weeks ($*p < 0.05$), whereas EFCT-implanted LV increased LV contraction ($*p < 0.05$, B). ENCT-implanted LV retained LV contraction by 8 weeks. EFCT-implanted LVs at 8 weeks after graft implantation significantly increased LV FAC compared to sham-operated LVs ($p = 0.009$ vs. sham-operated LV), whereas ENCT-implanted LV FAC tended to increase ($p = 0.07$). Data support that implanted EFCTs attenuate LV remodeling of postinfarcted LV. $*p < 0.05$ within group (ANOVA). 0 week, preimplantation; 4 weeks and 8 weeks, 4 weeks and 8 weeks after implantation. EDA, end-diastolic area; FAC, fractional area change.

collagen-extracellular matrix/cell mixture culture method, which we adapted for EFCT construction in the current study.²⁶ Their pioneering work and successful generation of ring-shaped EHT reported by Zimmermann *et al.* showed that EHT exhibits native mature myocardial-like tissue architecture and contractile properties.^{22,42} Cyclic mechanical stretch stimulation of EHT-induced CM hypertrophy (cellular growth) and increased the EHT contractile properties similar to mature adult myocardium.^{26,42} The implanted EHTs maintained myocardial tissue for 4 weeks after the EHT implantation, electrically coupled with the recipient myocardium, and attenuated further LV dilatation and preserved contractile function.²³ In the present study, active contractile force of culture day 7 ENCT was significantly greater than EFCT, whereas the contractile response to physiologically maximum β -adrenergic stimulation ($1 \mu\text{M}$, ISP) was similar in both constructs. Zimmermann *et al.* have shown that culture day 12 ring-shaped EHT from neonate rat cardiac cells responds to β -adrenergic stimulation similar to mature myocardium under the presence of 0.2 mM $[\text{Ca}^{2+}]$.^{23,27,42} In our study we did not see a dramatic inotropic response of EFCT or ENCT to the ISP. We measured the active contractile force and contractile response to ISP under the presence of 2 mM $[\text{Ca}^{2+}]$ ion. In a pilot study, both EFCT and ENCT did not generate substantial contractile force in the presence of only 0.2 mM extracellular $[\text{Ca}^{2+}]$ and we think that 2 mM $[\text{Ca}^{2+}]$ concentration represents a physiological extracellular $[\text{Ca}^{2+}]$ concentration. Although we could not conclude that ENCT contractile properties represent more mature myocardium than EFCT, CM proliferation activity and peak active force of EFCT may indicate that the CM phenotype within EFCT remains more similar to a proliferative fetal CM phenotype.^{1,37,38,40}

Li *et al.* showed that fetal cardiac cells maintain proliferative activity and form a myocardium-like tissue within a 3D gelatin-mesh scaffold *in vitro*. Their implanted gelatin mesh maintained the myocardial-like tissue architecture within postinfarcted myocardium and contributed to preservation

of postinfarcted LV contraction.²⁴ Their study, however, did not determine if the high cellular proliferation activity reflects CM proliferation. In the present study, we specifically analyzed CM proliferation and apoptosis activities. EFCT displayed higher CM proliferation activity before graft implantation than ENCT and the higher CM proliferation activity at 8 weeks after EFCT implantation was maintained at the same level as preimplantation, whereas CM proliferation activity at 8 weeks after ENCT implantation remained low. Our previous studies of chick EECT^{37,38} showed that cyclic mechanical stretch stimulation increases both EECT CM proliferation and contractile function, which mimics the adaptive capacity of the developing fetal myocardium to altered mechanical loads.^{41,43,44} We speculate that implanted EFCT exposure to active mechanical deformation by recipient myocardial contraction may be one of the mechanisms responsible for stimulating increased CM proliferation, supporting EFCT tissue survival, and the prevention of further negative LV remodeling.

Hypoxia and oxidative stresses induces CM apoptosis. Although immature fetal CMs are thought to be more tolerant than adult CMs to the hypoxia/oxidative stresses, the CM apoptosis is the common outcome.⁴⁵ Therefore, protection of CM from apoptosis and/or induction of CM proliferation under hypoxia/oxidative stresses are critical to prevent CM loss from the implanted graft. In the present study ENCT CM apoptosis activity was low at both 3 days and 8 weeks after implantation. We speculate that EFCT may maintain higher myocardial tissue survival (higher CM ratio of implanted graft at 8 weeks) by active CM proliferation with low CM apoptosis, whereas ENCT maintains myocardial tissue survival by protecting CM apoptosis, rather than CM proliferation at 8 weeks after graft implantation. Capillary density quantification within postinfarct myocardium showed that both EFCT and ENCT promoted capillary formation, whereas the capillary density within the donor graft did not show a difference between EFCT and ENCT groups, suggesting that capillary vessel formation was not directly

related to CM proliferation and CM apoptosis activities. In the current study, we did not have any evidence of increase in EFCT graft thickness after implantation though we found that α -sarcomeric actinin-positive ratio of EFCT displayed significantly higher than ENCT at 8 weeks after the implantation. These results suggest that the active CM proliferation of EFCT might compensate CM loss (apoptosis) for donor graft survival and functional contribution to the damaged recipient myocardium better than ENCT with less proliferating CMs.

Zimmermann *et al.* described that immunosuppression treatment was necessary to promote donor graft survival even in the syngeneic rat animal model.^{23,42} In the current study we did not treat animals with any immunosuppressive drugs before or after the graft implantation. Leor *et al.* reported that implanted fetal ventricular tissue fragments into postinfarcted rat myocardium survive and sustain cardiac function of recipient rat heart.³⁶ Roell *et al.* showed that embryonic CMs engraft well within postinfarcted myocardium sustaining recipient cardiac function.^{29,30} We note that although both EFCT and ENCT grafts were externally recognizable at 8 weeks after the graft implantation, the CM/non-CM ratio of EFCT was significantly higher than ENCT. We also found that ENCT-implanted heart sustained LV contraction at 8 weeks without immunosuppression treatment though the recipient LV functional recovery appeared to be less than EFCT-implanted LV. The underlying mechanisms of EFCT survival without immunosuppression and its functional contribution to the recipient myocardium remain to be elucidated, our results suggest that immature fetal type CMs have active CM proliferation and engraftment without immunosuppressive drug treatment, and functionally contribute to the recipient injured myocardium better than postnatal less proliferative CMs. Further studies are necessary to investigate whether the active CM proliferation is associated with immunotolerance for graft survival and additional immunosuppression treatment has beneficial effect on functional recovery of recipient myocardium.

Several limitations of the current study should be mentioned. We used Matrigel as supplemental extracellular matrices (ECMs) for EFCT construction. In our preliminary study, we found that Matrigel played an important role in facilitating cell aggregate expansion within EFCT. However, Matrigel is derived from mouse tumor cells, which is contraindicated for clinical use.⁴⁶ In addition, it remains unknown which ECM proteins and/or growth factors within Matrigel play a role in cell expansion and EFCT tissue formation. Further studies are necessary to replace the Matrigel with a clinically appropriate ECM/growth factor mixture. Second, ECM and/or growth factor treatment itself may have beneficial effects on myocardial regeneration and recipient cardiac function.^{47,48} In a preliminary study, we attempted acellular collagen/Matrigel construct (same concentrations as EFCT) implantation onto infarcted myocardium. However, acellular constructs were too fragile to maintain tissue integrity after implantation, and myocardial tissue formation was not observed at the implantation site. Therefore, we speculate that beneficial effects of EFCT implantation on recipient LV function is associated with survival of the EFCT, not the delivery of ECM proteins and/or growth factors. Further studies are required.

Constructed engineered cardiac tissue samples were of adequate size for the rat animal LV infarction model, but translation to larger preclinical models will require addressing the key issue of construct nutrient demand and supply. Simple nutrient/oxygen diffusion through EFCT may limit the size of constructs generated to repair larger injured hearts. CM proliferation activity and contractile function are greatly influenced by the oxygen and nutrient distribution of the tissue.^{28,49} It remains unknown whether the high CM proliferation activity would be preserved in a larger-scale engineered cardiac graft. Recent studies show that cell sheet cardiac graft is relatively easily constructed and also scalable to the human patients.^{13–17,19} Combination of proliferative CM and the cell sheet technology, such as layered cell sheet¹⁸ or cell aggregate patch,²⁰ may enable us to test whether high CM proliferation activity is maintained in a larger-scale engineered cardiac graft. Further studies are necessary to determine whether the high CM proliferation and low CM apoptosis activities within EFCT are purely intrinsic properties of fetal type CMs.

It remains to be determined whether our results of donor tissue survival and functional contribution to recipient myocardium will be maintained over extended time periods at which finite CM proliferation activity within EFCT may be lost. In the present study we found that GFP(+) EFCT cells did not migrate out from the graft tissue 2 weeks after implantation and the implanted Lewis rat EFCT survived at 8 weeks after implantation maintaining myocardial tissue structure. We speculate that cells within EFCT may stay within the graft, maintaining tissue structure. However, we could not track the fate of EGFP(+)EFCT cells for a prolonged period due to the risk of allogeneic EFCT graft rejection by host nude rat myocardium.¹⁰ Therefore, further study is necessary to track EGFP(+)EFCT cells using EGFP(+) graft implantation onto GFP(–) host infarction using inbred GFP transgenic rat implantation model.¹⁰

In the current study, we were not able to detect direct cell-to-cell connection by gap junction protein connexin-43 expression between donor CM and recipient CM at infarction border. We also did not investigate the electrocoupling between donor graft and host postinfarcted myocardium. Zimmermann *et al.* showed the implanted engineered cardiac graft has electrocoupling with host myocardium.²³ We observed that both EFCT and ENCT had spontaneous beating activities before graft implantation. Therefore, it remains unknown whether spontaneous beating CMs within the graft sense the recipient electrophysiological activities to synchronize their beating with recipient myocardium or the pacemaker CMs (spontaneously beating cells) are replaced by new CMs that electrical activities are regulated by recipient myocardium after the graft implantation. Further studies are necessary.

The underlying mechanisms that regulate postnatal CM proliferation *in vivo* and within EFCT *in vivo* after graft implantation are currently unknown and require further investigation. Various potential cell sources for cellular cardiomyoplasty, including stem/progenitor cell-derived CMs, are under investigation, and each cell source may have unique mechanisms that regulate cell survival, proliferation, and functional integration.^{5,7–9,50} Also remaining unknown is the tumorigenic potential associated with active donor CM proliferation. Therefore, elucidation of regulatory factors of

CM proliferation will play a key role for optimal CM preparation for cardiac repair.

In conclusion, implanted EFCTs onto postinfarcted LV myocardium maintained active CM proliferation with minimal CM apoptosis, preserved myocardial tissue structure, and attenuated the functional deterioration of postinfarcted LV. Our results suggest that active CM proliferation of donor cardiac tissue graft may play an important role for graft survival and functional contribution to the recipient injured myocardium.

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Disclosure Statement

No competing financial interests exist.

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