Scaffold-Free Human Cardiac Tissue Patch Created from Embryonic Stem Cells

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Progress in cardiac tissue engineering has been limited by (1) unfavorable cell and host responses to biomaterial scaffolds, (2) lack of suitable human cardiomyocyte sources, and (3) lack of fabrication techniques for scalable production of engineered tissue constructs. Here we report a novel and scalable method to generate scaffold-free human cardiac tissue patches. Human embryonic stem cells were differentiated to cardiomyocytes using activin A and BMP4 and placed into suspension on a rotating orbital shaker. Cells aggregated to form macroscopic disc-shaped patches of beating tissue after 2 days. Patch diameter was directly proportional to input cell number (approximately 11 mm with 12 million cells), and patches were 300–600 μm thick. Cardiomyocytes were concentrated around the patch edges and exhibited increased purity and maturation with time, comprising approximately 80% of total cells after 11 days. Noncardiac cell elements, primarily epithelium, were present at day 2 but were diminished markedly at later time points. Cardiomyocyte proliferation occurred throughout the patches at day 2 but declined by day 8. Patches exhibited automaticity and synchronous calcium transients, indicating electromechanical coupling. These novel scaffold-free human myocardial patches address critical challenges related to human cell sourcing and tissue fabrication that previously inhibited progress in cardiac tissue engineering.

Introduction

ADVANCES IN STEM CELL BIOLOGY have offered fresh hope for human myocardial regeneration after infarction. As one example, human myocardium has recently been formed in infarcted rodent hearts using human embryonic stem cell (hESC)–derived cardiomyocytes.[1–3] Unfortunately, small and highly variable cell-graft size currently limits the application of cell therapy for myocardial infarct repair.[4–6] Innovative methods to control intracardiac graft size would greatly enhance the efficacy of these therapies. Graft size is determined in large part by the percentage of cells retained in the myocardium after delivery, by the survival rate of those cells, and by their subsequent proliferation.[7–11] To date, most studies have injected enzymatically or mechanically dispersed cells directly into the injured left ventricular wall. This technique results in highly variable cell seeding efficiency, in which 0–90% of cells injected are successfully retained in the myocardium.[7] Further, only approximately 10% of cells that successfully engraft survive long-term in the heart, and injection of more cells simply results in greater cell death.[7,9–11] Cell delivery in a tissue-like structure that preserves cellular attachments could increase cell delivery efficiency and reduce cell death.[12]

Progress in cardiac tissue engineering, which seeks to generate myocardium-like tissue *in vitro* and then implant this tissue *in vivo*, provides a basis to improve technologies that control cell seeding efficiency and graft size.[13] Numerous groups have seeded embryonic or neonatal rat or chick cardiomyocytes into polymer or extracellular matrix scaffolds and gels[14–23] and demonstrated survival and engraftment of cardiac tissue constructs into rodent hearts.[15,16,20] Biomaterial-based and extracellular matrix–based tissue engineering, though promising, face several challenges: (1) polymer scaffolds and extracellular matrix–based xenograft materials elicit a host inflammatory response, (2) scaffold degradation products may release potentially toxic substances,[13,24] (3) scaffold compliance is often different from myocardial tissue,[25] and (4) cardiomyocytes often remain isolated from one another and do not form tissue that beats as a syncytium after forced seeding onto scaffolds.[13,25]

Scaffold-free tissue engineering seeks to create three-dimensional tissues without the use of polymers and exogenous matrix. Such tissues are composed only of cells and the matrix they secrete. Embryonic and neonatal cardiomyocytes from rat and chick hearts can form contractile, albeit small, spherical aggregates containing approximately 200 cells when cultured in suspension.[26,27] Scaffold-free neonatal rat cardiomyocyte sheets can be created and stacked to form interconnected three-dimensional tissues of up to 75 μm thickness.[28–31] To date, no macroscopic scaffold-free cardiac constructs have been created using human cardiomyocytes.

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Human cardiomyocytes derived from hESCs have recently been shown to successfully seed scaffolds[32] and to form very small (50–300 μm diameter) cell aggregates after culture in stationary suspension,[33] suggesting that these cells might be useful in cardiac tissue engineering. This work was limited by the laborious methods used to obtain hESC-derived cardiomyocytes and tissue constructs as well as by the small and variable size of cell aggregates. Treatment of human myocardial infarction, in which a billion cardiomyocytes may be lost in hundreds of thousands of patients annually,[13,34,35] will require the creation of tissue constructs composed of large numbers of human cardiomyocytes that can be mass produced with ease and consistency.

In the present study, we describe a new scalable method for creating macroscopic scaffold-free human cardiac tissue patches composed entirely of cells derived from hESCs. This technology overcomes several critical issues that previously inhibited the progress of cardiac tissue engineering.

Materials and Methods

Culture and derivation of cardiomyocytes from hESCs

The undifferentiated hESC H7[36] line (passages 43–53) was cultured in mouse embryonic fibroblast–conditioned media on tissue culture dishes coated with Matrigel (Growth Factor Reduced Matrigel; BD Biosciences, San Jose, CA).[1,6,37,38] The culture medium conditioned by mouse embryonic fibroblasts consisted of 80% Knockout Dulbecco's modified Eagle's medium (KO-DMEM; Invitrogen, Carlsbad, CA), 20% serum replacement, 1 mM L-glutamine (Gibco, Carlsbad, CA), 1% nonessential amino acids, 0.1 mM β-mercaptoethanol (Sigma, St. Louis, MO), and 8 ng/mL of basic fibroblast growth factor. Undifferentiated hESCs received media replacements daily and were passaged weekly (split 1:3 to 1:12).

hESC-derived cardiomyocytes were derived using the protocol recently described by Laflamme et al.,[1] which routinely yields greater than 30% cardiomyocytes.[1] Briefly, undifferentiated hESCs were dispersed to single cells using versene and mechanical trituration and then seeded onto Matrigel-coated plates at a density of 100,000 cells/cm. When the cells reached tightly packed confluency (typically about 5 days after plating), differentiation was induced by addition of activin A (50–100 ng/mL; R&D Systems, Minneapolis, MN) for 24 h followed by BMP4 (10 ng/mL; R&D Systems) for 4 days in RPMI medium (Gibco) supplemented with 1×B27 supplement (Gibco). Medium was then replaced every other day with RPMI-B27 until beating foci, indicative of cardiomyocyte differentiation, appeared.

System to create human cardiac tissue patches

Thirteen to 17 days after the addition of activin A to hESC cultures, differentiated hESC-derived cells were enzymatically dispersed using TrypLE (Gibco) and resuspended in high-serum medium (80% KO-DMEM, 1 mM L-glutamine, 0.1 mM β-mercaptoethanol, 1% nonessential amino acids stock, and 20% fetal bovine serum [Biomeda, Foster City, CA]) at a concentration of 0.25×10^6–3×10^6 cells/mL. Four milliliters of cell solution (1×10^6–12×10^6 cells) per well was cultured in suspension using a Teflon-coated ultra-low attachment six-well plate (Corning, Lowell, MA). Plates were placed on a rotating orbital shaker (1.9 cm orbital radius around center of gyration,

40 rpm; Barnstead, Dubuque, IA) at 37°C in a tissue culture incubator. Control plates were not rotated. The time at which cells were initially plated in suspension culture and placed on the rotating orbital shaker will be referred to as day 0.

Experiments to control patch size

Human cardiomyocytes were removed from activin/BMP culture 13 days after the addition of activin A, resuspended in 4 mL of high-serum medium, and cultured in low attachment plates (1, 4, 8, or 12 million cells per well; $n = 3$ for each group). Plates were placed on a rotating orbital shaker as described above. After 2 days, high-serum medium was carefully aspirated and replaced with PBS. Cardiac tissue patches were photographed, and diameter was measured.

Histological time course and bromodeoxyuridine incorporation experiments

HESC-derived cardiomyocytes were removed from differentiation culture 13 days after the addition of activin and seeded into rotating suspension culture at a concentration of 6×10^6 cells/well in high-serum medium (day 0). On days 2, 4, 8, and 11 ($n = 5$, 2, 3, and 3, respectively), patches were rinsed twice with PBS, fixed overnight in methanol and 10% v/v acetic acid, and then routinely processed for paraffin embedding and subsequent histological examination.

To test whether cardiomyocyte and noncardiomyocyte cell populations in patches were proliferative, six-well low attachment plates were seeded with 6×10^6 cells as described above. Patches were incubated with bromodeoxyuridine (BrdU, 10 μM; Roche, Nutley, NJ) for 24 h before fixation in methanol and 10% acetic acid on days 2, 4, 8, and 11 ($n = 2$, 2, 3, and 3, respectively) and routinely processed for histology.

Immunohistochemistry and microscopy

Sections parallel or perpendicular to the major patch surface area plane were cut from paraffin-embedded patch blocks. Sections were stained with hematoxylin and eosin. Patch thickness was measured from cross sections (sections perpendicular to major plane) using a Nikon 80i microscope equipped with an ocular reticle. Sections in a plane parallel to the major patch surface area plane were stained with a variety of antibodies to label cell populations. Sections were blocked with 1.5% normal goat serum in PBS and then incubated with primary antibodies against β-myosin heavy chain (β-MHC; clone A4.951; 1:10 dilution of hybridoma supernatant; ATCC) and/or Nkx2.5 (anti-human goat polyclonal, 1:1000 dilution; R&D Systems) overnight at 4°C to identify cardiomyocytes. Noncardiac graft cells were similarly identified by various histological markers, including pan-cytokeratin (epithelium; AE1/AE3, 1:150 dilution; Dako, Carpinteria, CA), α-fetoprotein (endoderm, 1:25,000 dilution; Dako), and β(III)-tubulin (neurons, 1:20,000 dilution; Sigma).[1,6,39] Sections were incubated with the appropriate species-specific secondary antibody for 1 h. Substrate-based staining was achieved using the chromagen diaminobenzidine (DAB, brown staining; Vector Laboratories, Burlingame, CA). All slides were counterstained with hematoxylin to mark all cell nuclei. Nkx2.5 and β-MHC double staining was performed by incubating slides with the antibody against Nkx2.5 followed by a biotinlyated secondary horse-anti-goat

secondary antibody (Jackson Laboratories, West Grove, PA) and streptavidin Alexa 555 (Invitrogen). Subsequently, the β-MHC primary antibody was followed by an Alexa 488-conjugated rabbit anti-mouse secondary (Invitrogen), and counterstaining was performed with Hoechst nuclear dye. To quantify the percentage of cells positive for each marker, cells were first binned into one of two categories. Cells on the patch edge were defined as within 200 μm of the edge of the patch, and cells in the patch center were greater than 200 μm from the patch edge. All positive and negative cells in each of 10 randomly selected 40× fields of view for patch edges and five 40× fields of view for patch centers were counted. Percentage of total positive cells was determined for each histological marker.

To identify proliferating cardiomyocytes, graft sections were double stained for β-MHC expression and BrdU incorporation. Sections were blocked and incubated with a primary antibody against β-MHC. β-MHC antibody binding was visualized with the chromagen Vector Red (Vector Laboratories), resulting in red cytoplasmic staining. Sections were incubated in 1.5 N HCl for 15 min at 37°C for BrdU antigen retrieval and then washed in 0.1 M borax buffer (pH 8.5) in PBS for neutralization. Sections were incubated with horseradish peroxidase–conjugated anti-BrdU antibody (1:25; Roche) overnight at 4°C. Nuclear incorporation of BrdU was visualized with DAB. Sections were counterstained with hematoxylin. BrdU-positive β-MHC double-positive nuclei were counted and recorded as a percentage of
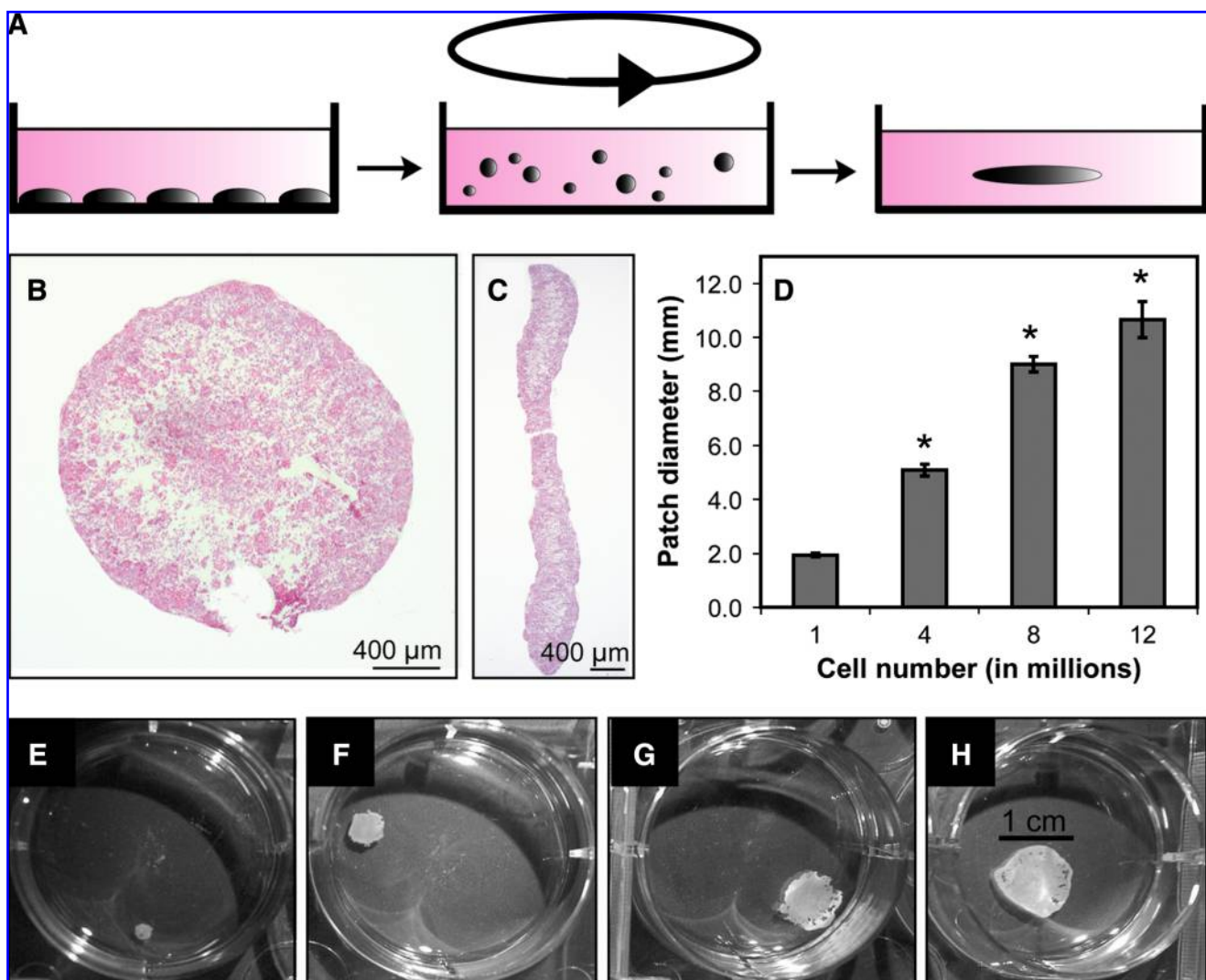

FIG. 1. Novel system to create scaffold-free human cardiac tissue patches of controlled size. HESCs were differentiated toward the cardiac lineage using activin A and BMP4 in adherent culture (**A**, left). At 13–17 days after addition of activin A, hESC-derived cardiomyocytes were removed from adherent differentiation culture, replated into suspension culture, and placed on a rotating orbital shaker (**A**, center). After 2 days of rotating suspension culture (day 2), hESC-derived cardiomyocytes aggregated to form macroscopic masses of beating human tissue (**A**, right). Representative hematoxylin and eosin–stained patches demonstrated a typical patch section parallel to the major patch plane (**B**) and cross section (**C**; section perpendicular to patch plane). Patch size increased (**D**; *$p < 0.05$ for 1 vs. 4, 8, or 12 million cells) with the addition of 1, 4, 8, or 12 million cells (**E–H**, respectively) to culture.

β-MHC–positive nuclei for all edge and center 40× fields of view. Photographs of stained patch sections were obtained using a light microscope (Nikon 80i, Tokyo, Japan) and QColor3 camera (Olympus, Center Valley, PA) or a confocal microscope (Zeiss LSM510 META, Thornwood, NY).

RNA Isolation and Quantitative RT-PCR

Human cardiomyocytes were removed from differentiation culture 17 days after the addition of activin A and seeded at a concentration of 2×10^6 cells per well in high serum medium. Triplicate patches were harvested after 2 or 10 days of culture on a rotating orbital shaker. RNA was isolated from each patch using an RNeasy Mini kit (Qiagen, Valencia, CA). Quality of isolated RNA was assessed by agarose gel electrophoresis. One microgram of total RNA per reaction was primed with random hexamers and reverse transcribed using Superscript II Reverse Transcriptase (Invitrogen). cDNA was amplified with a SensiMix DNA Kit containing SYBR green (Quantace, Norwood, MA) using primers for β-MHC (5′-GGGCAACAGGAAAGTTGGC-3′/5′-ACGGTGGTCTCT CCTTGGG-3′), Nkx2.5 (5′-CCAAGGACCCTAGAGCCG AA-3′/5′-ATAGGCGGGGTAGGCGTTAT-3′), AFP (5′-ACT GAATCCAGAACACTGCATAG-3′/5′-GCTTCTTGAACAA ACTGGGCAAA-3′), and HPRT (5′-TGACACTGGCAAAA CAATGCA-3′/5′-GGTCCTTTTCACCAGCAAGCT-3′). The copy number for each transcript was expressed relative to that of HPRT, which was used as an internal control, and normalized to a value of 1.0 at the day 2 (earliest) time point.

Confocal imaging of intracellular calcium concentration

HESC-derived cardiomyocytes were seeded at a concentration of 3×10^6 cardiomyocytes per well in high serum medium. After 2 days of culture on a rotating orbital shaker, cardiac tissue patches were loaded with 1 μL Fluo-4 AM solution (Molecular Probes, Carlsbad, CA) for 30 min. The incubation medium was removed, and patches were rinsed twice and then incubated in HEPES-modified Tyrode's solution (1.8 mM $CaCl_2$, 1.0 mM $MgCl_2$, 5.4 mM KCl, 140 mM NaCl, 0.33 mM NaH_2PO_4, 5 mM Glucose, 10 mM HEPES) for 30 min before microscopy to ensure complete de-esterification of the dye. Patches were imaged at room temperature using an inverted microscope (Zeiss LSM 510 Meta) coupled to a confocal laser scanning system (Zeiss LSM 510 Laser Module) with a Fluar 5×/0.25 objective. Fluo-4 AM was excited at 488 nm wavelength and filtered using a 505–550 nm band pass filter. Fluorescence was acquired in full-frame or line-scan mode. For full-frame images, pixel size was 4.97 μm and image size was 512×512 pixels. For line scans, patches were scanned in a line spanning patch surface area and perpendicular to the propagation of spontaneous intracellular calcium transients. Line scans had a temporal resolution of 3.07 ms per line and pixel size of 4 μm. Temporally sequential line scans were stacked vertically to produce line-scan images such that the horizontal axis designated distance along the patch scan line and the vertical axis designated time.

Statistical analysis

Statistical significance ($p < 0.05$) was determined using a two-tailed Student's t-test assuming unequal variance. Error bars represent standard error of the mean.

Results

Novel scaffold-free system for creating beating human cardiac tissue patches

We first sought to develop a scaffold-free technique to create macroscopic human cardiac tissue patches composed of cells derived from hESCs (Fig. 1A). After 2 days in suspension culture on the rotating orbital shaker, cells aggregated in the center of each well to form macroscopic discoid masses of tissue, which will be referred to as "human cardiac tissue patches" or simply "patches." Representative hematoxylin and eosin–stained patches sectioned in a plane parallel (Fig. 1B) or perpendicular (Fig. 1C, cross section) to the major patch plane show that the patches were typically discoid in geometry. Patches in some experiments had a shallow cup–like shape. In all conditions, patch thickness ranged from 300 to 600 μm and was not always homogeneous across a given patch (Fig. 1C). Rather, patch cross sections were sometimes slightly thicker at the edges and thinner in the center (Fig. 1C). Human cardiac tissue patches beat spontaneously and synchronously in culture (Supplemental Movie) until the termination of the experiment.

Control of human cardiac patch size

One advantage of conventional scaffold-based cardiac tissue engineering is the ability to control size and shape of engineered constructs.[13] We sought to test whether changing the number of hESC-derived cardiomyocytes placed into initial rotating suspension culture would result in different sized patches. Initial seeding of 1, 4, 8, or 12 million cells into rotating suspension culture resulted in patches with correspondingly increasing patch diameters that were remarkably consistent in size for each cell dose (Fig. 1B–F; $p < 0.05$ between 1 vs. 4 million cells and 4 vs. 8 or 12 million cells). Diameter ranged from 1.9 ± 0.1 mm to 10.7 ± 0.7 mm for 1 million to 12 million cells, respectively. We also noted that additional hESC-derived cardiomyocytes added to existing patches in suspension culture on day 2 did not incorporate into existing patches but rather formed small independent cardiac body aggregates similar to those formed in stationary suspension (data not shown). This likely indicates that intercellular adhesion molecules are no longer expressed on the surfaces of patches by day 2. Thus, varying the number of cells placed into rotating suspension culture provided a reproducible means to control patch diameter.

Increased cardiomyocyte purity with culture time

We next determined the cardiomyocyte composition of the beating tissue patches. Sections parallel to the major patch plane were stained with an antibody against β-MHC to identify cardiomyocytes and counterstained with hematoxylin to identify the nuclei of all cells. β-MHC transcript abundance was also determined by quantitative RT-PCR. Cardiomyocyte purity at patch edges increased over twofold between days 2 and 11 of rotation such that $79.1 \pm 9.9\%$ of cells were cardiomyocytes by day 11 (Fig. 2; $p < 0.05$ for day 2 vs. days 8 and 11). Cardiomyocytes appeared immature after only 2 days of patch culture, exhibiting very little cytoplasm or myofibrillar structure. Conversely, cardiomyocytes in patches after 8 or 11 days of culture exhibited increased myofibrillar organization, suggesting that they had reached
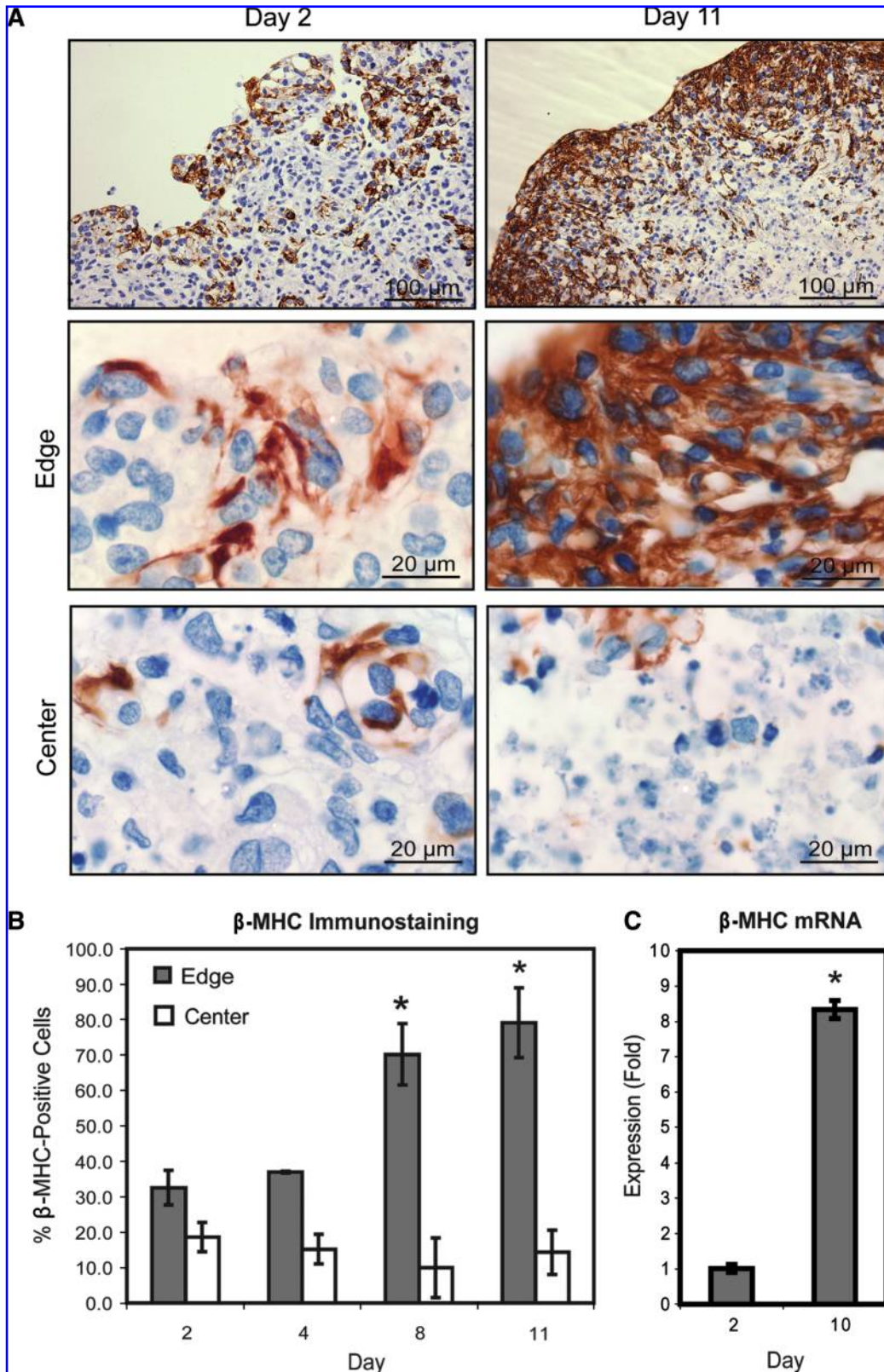
FIG. 2. Cardiomyocyte purity increases with extended culture time. Patches were sectioned parallel to the major patch plane, stained with an antibody against β-myosin heavy chain (brown) to identify cardiomyocytes, and counterstained with hematoxylin (**A**). After 2 days of culture, patch edges contained immature cardiomyocytes that exhibited very little cytoplasm and myofiber structure. Patch edges contained a highly enriched cardiomyocyte population exhibiting increased myofiber organization after 8 and 11 days of culture. Patch centers contained a noncardiac cell population at early culture time points (days 2 and 4). This population was largely replaced with fragmented cell nuclei characteristic of necrosis at later time points (days 8 and 11). The percentage of β-MHC–positive cardiomyocytes at patch edges increased significantly with increasing culture time (**B**; *$p < 0.05$ for day 2 vs. days 8 and 12). β-MHC mRNA expression determined by quantitative RT-PCR also increased significantly with culture time (**C**; *$p < 0.05$ for day 2 vs. day 10).
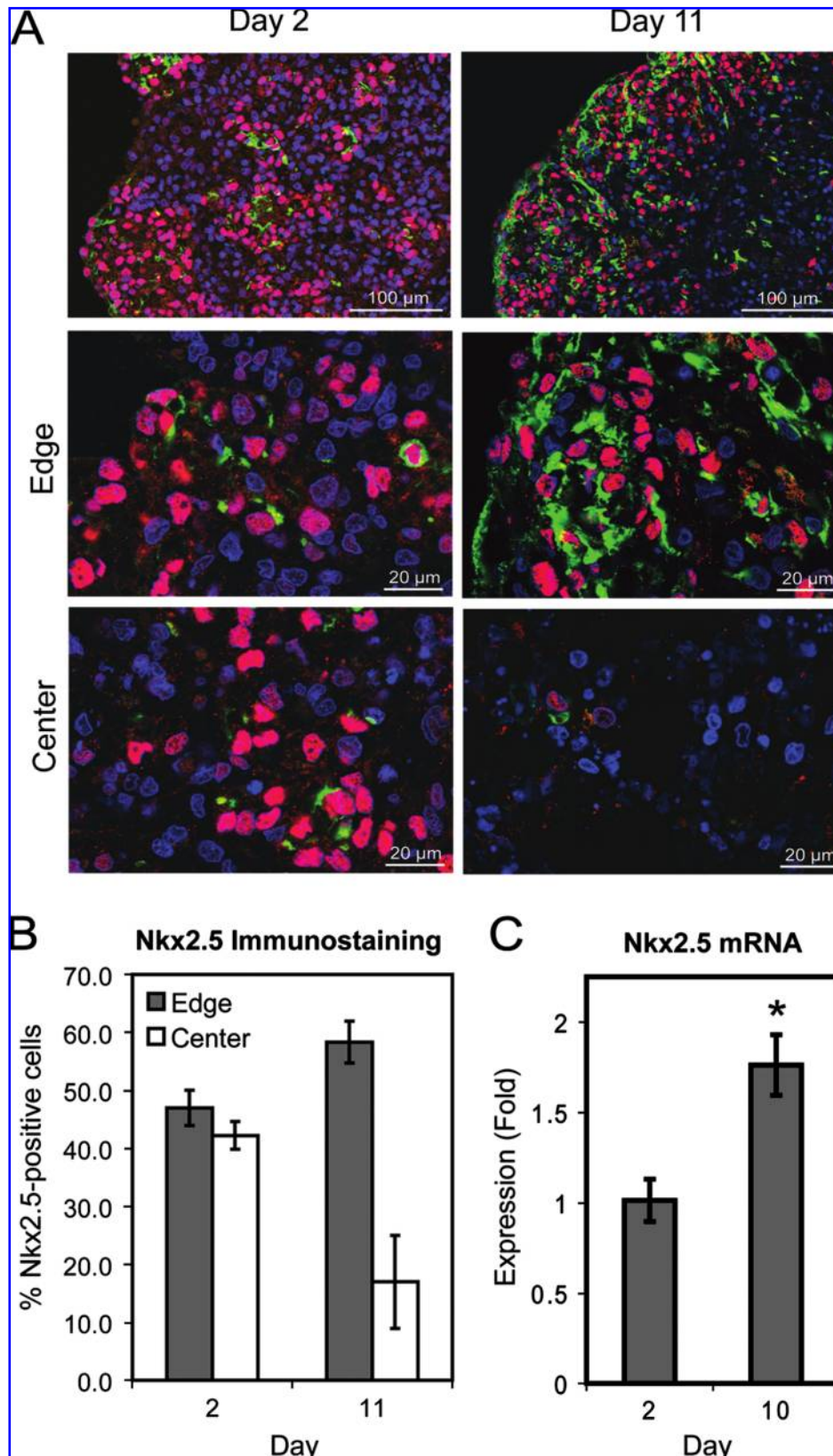
FIG. 3. Cardiac progenitors mature over time. Patch sections were stained with antibodies against Nkx2.5 (pink nuclei) and β-myosin heavy chain (β-MHC, green) to identify mature cardiomyocytes and progenitor cells (**A**). After 2 days, patch edges and centers were characterized by a large population of Nkx2.5-positive cardiomyocyte progenitor cells that were negative for β-MHC. Most Nkx2.5-positive cells at patch edges were double positive for β-MHC by day 11. Patch centers contained fragmented cell nuclei by day 11. The Nkx2.5-positive cardiomyocyte population comprised over 40% of the total cell population at both patch edges and centers at day 2, and the percentage of Nkx2.5-positive cardiomyocytes at patch edges increased slightly with culture time (**B**). Nkx2.5 mRNA expression determined by quantitative RT-PCR increased modestly with culture time (**C**; *$p < 0.05$ for day 2 vs. day 10).

a more mature state of differentiation. Cardiomyocyte purity at patch centers did not change with increased culture time and averaged $14.5 \pm 1.8\%$. Patch centers contained a robust cell population that was predominantly noncardiac at early culture time points and principally necrotic cell debris at later time points (Fig. 2). Similar to the increase observed in β-MHC protein with culture time, β-MHC transcript abundance increased over eightfold between days 2 and 10 ($p < 0.05$). Thus, human myocardial patches self-enriched over time.

Cardiac progenitor population matures with culture time

The absence of developed myofiber structure at early time points led us to hypothesize that early patches may contain a cardiomyocyte progenitor population that does not yet express β-MHC. To test this, we compared patches that were cultured for 2 or 11 days. Patch sections were stained using antibodies against both Nkx2.5 (a transcription factor expressed in both mature cardiomyocytes and cardiomyocyte progenitor cells; pink nuclei) and β-MHC (expressed only in mature cardiomyocytes; green) (Fig. 3). Alternatively, patch lysates were collected and Nkx2.5 transcript abundance was determined by quantitative RT-PCR. A large population of Nkx2.5-positive cardiomyocyte progenitor cells was observed at both the edge and the center of patches at day 2 ($47.0 \pm 3.1\%$ and $42.2 \pm 2.4\%$, respectively). Many of the Nkx2.5-positive cells were negative for β-MHC, suggesting that these cells had not yet matured to express β-MHC. Further, the percentage of Nkx2.5-positive cells in patch edges increased only modestly (from $47.0 \pm 3.1\%$ to $58.3 \pm 3.6\%$), while the β-MHC–positive cells increased more sharply (Fig. 2) between days 2 and 11. A similar trend was found in transcript abundance, in which Nkx2.5 transcript increased modestly (approximately 1.8-fold) between days 2 and 10 ($p < 0.05$), while β-MHC transcript abundance increased more substantially between days 2 and 10 (8-fold; Fig. 2). Cardiac tissue patches at early time points were thus composed of a high percentage of Nkx2.5-positive cardiomyocyte progenitor cells that had not been identified by β-MHC single staining. These cells matured and expressed β-MHC with increasing time in patch culture.

Decrease of noncardiomyocyte cell elements over time

HESCs can give rise to cells of all three germ layers, which could confound the therapeutic advantage of a myocardial patch. To identify noncardiac cell elements in the patches, sections were stained with antibodies against cytokeratin (an epithelial marker), α-fetoprotein (an endodermal marker), and βIII tubulin (a neuroectodermal marker). Patch lysates were also collected and analyzed for α-fetoprotein transcript abundance. A substantial cytokeratin-positive cell population was present in both patch edges and centers at day 2 (Fig. 4; $46.7 \pm 3.8\%$ at edge and $44.0 \pm 4.5\%$ at center). This contaminating cell population was significantly depleted throughout the patch with increasing culture time so that by day 11 it was nearly absent ($2.8 \pm 0.9\%$ at edge and $1.6 \pm 0.6\%$ at center; $p < 0.05$ for day 2 vs. day 11 for edge and center). Patches also contained a smaller α-fetoprotein–positive cell population at day 2 that comprised $3.2 \pm 0.9\%$ and $2.1 \pm 0.6\%$ of cells at edges and centers, respectively. Similar to cytokeratin-positive cells, this α-fetoprotein–positive

population disappeared almost entirely by day 11 of patch culture ($0.1 \pm 0.0\%$ at patch edge and $0.0 \pm 0.0\%$ at patch centers for day 11.) Less than 0.3% of cells in patch edges and centers for all time points were βIII tubulin positive. Similar to the protein data, transcript abundance of α-fetoprotein also decreased over threefold with time ($p < 0.05$). Taken together, these results showed that epithelial, endodermal, and neuroectodermal cell populations were depleted from patches with increasing culture time, which contrasted markedly with the enrichment observed for cardiomyocytes.

Proliferation of patch cardiomyocytes

Cellular proliferation after engraftment *in vivo* results in larger graft size and thus can be therapeutically advantageous. Unlike adult or neonatal cardiomyocytes, hESC-derived cardiomyocytes retain the ability to proliferate.[38,40] To test whether cardiomyocytes in human cardiac tissue patches were proliferative, patches were incubated with BrdU for 24 h. Immunostaining was performed using antibodies against β-MHC and BrdU. Representative patches in which both cardiomyocytes (red cells) and noncardiomyocytes were synthesizing DNA (cells with brown BrdU-positive nuclei) are shown in Figure 5. Human cardiomyocytes at the edges of human cardiac tissue patches were proliferative through 11 days of culture, but the percentage of proliferating cardiomyocytes decreased between days 2 and 11 ($5.4 \pm 1.1\%$ and $1.4 \pm 0.6\%$ at days 2 and 11, respectively). Cardiomyocytes at patch centers were generally less proliferative compared to edges, and proliferation in the center ceased entirely by day 11. Noncardiomyocyte proliferation decreased significantly at both the edge and center after 2 days of culture ($p < 0.05$). Cardiomyocyte proliferation was thus observed throughout the patches at day 2 after patch formation and concentrated to patch edges by days 8 and 11.

Cardiac tissue patches exhibit synchronous calcium transients

Electrical excitation of cardiomyocytes in normal cardiac tissue triggers intracellular calcium release and subsequent muscle contraction. Myocardium is electromechanically coupled such that synchronous activation occurs across the entire tissue with each beat. To test whether cells in the cardiac tissue patches were coupled, patches were cultured for 2 days on the rotating orbital shaker, loaded with the calcium indicator dye Fluo-4 AM, and imaged using confocal microscopy (Fig. 6). Increases in Fluo-4 AM fluorescence swept synchronously across the patch with each spontaneous beat (see Supplemental Movie). A representative full-frame confocal image of a patch loaded with Fluo-4 AM is shown in Figure 6A. Note the calcium wave sweeping approximately a third of the way across the patch. To gain temporal resolution for quantification of coupling, a single line across the patch (depicted by the red line in Fig. 6A) was scanned repeatedly over time. Each spontaneous beat resulted in a synchronous increase in Fluo-4 fluorescence across the entire patch. These results indicate that patch cells are electromechanically coupled.

Discussion

This study describes the first system for creating scaffold-free human cardiac tissue patches. These patches beat
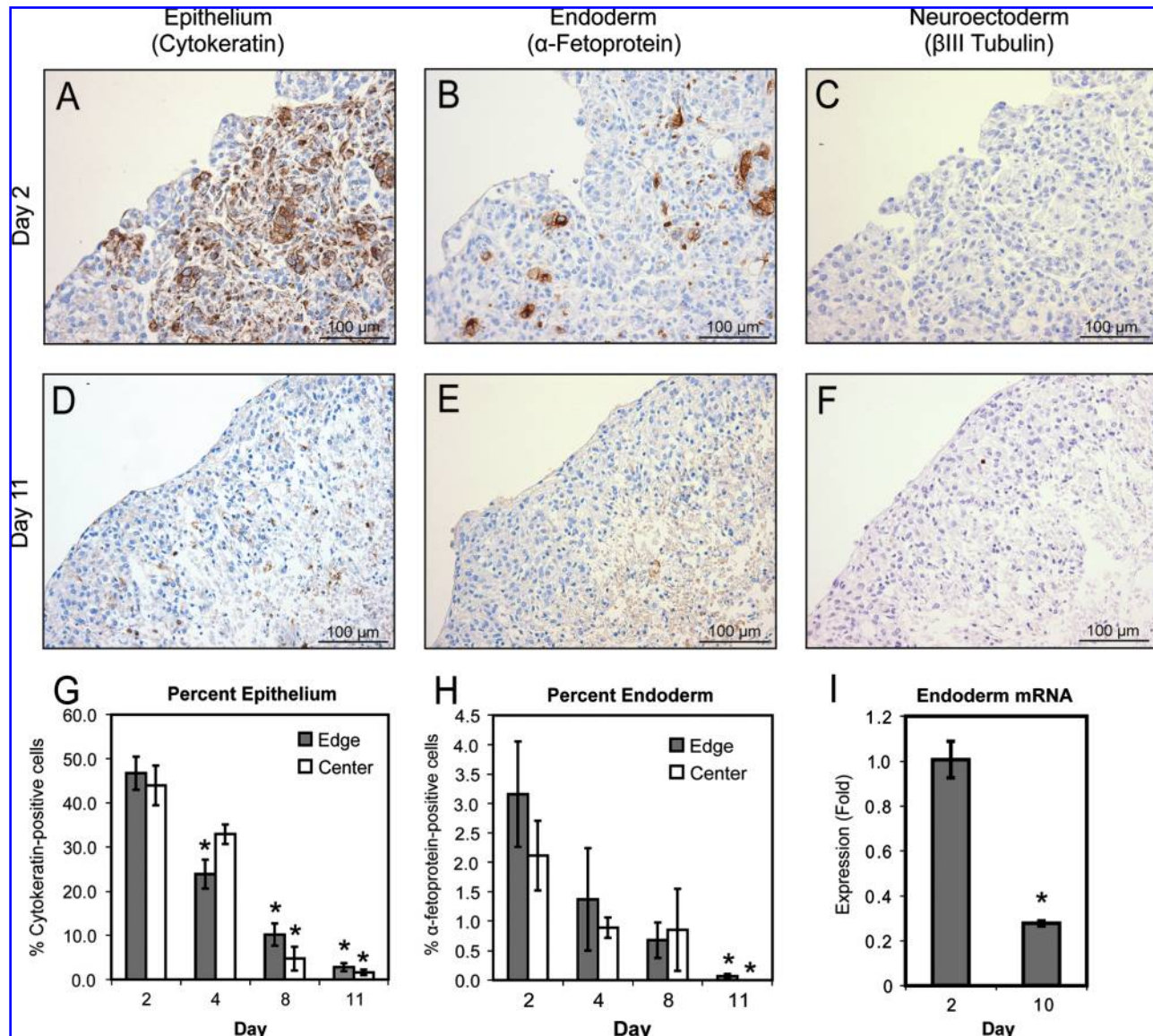
FIG. 4. Loss of contaminating cell elements over time. Patch sections were stained with antibodies against cytokeratin (epithelium), α-fetoprotein (endoderm), and βIII tubulin (neuroectoderm) to identify noncardiac cell elements in patches. Representative sections of patches fixed after 2 days of culture and stained for cytokeratin (**A**) or α-fetoprotein (**B**) show a substantial cytokeratin-positive cell population and a smaller α-fetoprotein–positive cell population. βIII tubulin–positive cells comprised less than 0.3% of the total cell population at day 2 (**C**). Patches cultured for 11 days show virtually no cytokeratin (**D**), α-fetoprotein (**E**), or βIII tubulin–positive (**F**) cell populations. Contaminating epithelium decreased significantly (**G**; $p < 0.05$ for day 2 vs. days 4, 8, and 11 at patch edge; $p < 0.05$ for day 2 vs. day 8 and 11 at patch center) with increasing culture time. Contaminating endoderm disappeared nearly entirely by day 11 of patch culture (**H**; $p < 0.05$ for day 2 vs. day 11 for both patch edge and center). Contaminating α-fetoprotein (endoderm) transcript decreased significantly with culture time (**I**; *$p < 0.05$ for day 2 vs. day 10).

spontaneously, and their size can be controlled precisely by varying the number of cells initially placed into culture. Cardiomyocytes were concentrated around patch edges, and cardiomyocyte purity and maturation increased with increasing culture time. Contaminating noncardiac cell elements were prevalent after 2 days of culture but almost entirely absent after 11 days of rotary suspension culture. Cardiomyocyte proliferation was observed throughout the patches after 2 days of culture and concentrated to patch edges by day 8. Patches transmitted synchronous calcium

transients, indicating that patch cells were electromechanically coupled.

The system developed in this study differs from previous work in two major ways. First, the majority of cardiac tissue engineering work rests on the foundation of using exogenous scaffolds or extracellular matrix–based materials in addition to cells. The scaffold-free system described in this study should reduce or eliminate some of the drawbacks associated with exogenous materials (e.g., foreign body reaction, toxic degradation products, material mechanical compliance that
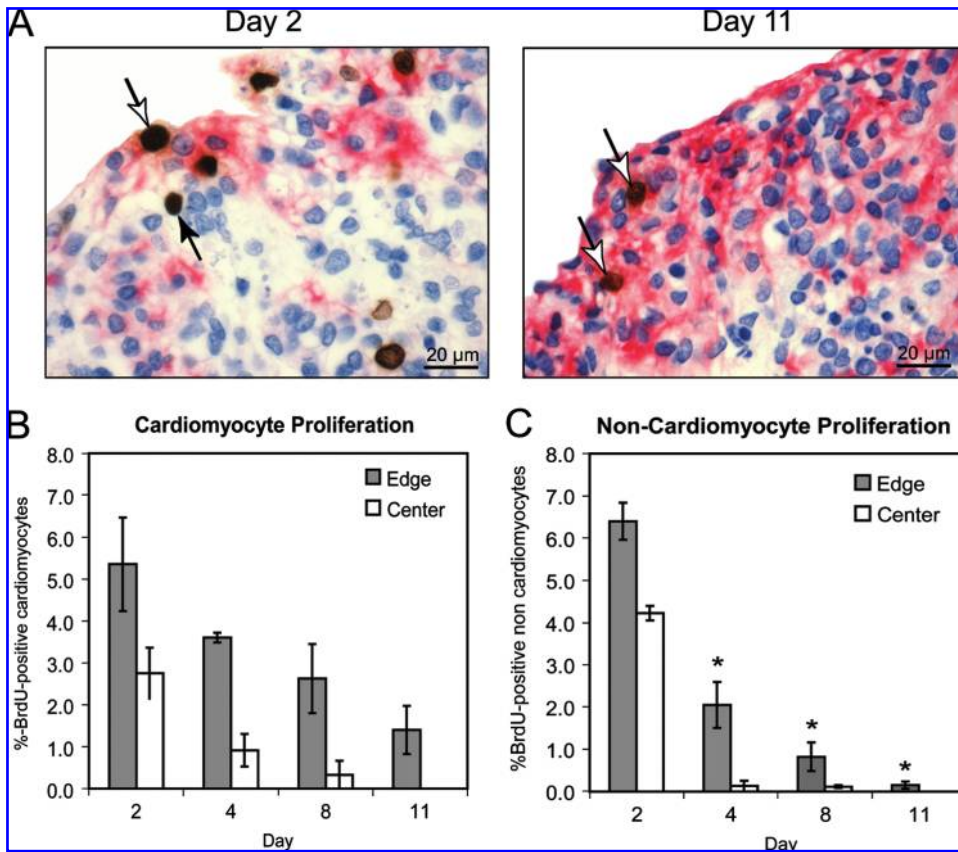
FIG. 5. Patch cardiomyocytes are proliferative. Patches were cultured in the presence of BrdU for 24 h. Sections were double-stained for β-myosin heavy chain (red; cardiomyocytes) and BrdU (brown; cells in S phase of cell cycle). Representative images of patches cultured from 2 and 11 days are shown (**A**; open and closed arrows point to representative proliferating cardiomyocytes and non-cardiomyocytes, respectively). Human cardiomyocytes at the edges of human cardiac tissue patches were proliferative through 11 days of culture, but the percentage of proliferating cardiomyocytes decreased between days 2 and 11 (**B**; not significant). Cardiomyocytes at patch centers were less proliferative than those at patch edges (**B**). Noncardiomyocyte proliferation was reduced significantly at both the edge and center culture periods that exceeded 2 days (**C**; *$p < 0.05$ for day 2 vs. days 4, 8, and 11 for both edge and center).
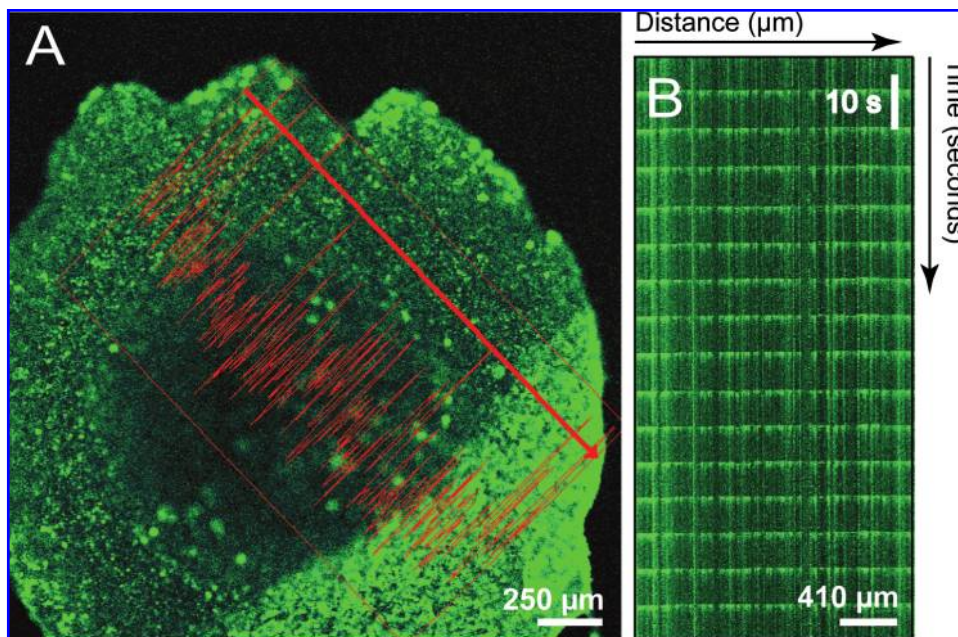

FIG. 6. Cardiac patches transmit synchronous intracellular calcium transients. Patches were loaded with the calcium indicator dye Fluo-4 AM and imaged using confocal microscopy. A representative full-frame patch image is shown (**A**). Note the simultaneous increase in Fluo-4 fluorescence that forms a line corresponding to a calcium wave occurring when the scan was approximately one-third of the way across the patch. The relatively darker center of the full-frame image was due to the slightly disc-shaped geometry of the patch such that the center was not in focus in this section. A line-scan image along the red line in (**A**) shows that increased Fluo-4 fluorescence occurs synchronously across the entire patch with each spontaneous beat, where each line of increased fluorescent intensity corresponds to a beat (**B**).

is stiffer than myocardium, and lack of uniform cell density associated with seeding on porous scaffolds[13,24,25]). In addition, scaffold-free cardiac tissue engineering work to date has utilized nonhuman cardiomyocyte populations as a proof of concept.[28–31] Use of human cells is an important step toward translation of engineered cardiac tissue to the clinic. hESC-derived cardiomyocytes possess numerous advantages for use in cardiac regeneration postmyocardial infarction: (1) hESCs can propagate indefinitely in the undifferentiated state in culture[36] and hence could be scaled for human therapy;[41] (2) hESC-derived cardiomyocytes have been shown to functionally couple with host myocardium after transplantation into pig and guinea pig hearts;[42,43] (3) recent advances in protocols to differentiate hESCs directly toward the cardiac lineage have made it possible to generate large quantities of hESC-derived cardiomyocytes.[1]

With regard to scalability, the scaffold-free system developed here is straightforward and efficient. It requires no equipment or materials other than standard tissue culture facilities and a rotating orbital shaker. Many dishes can be placed on one rotary shaker, providing capacity for the large-scale production of human cardiac tissue patches. Tailoring engineered patch size to need is commonly cited as an advantage of using solid material scaffolds, which can be manufactured in a predetermined size and shape, for tissue engineering.[44] Here, we have found that scaffold-free human cardiac tissue patch size could be consistently controlled by varying the initial number of cells seeded into culture. Patches described here ranged from approximately 0.2–1 cm in diameter, which is comparable to that of other cardiac tissue engineered constructs.[15,21,29,45]

As with patch size, we found that cellular composition between patches was consistent and reproducible. Importantly, the human cardiomyocytes proliferated in the patches, while noncardiomyocyte cell populations diminished with culture time, yielding patches composed of greater than 75% cardiomyocytes. Specifically, human cardiac tissue patch cardiomyocyte purity increased, while noncardiomyocyte cell populations decreased with culture time. Enrichment for cardiomyocytes was similarly found in a study in which cardiac bodies (approximately 50–300 μm diameter) created from hESC-derived cardiomyocytes were cultured in static suspension culture for more than 8 days.[33] The clearance of noncardiac elements such as epithelium over time in culture parallels the *in vivo* findings of Laflamme *et al.*, in which noncardiac elements were cleared after implantation into uninjured rat hearts.[6] Ongoing studies will work to further improve the purity of cell populations used to create human cardiac tissue patches and to rigorously assess whether such patches have any capability of forming teratomas or heterologous cell elements after implantation *in vivo*.

The composition of patches varied not only over time in culture, but also within each patch itself. Patch edges were substantially enriched for cardiomyocytes compared to patch centers, which largely contained necrotic cell debris. This agrees with previous cardiac tissue engineering work, in which the development of robust muscle reaching more than 100–200 μm into the engineered tissue is precluded by the diffusion limits of nutrients and oxygen.[13,25] Future studies will seek to improve tissue composition throughout the construct by (1) decreasing patch thickness to avoid cell ne-

crosis and/or (2) optimizing patch culture and composition biologically to reduce central necrosis. Patch thickness might be controlled, for example, by changing rotator rotational speed of the rotating orbital shaker, media viscosity, and/or particle size. Alternatively, patch culture and composition could be optimized to reduce necrosis biologically by optimizing patch culture medium, increasing cellular resistance to ischemia,[46] optimizing oxygen tension,[22,45] adding supporting noncardiomyocyte cell populations such as endothelial cells or fibroblasts,[23,32] or altering patch geometry such that surface area is increased relative to center area.[18]

Cardiomyocyte maturation also increased with culture time, as was evidenced by increasing expression of β-MHC and increasingly developed myofiber structure at later time points. Similarly, earlier studies suggest that embryonic chick or rat neonatal cardiomyocytes cultured in a collagen and/or matrigel matrix mature with increasing culture time. Specifically, Eschenhagen *et al.* and Zimmermann *et al.* found that the RNA yield and force of contraction generated by gel-casted embryonic chick and neonatal rat cardiomyocytes, respectively, increased with culture time despite decreasing proliferation rates.[17,21] We found a cardiomyocyte progenitor population that expresses Nkx2.5, an early cardiac transcription factor, but not β-MHC in human cardiac tissue patches. Such cells were prevalent throughout both the edges and centers of patches at early time points but were rare at later time points. It is possible that less-mature cardiac progenitor cell populations, which might be less aerobically active and more resistant to ischemia, could exhibit increased survival in the harsh ischemic environment. Development of systems in which the cardiomyocytes reproducibly mature over time in culture, such as in the present study, will allow testing of whether implantation of cardiac progenitor cell populations (e.g., Nkx2.5 positive and β-MHC negative) or more mature cardiomyocytes (e.g., β-MHC positive) will be optimal for cardiac repair applications.

Cell proliferation after implantation could expand patch size, allowing patches to be further scaled *in vivo.* In this situation, a smaller number of cells would be required in the initial creation of tissue constructs. Several groups have reported that hESC-derived cardiomyocytes are proliferative both in culture and after implantation into animals.[1,6,32,38,40] Here, we show that hESC-derived cardiomyocytes retain the ability to proliferate in scaffold-free human cardiac tissue patches after extended culture *in vitro.* Methods to control the proliferation of graft cells after implantation are under development.[47] Such methods could allow the expansion of human cardiac tissue patches in a controlled fashion after implantation such that host vasculature infiltrates and supports the engineered tissue as it grows.

Importantly, we found that patches were able to synchronously conduct calcium impulses. In normal cardiac tissue, electrical excitation of each cardiomyocyte triggers calcium release from intracellular stores and subsequent muscle contraction. Electromechanical interconnection of cardiomyocytes to their neighbors ensures that this process occurs in a synchronous fashion. Engineered cardiac tissue must also beat and conduct impulses synchronously if it is to optimally contribute to host contraction. Previous work has shown that rat neonatal cardiomyocyte sheets can conduct electrical impulses[29] and that collagen-embedded neonatal cardiomyocytes can integrate with host myocardium.[20] Here, we show

that scaffold-free human cardiac tissue patches synchronously transmit intracellular calcium transients and hence are electromechanically coupled. These data suggest that patches have the potential to connect with and synchronously transmit impulses with host human myocardium. The human cardiac tissue patches described herein could have significant impact on the development of drugs and therapies to treat human heart disease.

Summary

Scaffold-free human cardiac tissue patches may be useful in decreasing cell death and increasing cell retention compared to traditional methods of graft cell injection. Coupling of a simple, scalable technology for creating macroscopic scaffold-free patches with a large source of hESC-derived cardiomyocytes, as in the present study, could be broadly applicable to both cell grafting and drug development studies.

Acknowledgments

Disclosure Statement

No competing financial interests exist.

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