Three-Dimensional Culture Alters Primary Cardiac Cell Phenotype

Robert E. Akins, Jr., Ph.D.,¹ Danielle Rockwood, Ph.D.,^{1,2} Karyn G. Robinson, M.S.,¹
Daniel Sandusky, B.A.,¹ John Rabolt, Ph.D.,² and Christian Pizarro, M.D.³

The directed formation of complex three-dimensional (3D) tissue architecture is a fundamental goal in tissue engineering and regenerative medicine. The growth of cells in 3D structures is expected to influence cellular phenotype and function, especially relative cell distribution, expression profiles, and responsiveness to exogenous signals; however, relatively few studies have been carried out to examine the effects of 3D reaggregation on cells from critical target organs, like the heart. Accordingly, we cultured primary cardiac ventricular cells in a 3D model system using a serum-free medium to test the hypothesis that expression profiles, multicellular organizational pathways, tissue maturation markers, and responsiveness to hormone stimulation were significantly altered in stable cell populations grown in 3D versus 2D culture. We found that distinct multi-cellular structures formed in 3D in conjunction with changes in mRNA expression profile, up-regulation of endothelial cell migratory pathways, decreases in the expression of fetal genes (*Nppa* and *Ankrd1*), and increased sensitivity to tri-iodothyronine stimulation when compared to parallel 2D cultures comprising the same cell populations. These results indicate that the culture of primary cardiac cells in 3D aggregates leads to physiologically relevant alterations in component cell phenotype consistent with cardiac ventricular tissue formation and maturation.

Introduction

TISSUES AND ORGANS DEPEND ON complex, three-dimensional (3D), multi-cellular structures to function. Reestablishing these tissue architectures *ex vivo* or *in situ* is a central goal in tissue engineering and regenerative medicine. The heart is a principal target for research in these areas, but the effects of 3D organization on the cells that make up the heart are not well understood. Encouragingly, several groups^{1–12} have described the self-organization and neovascularization of 3D heart cell reaggregates *in vitro*. These studies demonstrate that populations of primary cardiac cells can reestablish aspects of tissue-level architecture when grown in supportive culture systems; however, the effects of 3D culture on the molecular phenotype of the component cells remain largely unexplored. With mounting evidence that multi-cellular organization has profound effects on cell function,^{13,14} there is a need to characterize associations between multi-cellular structure and cell phenotype *ex vivo* so that issues of cell responsiveness to different culture conditions can be included in the design of advanced scaffolds for tissue engineering.

Primary neonatal rat cardiac ventricle cells (NRCVCs) represent a useful and accessible model system for tissue engineering and molecular studies. Isolated NRCVCs comprise a

mixed population of cells representative of the intact tissue and include 75–80% cardiac muscle cells (cardiomyocytes [CMs]), which are widely recognized to be nonproliferative, with the remainder made up largely of endothelial cells (ECs) and cardiac fibroblasts,^{15–18} which are highly proliferative. The growth of such mixed cell populations in serum-containing media often leads to fibroblast overgrowth. Serum-free media, on the other hand, can severely restrict cell proliferation, and in the present study, NRCVCs were grown in a custom serum-free medium to investigate phenotypic differences associated with 3D versus two-dimensional (2D) culture systems. Specifically, we investigated alterations in multi-cellular architecture, differences in gene expression profile, and changes in hallmarks of ventricular tissue development and maturation, including (i) the distribution of ECs within 3D aggregates, (ii) the maturational status of the ventricular myocytes detected as decreases in fetal gene expression, and (iii) the responsiveness of the cellular system to hormonal stimulation by triiodothyronine (T₃).

Materials and Methods

Cell isolation and culture

NRCVCs were isolated using Neonatal Cardiomyocyte Isolation System kits (Worthington Biochemical, Lakewood,

¹Nemours Biomedical Research Center, A.I. duPont Hospital for Children, Wilmington, Delaware.

²Department of Materials Science and Engineering, University of Delaware, Newark, Delaware.

³Nemours Cardiac Center, A.I. duPont Hospital for Children, Wilmington, Delaware.

NJ) as in previous studies by our group.⁵ Viable cells were enumerated by Trypan-blue exclusion (Invitrogen, Carlsbad, CA). To support the inclusion of the same cell subpopulations in 3D and 2D cultures, Nunclon™ Δ-Surface tissue culture polystyrene (TCPS) were used in both cases for cell seeding: microcarriers were used as seeding surfaces to initiate 3D cultures and Nunclon Δ-Surface plates were used for 2D (Nunc/Thermo-Fisher Scientific, Rochester, NY). To encourage cell–cell interactions and to reduce cell proliferation by contact inhibition, cultures were generally inoculated at a high density (1×10^6 cells per 4.8 cm^2 of TCPS per mL of medium) although in some experiments, cultures were inoculated at a medium (0.5×10^6 cells per 4.8 cm^2) or low (0.2×10^6 cells per 4.8 cm^2) density to encourage fibroblast proliferation as noted in the text. In addition, a defined serum-free medium that had been used previously by our group (AI-1 medium)^{5,19} was employed to limit mitogenic effects from serum. AI-1 medium comprised a 1:1 mixture of Dulbecco's modified Eagle's and Ham's F-12 media, to which was added $1 \times$ modified Eagle's medium (MEM) nonessential amino acids, $0.1 \times$ MEM vitamin solution, 2.45 mg/mL sodium bicarbonate, 100 U/mL penicillin, 100 U/mL streptomycin (all from Invitrogen), 20 $\mu\text{g/mL}$ sodium ascorbate, 0.25 mg/mL fetuin, 0.0835 mg/mL calcium chloride, 0.001 $\mu\text{g/mL}$ sodium selenate, 1.35 mg/mL additional dextrose, 0.01 $\mu\text{g/mL}$ epidermal growth factor, 75 pg/mL triiodothyronine (unless otherwise indicated in the text), 0.05 $\mu\text{g/mL}$ hydrocortisone, 0.05 mU/mL insulin (all from Sigma-Aldrich, St. Louis, MO), 5 $\mu\text{g/mL}$ holo-transferrin (BD Biosciences, Bedford, MA), 10 mg/mL bovine serum albumin (BSA; Roche Diagnostics, Indianapolis, IN), and 1 μM palmitate (Sigma-Aldrich) pre-conjugated with 0.12 mg/mL fatty-acid-free BSA (Roche Diagnostics) as carrier. In addition to the use of fetuin in the medium, TCPS surfaces were coated with human fibronectin (BD Biosciences; 1 μg per cm^2) to encourage cell adhesion in the absence of serum. For 3D cultures, microcarriers were suspended in polytetrafluoroethylene VueLife™ vessels (American FluorSeal, Gaitersburg, MD) by rotation as in our previous work.⁵ Polytetrafluoroethylene culture vessels were rotated about their long axes at 30 rpm to suspend cells and microcarrier beads without excessive fluid shear, which can be damaging to cells.²⁰ Cultures were given a complete change of medium 24 h after initiation and at 96-hour intervals thereafter. Cultures were assessed throughout to assure spontaneous beating, which is characteristic of healthy NRCVC cultures.

Assessment of culture composition and cell distribution

Fluorescence microscopy was carried out using methods previously established by our group.^{5,6,21} In brief, parallel 2D and 3D culture samples were fixed in buffered, 2% paraformaldehyde (for sarcomeric myosin, vimentin, and BrdU staining) containing 0.1% Triton X-100 (Sigma-Aldrich) or -20°C methanol (for platelet/endothelial cell adhesion molecule [PECAM] staining), blocked with 3% BSA in phosphate-buffered saline. Samples were stained with *bis*-benzimidazole (Hoechst 33258, Sigma-Aldrich) to detect nuclei and anti-sarcomeric myosin heavy chain (MyHC; monoclonal A4.1025, Developmental Studies Hybridoma Bank, Iowa City, IA, to detect CMs), anti-PECAM (BD-Pharmingen, Franklin Lakes, NJ, to detect EC clusters), or anti-vimentin (monoclonal V9;

Sigma-Aldrich, to detect mesenchymal cells) with a Texas-Red-conjugated secondary antibody (Jackson Immuno Research, West Grove, PA). Selected samples were also stained with Alexa-Fluor-488-conjugated phalloidin (Invitrogen) to detect filamentous actin, which is dramatically elevated in CMs due to the presence of sarcomeres. Viable cell content was estimated using Cell Titer Blue Assays (Promega, Madison, WI) following the manufacturer's recommendations. To estimate cell proliferation by BrdU (5-bromo-2-deoxyuridine; Sigma-Aldrich) incorporation, cells were labeled with 100 μM BrdU for 6 h on day 4 of culture. DNA was then denatured with 2 N HCl for 30 min at 37°C followed by neutralization in 0.1 M borate buffer (pH 8.0) to expose BrdU. Anti-BrdU antibody conjugated to Alexa Fluor 594 (Invitrogen) was used for observation. BrdU-positive cells were enumerated in 10 randomly selected microscope fields at $200\times$ total magnification. Light microscopic observations were carried out using an Evolution QEi, 12-bit, cooled CCD camera (Media Cybernetics, Silver Spring, MD) mounted to an Olympus model BX-60 epi-fluorescence microscope operated using Image Pro Plus software (Media Cybernetics); fluorescence images were pseudo-colored for presentation. Scanning electron microscopy (SEM) was performed on a JEOL 840A scope operating at 10 kV. Samples for SEM were fixed at 4°C with 2% glutaraldehyde in 0.1 M cacodylate buffer, rinsed and dehydrated in graded acetonitrile solutions,²² critical point dried, and sputter coated with gold before observation.

Biochemical assays for creatine kinase, glucose-6-phosphate dehydrogenase, malate dehydrogenase, nicotinamide adenine dinucleotide (NAD)-dependent cytochrome-c reductase, and pyruvate kinase (PK) were carried out as previously described.^{21,23,24} Briefly, cells were rinsed and lysed to release cytosolic and mitochondrial enzymes. Specific activities were measured using direct or coupled assays and expressed in Units per mL of homogenate, with 1 U being defined as the amount of enzyme that reduces 1 nmol of substrate (NAD, NAD phosphate [NADP], or cytochrome c as appropriate for the enzyme) per mg of protein per minute. Protein and DNA levels in homogenates were determined using BCA (Pierce Chemical, Indianapolis, IN) and Hoechst 33258 (Sigma-Aldrich), and specific activities were calculated as Units of enzyme per mg of protein; the amount of protein per DNA was calculated as an index of hypertrophy.

Levels of MyHC, which is expressed in CMs but not in other cardiac-derived cells, and filamentous actin (f-actin), which is more abundant in CMs than in other cells due to the presence of sarcomeres, were quantified fluorimetrically and normalized to nucleic acid staining. To accomplish this, samples were fixed, rinsed, and stained as above to detect MyHC (A4.1025 antibody with a Texas-Red-conjugated secondary), f-actin (Alexa-Fluor-488-conjugated phalloidin), and nuclei (Hoechst 33258). Fluorescence levels were quantified using a Cytofluor 4000 fluorescence reader (Applied Biosystems, Foster City, CA).

Assessment of gene expression

Real-time quantitative polymerase chain reaction (PCR) was carried out using a single-color BioRad My-iQ detection system and iScript One-Step reverse transcription PCR Kits with SYBR Green (BioRad, Hercules, CA). Primers are listed in Table 1. RNA was isolated using TRI Reagent

TABLE 1. POLYMERASE CHAIN REACTION PRIMER PAIRS

Gene	Forward primer	Reverse primer
<i>Bmp-2</i>	CCATCACGAAGAAGCCATCG	AACCTGGTGTCCAATAGTCTGGTC
<i>Id-3</i> (25)	GTGATCTCCAAGGACAAGAGGA	GGGCTGTGGCAAGATCGA
<i>Myh-6</i>	AGAAGCACTTGAAGAACGCC	ATAGCAACAGCGAGGCTCTTTC
<i>Myh-7</i>	GAAGATCCGAAAGCAACTGG	TTTCAAAGGCTCCAGGTCTC
<i>Thbs-1</i> (26)	GACACACGACTGCAACAAGAA	GTCTCCCACATCATCTCTGTCA
<i>Tnni-3</i> (5)	GCTCGTGTGGACAAAGTGGATG	TTATTCCTCAGAGATGCCCTTCAG

All sequences are listed 5' to 3'. *Bmp-2*, bone morphogenetic protein 2; *Id-3*, inhibitor of DNA binding 3; *Myh-6*, myosin heavy chain 6/cardiac α ; *Myh-7*, myosin heavy chain 7/cardiac β ; *Thbs-1*, thrombospondin 1; *Tnni3*, cardiac troponin I.

(Molecular Research Center, Cincinnati, OH). For 2D cultures, 1.0 mL TRI Reagent was added to each 35 mm dish. Three-dimensional aggregates were collected in 15 mL conical tubes, and the medium was aspirated; 1 mL of TRI Reagent was added, and the sample vortexed. The TCPS support beads floated to the surface, and the volume of liquid below was collected in sterile Eppendorf tubes. The manufacturer's directions were followed for further purification of RNA. For analysis of α - and β -MyHC expression, multiplex reverse transcription PCR was employed due to the significant homology between the nucleotide sequences and the large size of the PCR products needed to discriminate the two isoforms. Briefly, 1 μ g of RNA was used for each 20 μ L reaction, which consisted of 5 mM MgCl₂, 2.5 mM dNTP mix, 20 units of RNasin, 12.5 μ g/mL Oligo dT 15, and 40 units of avian myeloblastosis virus reverse transcriptase (all from Promega). Ten microliters of the reverse transcription reaction was subsequently used in 50 μ L PCRs with Taq polymerase (5000 μ /mL; Invitrogen), and 25 pmol/ μ L of each primer as listed in Table 1. Reactions containing all four primers were run for 30 PCR cycles. The product was separated on 1% agarose gels and observed by ethidium bromide staining. The fluorescence intensity of each band was quantified using an Eagle-Eye gel documentation system (Stratagene, LaJolla, CA).

Expression data derived from Affymetrix chips used in a previous experiment⁵ were analyzed to identify differences in expression associated with 2D versus 3D culture using The Institute for Genomic Research Multiple Experiment Viewer (TMeV) version 4.3.02 from the TM4 microarray software suite.²⁵ Briefly, cells were grown in 2D and 3D culture for 1, 4, and 6 days in two separate experiments generating 12 separate samples. RNA was isolated using TRI Reagent (Molecular Research Center) with bromochloropropane and purified using RNEasy kits (Qiagen, Valencia, CA). Double-stranded cDNA was prepared using a T₇-oligo-dT primer (T₇-dT₂₄, Integrated DNA Technologies, Coralville, IA) and SuperScript Double Stranded cDNA Synthesis Kits (Invitrogen). T₇-polymerase *in vitro* transcription and biotin-labeling were performed using BioArray HighYield RNA Transcription Kits (Enzo Life Sciences, Farmingdale, NY). Labeled cRNAs were hybridized to GeneChip Rat Genome U34A (RG-U34A) Arrays (Affymetrix, Santa Clara, CA), washed, and stained with R-phycoerythrin streptavidin (Molecular Probes) using the GeneChip Fluidics Station 400 and the EukGE-WS2 fluidics protocol. Signals were acquired using an Affymetrix GeneArray Scanner, and numeric data were derived from raw data images and normalized using Microarray Suite 5 software (Affymetrix). Signals on each chip were mean centered to a value of 500 based on a scaling

factor calculated from the middle 96% of all signal values. The entire data set of 8799 test probesets across all samples was loaded into TMeV for analysis.

Assessment of atrial natriuretic peptide and cardiac adriamycin response protein expression

Atrial natriuretic peptide (ANP), which exhibits decreasing expression during ventricular CM maturation, was detected in cellular extracts as previously described²⁶ using a high-sensitivity enzyme immunoassay for rat ANP containing amino-acids 1–28 (Bachem, San Carlos, CA) following the manufacturer's recommendations. This assay detected total ANP including pre-pro-ANP, pro-ANP, and ANP. Expression of Ankrd1, which encodes cardiac adriamycin response protein (CARP; also called cardiac ankyrin repeat protein), another marker of immature CMs, was assessed by Western blot using a polyclonal antibody generously provided by Dr. Yimin Zou.²⁷ Cultures were rinsed, total protein was extracted using lithium dodecyl sulfate-containing sample buffer, and protein content was determined by bicinchoninic acid (BCA) assay. Equivalent amounts of protein (10 μ g) were separated on NuPage 4–12% Bis-Tris PAGE gels (Invitrogen) in NuPage MOPS Running Buffer (Invitrogen). Proteins were transferred to nitrocellulose membranes (GE-Amersham, Piscataway, NJ) in NuPage Transfer Buffer (Invitrogen) at 30 V for 1 h. Membranes were rinsed and blocked for 1 h in 8% nonfat dry milk (BioRad) and incubated overnight in 1:1000 polyclonal anti-CARP antibody followed by horseradish peroxidase-conjugated goat anti-rabbit secondary (BioRad). Bands were detected using enhanced chemi-luminescence (ECL) reagents from GE-Amersham (Piscataway, NJ) and Kodak Biomax-Light film (Rochester, NY).

Results

Organization of cells in 3D versus 2D culture

Populations of isolated NRCVCs contain all the cells found in the intact tissue in the same relative proportions.^{15,16,28} Since the adhesion of cells is affected by the physico-chemical nature of the culture substrate, we sought to assure that the same subpopulations of cells were present in the 2D and 3D systems employed here. Cultures were initiated on identical TCPS surfaces coated with fibronectin. Nonadherent cells were then removed by medium exchange. Performing this TCPS-based collection of cells using micro-carrier beads within rotating bioreactors resulted in the same degree of total cell adhesion and CM-specific adhesion as

FIG. 1. Assessment of homogeneity between two-dimensional (2D) and three-dimensional (3D) cultures. Samples were collected on day 1 or 6 of culture as indicated and assessed for bulk differences in culture composition, hypertrophy, and metabolic function. (A) Cell attachment efficiency was calculated as the number of cells attached to tissue culture polystyrene (TCPS) surfaces after 24 h divided by the total number of viable cells in the original inoculum. The proportion of attached cardiomyocytes (CMs), which are positive for myosin heavy chain (MyHC) immunostaining, per total adherent cells was also calculated. (B) On day 6, MyHC and filamentous actin (f-Actin) levels, which are indicative of CM hypertrophic status, were determined in fixed samples by fluorescence *in situ* quantitation assay and are presented as fluorescence ratios normalized to DNA. Total protein content, which is a key indicator of tissue hypertrophy, was measured in culture homogenates and normalized to DNA. Units presented are μg protein per 10 ng DNA. (C) Intermediary metabolic enzyme activities in day 6 cell extracts calculated as units of enzyme activity per mg of total protein per minute. No significant differences were found in (A), (B), or (C). Data are mean values $\pm$ standard deviation for $n = 6$ samples.

FIG. 2. Cell proliferation in the serum-free medium. (A) Effects of serum on relative cell numbers were estimated by Cell Titer Blue (CTB) assays performed at intervals over 13 days of 2D culture. Cells grown in the serum-containing medium ($\diamond$) showed increasing CTB results, whereas cells grown in the serum-free medium (Δ) showed stable CTB levels over the same timeframe. Data are mean values $\pm$ standard deviation for $n = 3$ samples measured repeatedly over time. Serum-free and serum-containing time courses were found to be significantly different by repeated measures analysis of variance ($p < 0.05$). (B) BrdU incorporation over 6 h on day 4 of culture. Both the absence of serum and the culture at higher initial cell density resulted in decreased cell proliferative activity. Data presented are mean values $\pm$ standard deviation for $n = 3$ samples each assessed across 10 random microscope fields.

that seen in control tissue culture plates (Fig. 1A). Three-dimensional cultures on TCPS beads and 2D cultures on TCPS plates collected on day 6 of culture were nearly identical in their sarcomeric MyHC, filamentous actin (f-Actin), and total protein content (Fig. 1B). Intermediary metabolic enzyme activities in extracts collected from day 6 cultures were also evaluated and found to be nearly identical in the two systems (Fig. 1C). These data indicate that the constituent cells comprising the 3D/bioreactor cultures and the 2D/plate cultures were the same.

Fibroblast overgrowth is a critical issue in NRCVC culture, and we sought to limit cell proliferation by using high seeding densities and by employing a serum-free medium. To verify that cell proliferation was held in check in the serum-free medium, Cell Titer Blue assays were used to estimate changes in the number of cells present in cultures over time. As seen in Figure 2A, the total number of cells present

FIG. 3. Distribution of cells in 3D aggregates. (A) Phase contrast image of a standard 2D culture on TCPS culture wells. Arrows indicate patches of elongated cells (lower arrow) indicative of cardiac muscle cells interspersed with nonmuscle cells (upper arrow). (B) Phase contrast image of 3D aggregates initiated on TCPS support beads. Arrow indicates typical 3D cell mass. Inset is a lower magnification view of a multi-cell/multi-bead aggregate. (C) Two-dimensional culture stained for MyHC to indicate CMs and DNA. Similar to (A), patches of cardiac muscle cells are interspersed with nonmuscle cells (arrows) in a mosaic pattern. (D) Three-dimensional culture stained for MyHC to indicate CMs and DNA. The exterior-most cells do not contain MyHC (arrows indicate nuclei of these cells). (E) Three-dimensional culture stained for vimentin and DNA, verifying the presence of mesenchymal/endothelial cells (ECs) on the aggregate surface (arrows); this staining is consistent with an EC layer. (F) Two-dimensional culture stained for platelet/endothelial cell adhesion molecule (PECAM) (CD31) and DNA showing an island of PECAM-positive ECs amidst PECAM-negative cells (arrow). (G) Three-dimensional culture stained for PECAM showing that exterior-most nuclei are within cells positive for PECAM (arrow), a marker for ECs in contact with each other. (H) Scanning electron micrograph (SEM) of a 2D culture. Lower arrow indicates an elongated, binucleate cell, which is consistent with a CM. Upper arrow indicates a mononucleated cell with a more spread morphology. Nucleoli are readily apparent. (I) SEM of a 3D culture surface showing only cells with an endothelial appearance are present (arrows). (J) SEM of the interior aspect of a rat heart lumen showing the configuration of the ECs lining the organ lumen. Bar = 50 μm except for inset figure in (B), where bar = 100 μm . Color images available online at www.liebertonline.com/ten.

FIG. 4. Relative adhesion of muscle versus nonmuscle cells. The proportion of CMs to non-CMs in cultures that were plated for (A) 5 min, (B) 15 min, (C) 30 min, or overnight (inset in C) and then rinsed was tabulated based on the proportion of MyHC-positive CMs to total nuclei present after 24 h of culture. It is well established that non-myocytes adhere more rapidly than myocytes in serum-containing media, and here we show that the same phenomenon occurs in our serum-free system. (D) Graph showing the percent of MyHC-positive (CMs) and MyHC-negative cells (non-CMs) present on TCPS as a function of time. Squares indicate non-CMs present at the time indicated divided by the total non-CMs after 16 h; diamonds indicate CMs present at the given time to total CMs present after 16 h; triangles indicate the percent of adherent cells that were CMs. Under the conditions used, all of the non-CMs stuck in ~45 min. In this timeframe, only about 10–15% of available CMs adhered; nevertheless, the cell population present at 45 min comprised about 30% CMs. Color images available online at www.liebertonline.com/ten.

in NRCVC cultures grown without serum remained relatively constant over a full 13 days, whereas cells grown with 10% fetal calf serum added to the AI-1 medium continually increased in number. To specifically quantify proliferation, cells were exposed to BrdU (Fig. 2B). NRCVCs seeded at a high density and those grown in the serum-free medium had significantly attenuated levels of BrdU staining compared to cells grown with serum or at a lower initial density. Human vascular smooth muscle cells (T/G-HA vSMC, CRL-1999, American Type Culture Collection, Manassas, VA), which were assayed as a positive control for a proliferative cell type, demonstrated the highest degree of BrdU incorporation.

As seen in Figure 3, the cells grown in bioreactor cultures for 6 days self-organized 3D masses that remained associated with the beads but were elevated from the microcarrier surfaces. These elevated cell masses approached a depth of 100 microns, and appeared between beads in all multi-bead aggregates; on average, clusters contained 8.2 (+5.2) TCPS beads based on a random sampling of 100 clusters. Overall, we saw no signs of necrosis within clusters. Three-dimensional architectures were typified by an exterior layer of ECs similar to the cardiac luminal epithelium. The same cells collected on TCPS in static, 2D cultures did not form such structures and were typified by isolated islands of ECs surrounded by other cell types in a mosaic-like pattern. In addition, observation of cultures at the SEM level indicated dramatic differences such that the superficial layer of ECs in 3D closely resembled the cardiac lumen in the appearance of cell junctions and surfaces, whereas 2D cultures exhibited prominent nuclei. The emergence of an EC sheet on the exterior of 3D aggregates that are principally made up of CMs was a striking and unexpected finding. In typical culture systems, nonmuscle cells like ECs adhere to TCPS surfaces more rapidly than CMs.^{29,30} This phenomenon forms the basis of the widely employed strategy of preplating to remove non-CMs from NRCVC suspensions before cul-

ture.^{31–33} We investigated the possibility that the absence of serum resulted in a reversal of the typical relative adhesion order using timed adhesion assays in which cells were allowed to adhere for the time indicated then were rinsed and allowed to spread overnight. Staining for MyHC and nuclei was carried out, and CM (MyHC-positive) versus non-CM (MyHC-negative) cells were enumerated. As seen in Figure 4, CMs adhered more slowly than non-CMs in our serum-free system just as they do in other cell culture systems.

Differences in EC migratory pathways and gene expression patterns in 3D versus 2D cultures

The appearance of ECs on the surface of 3D reaggregate cultures principally containing CMs suggested that cell migration may have taken place; accordingly, we assessed the activation of a critical EC migratory pathway in 3D versus 2D culture. Upregulation of Id3 and the associated Id1, which acts in concert with Id3 to control EC migration,^{34,35} is considered necessary and sufficient to mediate bone morphogenetic protein-induced EC migration, which functions via attenuation of thrombospondin (Thbs).³⁶ We, therefore, measured BMP, Id, and Thbs expression in our culture systems using real-time quantitative PCR. Fold differences were calculated using the $\Delta\Delta C_t$ method with Tnni3 (i.e., cardiac troponin I) as the loading reference within each sample and expression in freshly isolated cells as the baseline. As shown in Figure 5A, Bmp-2 levels increased slowly in the 2D control cultures reaching a peak on day 4, whereas Bmp-2 level increased dramatically on day 1 of 3D cultures and returned to near control levels on days 2 and 3 before peaking again on day 4. Id3 levels (Fig. 5B) in the 2D controls stayed low until day 4 when they started to rise, reaching a peak on day 5; in 3D, Id3 peaked on day 1 then again on day 5. Thbs levels (Fig. 5C) in the 2D samples were elevated on day 1, peaked on day 2, and then declined; Thbs in 3D cultures, on the other

hand, remained low on day 1, consistent with the elevated Id3, but peaked on day 2. These differences occurred within samples that had consistent levels of the Tnni3 housekeeping gene throughout the experiment (Fig. 5D) and suggest that BMP-induced EC migratory pathways were active in early NRCVC cultures grown in 3D. This interpretation was supported by the observed loss of 3D structure formation when

Noggin, a BMP antagonist, was included in the culture medium (Fig. 5E, F). Thus, genes in EC migratory pathways were differentially activated in 3D culture, and these pathways may have played central roles in the formation of 3D aggregate structure, especially during the initial phases of culture establishment. It should be noted that BMPs, including BMP-2, also play important roles in CM differentiation and cardiac morphogenesis *in vivo*,³⁷ and the upregulation of *Bmp-2* expression early in 3D culture may exert effects on multi-cellular organization beyond the potential activation of EC migration.

To assess general differences in gene expression patterns between 2D and 3D cultures, we employed a genomic analysis approach. Previous work in our lab had been carried out to compare bioreactor-cultured NRCVCs with parallel cells grown in standard tissue culture dishes over the same time period. We found differential expression of genes associated with tissue morphogenesis and blood vessel formation.⁵ Data from that study were reanalyzed here to identify genes that were differentially expressed at statistically significant levels between the 2D and 3D arms of the study. RNA from days 1, 4, and 6 of 2D and parallel 3D cultures were analyzed from two separate experiments (i.e., 12 RNA samples) using Affymetrix RG-U34A Arrays. The data were compared using a nonparametric, Two Factor Mack-Skillings Test to separate time-dependent and culture-dependent differences. This approach identified 158 genes (Table 2) represented by 167 probesets that were significantly different in 3D versus 2D culture ($p < 0.01$). Based on a figure of merit analysis, K-Means clustering was carried out on these probesets to identify six clusters among the culture-type significant genes (Figure 6). Our analysis demonstrates that fundamental differences in the molecular phenotype of primary cardiac cells exist between our 3D and 2D culture systems.

Tissue maturation in 3D culture

In heart tissue, EC structure and CM structure formation, maturation, and maintenance are linked, especially in the developing fetus.^{38–40} Since comparisons of our 2D and 3D culture systems demonstrated alterations in EC distribution

FIG. 5. Mediators of EC migration by real-time quantitative polymerase chain reaction. **(A)** Bmp-2 levels were substantially elevated in 3D on day 1 of culture and then attenuated on days 2 and 3 before peaking again on day 4. In 2D controls, there was a consistent rise in Bmp-2 until day 4 and then a decrease. **(B)** Id3, which mediates bone morphogenetic protein (BMP)-induced EC migration by inhibiting thrombospondin (Thbs), was also elevated on day 1 in 3D and then again on day 5. **(C)** Thbs1 remained low in 3D on day 1, as expected from the Id3 data, and then increased on day 2 and again on day 6. **(D)** Troponin I (Tnni3) expression, on the other hand, was evenly expressed in 3D and 2D cultures over time in as shown here by plotting actual Ct. **(E)** Three-dimensional masses formed along and between the TCPS beads used to initiate culture; these masses had a smooth surface of ECs (also see Fig. 3). **(F)** In cultures grown in the presence of 1 µg/mL Noggin, which inhibits receptor binding by BMP, cells grow on the TCPS bead surfaces but 3D masses do not form. Plotted data are mean values $\pm$ standard deviation for $n = 3$ determinations.

TABLE 2. DIFFERENTIALLY EXPRESSED GENES IN THREE-DIMENSIONAL VERSUS TWO-DIMENSIONAL CULTURE

Cluster	Genes included in cluster
1	<i>Actc1</i> (x2), <i>Asb2</i> , <i>Atp1b1</i> , <i>Bckdhlb</i> , <i>Bdh1</i> , <i>Ckmt2</i> , <i>Cnn1</i> , <i>Cst3</i> , <i>Dpep1</i> , <i>Edg2</i> , <i>Eln</i> , <i>Fmo1</i> , <i>Gpx1</i> , <i>Hspb6</i> (x2), <i>LOC364241</i> , <i>Map2k5</i> , <i>Mmp23</i> , <i>Myl2</i> (x2), <i>Myl9_predicted</i> (x2), <i>M81183Exon_UTR_at</i> , <i>Slc25a26_predicted</i> , <i>Ndrg4</i> , <i>Nppa</i> (x3), <i>Pam</i> , <i>Prss35</i> , <i>Ptk2b</i> , <i>Pygb</i> , <i>RGD1307150_predicted</i> , <i>RGD1564935_predicted</i>
2	<i>Abo</i> , <i>Akap12</i> , <i>Blmh</i> , <i>Bmp2</i> , <i>Btbd14b</i> , <i>Calr</i> , <i>Cd44</i> , <i>Cdk7</i> , <i>Comt</i> , <i>Copb2</i> , <i>Dnajb1_predicted</i> , <i>Eif2b1</i> , <i>Fdxr</i> , <i>Fkbp4</i> , <i>Folh1</i> , <i>Gmfb</i> , <i>Hsph1</i> , <i>Inhba</i> , <i>Irf3</i> , <i>Mdm2_predicted</i> , <i>Nfkb1</i> , <i>Nsfl1c</i> , <i>Nsfl1c</i> , <i>Nudc</i> , <i>Nup153</i> , <i>Pafah1b2</i> , <i>Prim2</i> , <i>Psmc2</i> , <i>RGD1359684</i> , <i>RGD1308350</i> , <i>Rps15</i> , <i>Slc7a1</i> , <i>Tcf1</i> , <i>Tnfrsf11b</i> , <i>Tpm4</i> , <i>Wdr39</i>
3	<i>Af6</i> , <i>Alcam</i> , <i>ANXA3</i> , <i>Arl1</i> , <i>Aven_predicted</i> , <i>Dad1</i> , <i>Dnajc17_predicted</i> , <i>FN1</i> , <i>Hmgb2</i> , <i>HSPCB</i> , <i>Kb1</i> , <i>Kcna1</i> , <i>Kif4</i> , <i>LOC493574</i> , <i>LOC689128</i> , <i>LOC690263</i> , <i>NR3C2</i> , <i>Prss2</i> , <i>Psmas5</i> , <i>Rab2</i> , <i>rc_AA893932_at</i> , <i>rc_AI639520_at</i> , <i>Rgc32</i> , <i>RGD1359684</i> , <i>RGD1559939_predicted</i> , <i>RGD620382</i> , <i>Robo2</i> , <i>Rpl21</i> , <i>Rpl5</i> , <i>Sfrs2</i>
4	<i>Abcb1b</i> , <i>Ahr</i> , <i>Ass</i> , <i>Csk</i> , <i>Dnajc5</i> , <i>Fn1</i> , <i>Fn1</i> , <i>Gpc3</i> , <i>Gria3</i> , <i>Homer1</i> , <i>LOC246137</i> , <i>LOC246295</i> , <i>Pem</i> , <i>Phyh2</i> , <i>Pik4cb</i> , <i>Pik4cb</i> , <i>Prdm2</i> , <i>rc_AA866369_at</i> , <i>rc_AA892204_at</i> , <i>Tra1_predicted</i> , <i>Zmym4_predicted</i>
5	<i>Bst1</i> , <i>Clcn2</i> , <i>Crry</i> , <i>Dad1</i> , <i>Gch</i> , <i>Gda</i> , <i>Gstt1</i> , <i>LOC501282</i> , <i>Spata9_predicted</i> , <i>Plau</i> , <i>RGD1311155</i> , <i>Rpn1</i>
6	<i>Acta1</i> , <i>Actg2</i> , <i>Ankrd1</i> , <i>Argbp2</i> (x2), <i>Cav3</i> , <i>Cryab</i> , <i>Csrp3</i> , <i>Cyc1_predicted</i> , <i>Des</i> , <i>Flnc_predicted</i> , <i>JPH2</i> , <i>Lipe</i> , <i>LOC360618</i> , <i>MGC109519</i> , <i>Myh6</i> , <i>Myh7</i> , <i>Ndufab1_predicted</i> , <i>Nes</i> , <i>Neurl2_predicted</i> , <i>Pde2a</i> , <i>Ppp1cb</i> , <i>Psat1</i> (x2), <i>Ptprc</i> , <i>rc_AI010292_s_at</i> , <i>RGD1306714</i> , <i>Stxbp1</i> , <i>Synj2</i> , <i>Tagln</i> , <i>Tpm1</i> (x2), <i>Ucp2</i>

Cluster designations correlate with the results of the K-Means Clustering shown in Figure 6. If more than one probeset for the same gene was identified, the total number is indicated in parentheses. Gene symbols were obtained from the online NetAffx™ Analysis Center (www.affymetrix.com/analysis/index.affx); in cases where specific gene symbols were not available, designations from the Rat Genome Database (RGD) or the original Affymetrix probeset IDs are provided.

and EC migratory pathways, we further investigated the effects of 3D culture on NRCVC maturational status by evaluating protein markers associated with immature tissue.

ANP is an endocrine mediator that is highly expressed in mature cardiac atria. In cardiac ventricles, ANP expression decreases with CM maturation so that mature CMs express very little ANP.²⁶ The process of ANP production differs between atrial and ventricular CMs. In both cell types, *Nppa* (the gene encoding ANP) mRNA is translated into a 152-amino acid pre-pro-peptide, which is cleaved to form the 126-amino acid pro-ANP. Atrial CMs store pro-ANP in granules from which ANP is released via a regulated pathway when intermittent stretch increases in the tissue, usually due to elevated blood volume. In contrast, ventricular CMs secrete ANP via a constitutive pathway in which ANP is released shortly after synthesis, and pro-ANP is generally not stored in granules.^{41,42} Significant production of ANP by ventricular CMs occurs only in immature tissue and is only reexpressed in adult tissue that is hypertrophic. Since immature and hypertrophic ventricular cells actively produce ANP, they contain elevated steady state levels of immunoreactive ANP compared to mature ventricular cells. We used this pattern of ANP production to assess the status of our NRCVCs, and as seen in Figure 7A, cells grown in 3D culture had substantially depressed ANP levels, which is consistent with a more mature CM phenotype.

Similar to *Nppa*, expression of the *Ankrd1* gene, which encodes cardiac ankyrin repeat protein (CARP),^{27,43} is also down-regulated in the adult heart⁴⁴ but reexpressed in cardiac pathogenesis.^{45,46} Western blotting for CARP expression at days 1, 4, and 6 of 2D versus 3D culture indicated that *Ankrd1* expression was substantially reduced in 3D culture, further supporting a difference in NRCVC maturational status associated with 3D culture (Fig. 7B).

Hormone responsiveness in 3D culture

Finally, we investigated whether culture in 3D affected the physiologic responsiveness of the component cells to exogenous stimulation.

Previous work by Armstrong and co-workers established that chick embryonic CMs can proliferate and respond to growth factor stimulation when cultured in 3D, but not when grown in 2D.⁴⁷ In those studies, factors associated with hyperplastic growth of the embryonic myocardium were used to assess effects on cell proliferation. In the present study, responsiveness to T₃, which is associated with postnatal CM phenotype in mammalian tissue, was assessed. Early postnatal, ventricular maturation *in vivo* is typified by a conversion in the MyHC isoform composition of CMs so that the relative level of α -MyHC (i.e., *Myh6*) increases while β -MyHC (i.e., *Myh7*) decreases. This conversion occurs in conjunction with increasing levels of circulating T₃, and T₃ has been shown to antithetically regulate α - and β -MyHC expression.⁴⁸ The switch in MyHC isoform in response to T₃ elevation occurs rapidly in mature, native tissue with complete conversion seen in 24 h when hypothyroid rats are made replete with T₃.⁴⁹ Accordingly, we investigated the responsiveness of 3D tissue-like constructs to T₃ exposure by evaluating the level of α - and β -MyHC present 24 h after a switch from T₃-free to T₃-containing media. Our serum-free culture system allows us to control the specific level of hormone present, and as can be seen in Figure 8, 2D and 3D cultures both respond to increasing hormone exposure by reexpressing α -MyHC. The cells grown in 3D culture, however, switched their expression to a much greater degree in the time-frame of the experiment. This increased rate of conversion to α -MyHC is consistent with what is seen in mature, native tissue, suggesting that 3D culture elicits a shift in hormone responsiveness to a more mature, native tissue level.

Discussion

We found that NRCVCs cultured in 3D aggregates comprising the same cell population as parallel 2D cultures exhibit subtle but physiologically important alterations in phenotype. Cells cultured in 3D self-assembled into a tissue-like conformation with a superficial layer of ECs resembling

FIG. 6. Cluster analysis of differentially expressed genes. Average normalized expression values across the two experiments are given on the Y-axis where normalized value = (raw value – mean value for 12 samples) / (standard deviation for 12 samples). The time course (days 1, 4, and 6) is presented for the 3D and 2D conditions. Probesets were grouped using K-Means Clustering with the number of clusters set at six based on figure of merit analysis. The expression patterns are shown for each of the six clusters on days 1, 4, and 6 from left to right under 3D and 2D conditions. The number of probesets included is given by *n*; error bars are standard deviations. Cluster members are given in Table 2.

the luminal epithelium of native heart tissue. Three-dimensional culture was associated with altered gene expression profiles relative to 2D, and 3D culture resulted in the differential activation of EC migratory pathways. Three-dimensional cultures exhibited decreased ANP and CARP protein levels indicative of improved tissue maturation, and 3D aggregates responded more rapidly to T_3 exposure than parallel 2D cultures, consistent with a mature, native-tissue phenotype. These data indicate that 3D culture in the serum-free medium results in the reorganization and phenotypic alteration of the component cells into structures and behaviors similar to those seen *in vivo*.

Three-dimensional culture systems have significantly advanced our understanding of fundamental relationships between tissue-level organization and component cell function,^{13,14} and substantial progress has been made in the use of 3D methods to investigate the formation of cardiac tissue.^{1–12} The phenotypic adaptations of cells when organized in engineered tissues, however, have not been well elucidated. To study aspects of *ex vivo* cardiogenesis, we disso-

ciated functional tissue and obtained a mixed cell population representing the original tissue's composition but lacking its multi-cellular organization. It is noteworthy that this cell population is the target population for stem cell and other cell procurement strategies in cardiovascular regenerative therapies and that understanding the controlled assembly of tissue level structures by this population may be critical to the ultimate success of cardiac tissue engineering efforts. In the present study, the formation of 3D aggregates is not entirely surprising as other investigators have used similar low-shear suspension culture models to generate 3D aggregates. The presented work, however, is distinguished by the use of a serum-free medium and highly controlled culture conditions, which severely restrict cell overgrowth (Figs. 1 and 2) and which allow the identification of phenotypic differences between component cells in parallel 3D and 2D models.

The up-regulation of EC migratory pathways (Fig. 5) and the appearance of a luminal endothelial sheet in the 3D cultures (Fig. 3) were striking and unexpected given the

FIG. 7. Markers of ventricular maturation. (A) Immunoreactive atrial natriuretic peptide (ANP) levels determined by enzyme immunoassay assay. Data are presented as ng of total ANP (ANP plus pro-ANP plus pre-pro-ANP) per mg extracted protein. Three-dimensional cultures had substantially less ANP than parallel 2D cultures. Tissue levels are shown for reference. Data are means of duplicate determinations for each time point. (B) Representative Western blot of samples from 3D and 2D cultures collected at 1, 4, and 6 days after culture initiation showing substantial elevation in cardiac adriamycin response protein (CARP) levels in 2D culture.

propensity of CMs to adhere to surfaces later than other cells (Fig. 4). One possibility is that fluid shear in our 3D suspension-culture system activated the CMs or ECs, which are known to be sensitive to mechanical stimulation.^{50–54} The level of fluid shear determined for systems like the one employed here, however, is low (< 10 dynes/cm²),^{55,56} and the shear level may not be sufficient to trigger a cellular response. In addition, fluid shear is continuously present throughout the duration of 3D culture, but the *Bmp-2*, *Id3*, and *Thbs1* expression levels increase and then decrease in both systems (Fig. 4). Finally, *Bmp-2* expression, which may drive EC migration, is reportedly unaffected by shear stress in other experiments using similar cells,⁵⁷ and preliminary studies in our lab to induce *Bmp-2* expression in 2D cultures by low level shear exposure have been unsuccessful (data not shown). Thus, although a role for fluid shear cannot be completely ruled out in EC redistribution, it is unlikely that shear contributes to differences in *Bmp-2* expression. A second possibility is that differences in *Bmp-2* RNA level and EC migration result from coupled interactions between CMs and ECs activated by the 3D contacts permitted in the suspension culture system. BMP-2 is a potential mediator of coordinated EC and CM structure formation *in vivo*,³⁸ and CM function is suspected of driving aspects of cardiac morphogenesis during normal development via BMP.⁵⁸ This is an attractive alterna-

FIG. 8. Responsiveness to triiodothyronine (T₃). (A) Representative ethidium-bromide-stained gel of multiplex polymerase chain reaction of reverse-transcribed RNA samples using combined primers for α - and β -MyHC. Atrial tissue, which contains α -MyHC but not β -MyHC, and soleus muscle, which contains β -MyHC but not α -MyHC, are shown as controls. (B) Quantification of α - to β -MyHC levels from (A) showing the higher level of sensitivity to physiologic doses of triiodothyronine in the 3D cultures.

tive, but further experiments with 3D models are needed to clarify potential multi-cellular interactions and the mechanisms driving multi-cellular structure formation *in vitro*.

Interestingly, researchers using NRCVC reaggregate culture systems based on serum-containing media do not report exteriorized ECs,^{7–12} and we initially suspected that the absence of serum in our system may have resulted in a reversal of relative adhesiveness such that CMs adhered first forming a core of cells onto which ECs subsequently adhered. Experiments shown in Figure 4, however, indicate that CMs adhere later than other cells in our serum-free medium; thus, the appearance of a superficial epithelial layer is not easily accounted for by early CM adhesion to TCPS. Our observations suggest that NRCVCs possess a latent ability to organize an EC sheet after culture initiation and that this ability is accessed in 3D/bioreactor cultures but not in 2D/plate cultures when a serum-free medium is used.

Genomic expression analysis using Affymetrix Chips indicated that a number of genes were differentially expressed in 3D versus 2D culture and that these clustered into six groups (Fig. 6). Evaluation of either the entire 167 genes or the genes in each of the six clusters separately was carried out using the online database for annotation, visualization, and integrated discovery (DAVID) informatics resource from the National Institutes of Health.^{59,60} After controlling for false discovery using Benjamini-Hochberg corrections, DAVID analysis revealed no thematic relationships among the differentially expressed genes beyond ontologies associated with muscle contraction and muscle development (not shown). Nonetheless, there were physiologically significant

differences in gene expression associated with 3D culture, and several of the genes identified in the genomic screening, including *Bmp-2* (Cluster 2; also in Fig. 5), as well as *Nppa* and *Ankrd1* (Clusters 1 and 6, respectively; also in Fig. 7), were analyzed elsewhere in this article. Determining the functional significance of the other expression differences will require additional molecular and proteomic analyses.

The observed differences in ANP and CARP levels suggest the acquisition of a different and possibly more mature cell phenotype in 3D versus 2D culture. ANP expression from the *Nppa* gene has been well studied. ANP is expressed in immature ventricular CMs, but expression decreases over time. In the mature heart, ANP expression is largely restricted to atrial CMs except under pathologic conditions associated with increased ventricular CM stretch (i.e., preload) and hypertrophy.^{61,62} Since there is no preloading of CMs in our system and no evidence for hypertrophy (Fig. 2), the decrease in ANP is suggestive of CM maturation in the 3D cultures. Similarly, differences in CARP expression from the *Ankrd1* gene, which is developmentally down-regulated but reexpressed in hypertrophic ventricular tissue,^{44,63} suggest that 3D culture encourages CM maturation. As with *Bmp-2*, a possible explanation for these differences lies in alteration of cell-cell contacts in 3D. Interestingly, recent work on signaling mechanisms involving Notch pathways, which are triggered by specific cell-cell interactions, has shown that both ANP^{64,65} and CARP⁶⁶ levels are controlled by the Notch signaling intermediate Hey-2. Thus, differences in ANP and CARP may be associated with altered cell-cell interactions and the acquisition of a layered, tissue-like geometry in 3D.

Ventricular CMs respond to changes in T_3 levels by altering the ratio of α - and β -MyHC.⁴⁸ *In vivo*, this isotype switch occurs rapidly when T_3 is given to hypothyroid animals.⁴⁹ Developmentally, the complement of α - and β -MyHC in rodent cardiac ventricles shifts from ~50% α at birth to nearly 100% α by 3 weeks postnatal.⁶⁷ Thus, the rapid and nearly complete conversion of MyHC mRNA to the α form when 3D cultures were given 3 nM T_3 (Fig. 8) is consistent with a shift in hormone sensitivity and the adoption of a more mature CM phenotype.

Taken together, our data indicate that the growth of cardiac ventricular cells in 3D resulted in subtle but significant alterations in cell organization and function. In particular, 3D culture was associated with differences in gene expression, cell migratory signaling, tissue maturation, and responsiveness to hormonal stimulation; these are all differences that impact directly on the design of functional, 3D cardiac tissue. The mechanisms driving alterations in cell function require further investigation but likely involve specific cellular interactions in the 3D environment. This concept is supported by our studies, which emphasized 3D cell masses and did not employ 3D scaffolds. Consideration of the cell interactions that drive the functional alteration of cells and tissues may impact heavily on scaffold design and strategies for tissue formation and may prove critical as the field approaches clinical applications for complex 3D tissues.

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Disclosure Statement

No competing financial interests exist.

References

1. Jakab, K., Norotte, C., Damon, B., Marga, F., Neagu, A., Besch-Williford, C.L., Kachurin, A., Church, K.H., Park, H., Mironov, V., Markwald, R., Vunjak-Novakovic, G., and Forgacs, G. Tissue engineering by self-assembly of cells printed into topologically defined structures. *Tissue Eng Part A* **14**, 413, 2008.
2. Yildirim, Y., Naito, H., Didie, M., Karikkineth, B.C., Biermann, D., Eschenhagen, T., and Zimmermann, W.H. Development of a biological ventricular assist device: preliminary data from a small animal model. *Circulation* **116**, I16, 2007.
3. Masuda, S., Shimizu, T., Yamato, M., and Okano, T. Cell sheet engineering for heart tissue repair. *Adv Drug Deliv Rev* **60**, 277, 2008.
4. Sekine, H., Shimizu, T., Hobo, K., Sekiya, S., Yang, J., Yamato, M., Kurosawa, H., Kobayashi, E., and Okano, T. Endothelial cell coculture within tissue-engineered cardiomyocyte sheets enhances neovascularization and improves cardiac function of ischemic hearts. *Circulation* **118**, S145, 2008.
5. Akins, R., Gratton, K., Quezada, E., Rutter, H., Tsuda, T., and Soteropoulos, P. Gene expression profile of bioreactor-cultured cardiac cells: activation of morphogenetic pathways for tissue engineering. *DNA Cell Biol* **26**, 425, 2007.
6. Akins, R.E., Boyce, R.A., Madonna, M.L., Schroedl, N.A., Gonda, S.R., McLaughlin, T.A., and Hartzell, C.R. Cardiac organogenesis *in vitro*: reestablishment of three-dimensional tissue architecture by dissociated neonatal rat ventricular cells. *Tissue Eng Part A* **5**, 103, 1999.
7. Baar, K., Birla, R., Boluyt, M.O., Borschel, G.H., Arruda, E.M., and Dennis, R.G. Self-organization of rat cardiac cells into contractile 3-D cardiac tissue. *FASEB J* **19**, 275, 2005.
8. Birla, R.K., Borschel, G.H., and Dennis, R.G. *In vivo* conditioning of tissue-engineered heart muscle improves contractile performance. *Artif Organs* **29**, 866, 2005.
9. Birla, R.K., Borschel, G.H., Dennis, R.G., and Brown, D.L. Myocardial engineering *in vivo*: formation and characterization of contractile, vascularized three-dimensional cardiac tissue. *Tissue Eng* **11**, 803, 2005.
10. Just, L., Kursten, A., Borth-Bruhns, T., Lindenmaier, W., Rohde, M., Dittmar, K., and Bader, A. Formation of three-dimensional fetal myocardial tissue cultures from rat for long-term cultivation. *Dev Dyn* **235**, 2200, 2006.
11. Kelm, J.M., Ehler, E., Nielsen, L.K., Schlatter, S., Perriard, J.C., and Fussenegger, M. Design of artificial myocardial microtissues. *Tissue Eng* **10**, 201, 2004.
12. Watzka, S.B., Steiner, M., Samorapoompichit, P., Gross, K., Coles, J.G., Wolner, E., and Weigel, G. Establishment of vessel-like structures in long-term three-dimensional tissue culture of myocardium: an electron microscopy study. *Tissue Eng* **10**, 1684, 2004.
13. Griffith, L.G., and Swartz, M.A. Capturing complex 3D tissue physiology *in vitro*. *Nat Rev* **7**, 211, 2006.

14. Lee, G.Y., Kenny, P.A., Lee, E.H., and Bissell, M.J. Three-dimensional culture models of normal and malignant breast epithelial cells. *Nat Methods* **4**, 359, 2007.
15. Harding, S.E., Davies, C.H., Wynne, D.G., and Poole-Wilson, P.A. Contractile function and response to agonists in myocytes from failing human heart. *Eur Heart J* **15 Suppl D**, 35, 1994.
16. Wollenberger, A. Isolated heart cells as a model of the myocardium. *Basic Res Cardiol* **80 Suppl 2**, 9, 1985.
17. Speicher, D.W., Peace, J.N., and McCarl, R.L. Effects of plating density and age in culture on growth and cell division of neonatal rat heart primary cultures. *In Vitro* **17**, 863, 1981.
18. Millart, H., and Seraydarian, M.W. Influence of plating density on individual cell growth, cell division and differentiation of neonatal rat heart primary cultures. *Tissue Cell* **18**, 209, 1986.
19. Akins, R.E. Cardiac tissue. In: Atala, A., and Lanza, R., eds. *Methods of Tissue Engineering*. San Diego, CA: Academic Press, 2002, pp. 915–925.
20. Vunjak-Novakovic, G. Cardiac tissue engineering: effects of bioreactor flow environment on tissue constructs. *J Chem Technol Biotechnol* **81**, 485, 2006.
21. Akins, R., McLaughlin, T., Boyce, B., Gilmour, L., and Gratton, K. Exogenous metalloporphyrins alter the organization and function of cultured neonatal rat heart cells via modulation of heme oxygenase activity. *J Cell Physiol* **201**, 26, 2004.
22. Holshek, J., and Akins, R. Acetonitrile is better than ethanol as a dehydrating agent for cells prepared for SEM. *Proc Microsc Soc Am* **52**, 324, 1994.
23. Akins, R., Schroedl, N., Gonda, S., and Hartzell, C. Neonatal rat heart cells cultured in simulated microgravity. *In Vitro Cell Dev Biol Anim* **33**, 337, 1997.
24. Schroedl, N.A., Bacon, C.R., Huang, Y.C., and Hartzell, C.R. Protein metabolism during nutrient deprivation and re-feeding of neonatal heart cells. *Am J Physiol* **257 (5 Pt 1)**, C913, 1989.
25. Saeed, A.I., Sharov, V., White, J., Li, J., Liang, W., Bhagabati, N., Braisted, J., Klapa, M., Currier, T., Thiagarajan, M., Sturn, A., Snuffin, M., Rezantsev, A., Popov, D., Ryltsov, A., Kostukovich, E., Borisovsky, I., Liu, Z., Vinsavich, A., Trush, V., and Quackenbush, J. TM4: a free, open-source system for microarray data management and analysis. *Biotechniques* **34**, 374, 2003.
26. Rockwood, D.N., Akins, R.E., Jr., Parrag, I.C., Woodhouse, K.A., and Rabolt, J.F. Culture on electrospun polyurethane scaffolds decreases atrial natriuretic peptide expression by cardiomyocytes *in vitro*. *Biomaterials* **29**, 4783, 2008.
27. Zou, Y., Evans, S., Chen, J., Kuo, H.C., Harvey, R.P., and Chien, K.R. CARP, a cardiac ankyrin repeat protein, is downstream in the Nkx2-5 homeobox gene pathway. *Development* **124**, 793, 1997.
28. Sperelakis, N. Cultured heart cell reaggregate model for studying cardiac toxicology. *Environ Health Perspect* **26**, 243, 1978.
29. Kasten, F.H. Rat myocardial cells *in vitro*: mitosis and differentiated properties. *In Vitro* **8**, 128, 1972.
30. Polinger, I.S. Separation of cell types in embryonic heart cell cultures. *Exp Cell Res* **63**, 78, 1970.
31. Gharaibeh, B., Lu, A., Tebbets, J., Zheng, B., Feduska, J., Crisan, M., Peault, B., Cummins, J., and Huard, J. Isolation of a slowly adhering cell fraction containing stem cells from murine skeletal muscle by the preplate technique. *Nat Prot* **3**, 1501, 2008.
32. Rando, T.A., and Blau, H.M. Primary mouse myoblast purification, characterization, and transplantation for cell-mediated gene therapy. *J Cell Biol* **125**, 1275, 1994.
33. Richler, C., and Yaffe, D. The *in vitro* cultivation and differentiation capacities of myogenic cell lines. *Dev Biol* **23**, 1, 1970.
34. Benezra, R., Rafii, S., and Lyden, D. The Id proteins and angiogenesis. *Oncogene* **20**, 8334, 2001.
35. Glienke, J., Schmitt, A.O., Pilarsky, C., Hinzmann, B., Weiss, B., Rosenthal, A., and Thierauch, K.H. Differential gene expression by endothelial cells in distinct angiogenic states. *Eur J Biochem* **267**, 2820, 2000.
36. Valdimarsdottir, G., Goumans, M.J., Rosendahl, A., Bruggman, M., Itoh, S., Lebrin, F., Sideras, P., and ten Dijke, P. Stimulation of Id1 expression by bone morphogenetic protein is sufficient and necessary for bone morphogenetic protein-induced activation of endothelial cells. *Circulation* **106**, 2263, 2002.
37. van Wijk, B., Moorman, A.F., and van den Hoff, M.J. Role of bone morphogenetic proteins in cardiac differentiation. *Cardiovasc Res* **74**, 244, 2007.
38. Bhattacharya, S., Macdonald, S.T., and Farthing, C.R. Molecular mechanisms controlling the coupled development of myocardium and coronary vasculature. *Clin Sci (Lond)* **111**, 35, 2006.
39. Tirziu, D., Chorianopoulos, E., Moodie, K.L., Palac, R.T., Zhuang, Z.W., Tjwa, M., Roncal, C., Eriksson, U., Fu, Q., Elfenbein, A., Hall, A.E., Carmeliet, P., Moons, L., and Simons, M. Myocardial hypertrophy in the absence of external stimuli is induced by angiogenesis in mice. *J Clin Invest* **117**, 3188, 2007.
40. Tirziu, D., and Simons, M. Endothelium as master regulator of organ development and growth. *Vasc Pharm* **50**, 1, 2008.
41. Bloch, K.D., Seidman, J.G., Naftilan, J.D., Fallon, J.T., and Seidman, C.E. Neonatal atria and ventricles secrete atrial natriuretic factor via tissue-specific secretory pathways. *Cell* **47**, 695, 1986.
42. Irons, C.E., Sei, C.A., and Glembofski, C.C. Regulated secretion of atrial natriuretic factor from cultured ventricular myocytes. *Am J Physiol* **257 (5 Pt 1)**, C913, 1989.
43. Jeyaseelan, R., Poizat, C., Baker, R.K., Abdishoo, S., Isterabadi, L.B., Lyons, G.E., and Kedes, L. A novel cardiac-restricted target for doxorubicin. CARP, a nuclear modulator of gene expression in cardiac progenitor cells and cardiomyocytes. *J Biol Chem* **272**, 22800, 1997.
44. Kuo, H., Chen, J., Ruiz-Lozano, P., Zou, Y., Nemer, M., and Chien, K.R. Control of segmental expression of the cardiac-restricted ankyrin repeat protein gene by distinct regulatory pathways in murine cardiogenesis. *Development* **126**, 4223, 1999.
45. Wei, Y.J., Cui, C.J., Huang, Y.X., Zhang, X.L., Zhang, H., and Hu, S.S. Upregulated expression of cardiac ankyrin repeat protein in human failing hearts due to arrhythmogenic right ventricular cardiomyopathy. *Eur J Heart Fail* **11**, 559, 2009.
46. Zolk, O., Frohme, M., Maurer, A., Kluxen, F.W., Hentsch, B., Zubakov, D., Hoheisel, J.D., Zucker, I.H., Pepe, S., and Eschenhagen, T. Cardiac ankyrin repeat protein, a negative regulator of cardiac gene expression, is augmented in human heart failure. *Biochem Biophys Res Commun* **293**, 1377, 2002.
47. Armstrong, M.T., Lee, D.Y., and Armstrong, P.B. Regulation of proliferation of the fetal myocardium. *Dev Dyn* **219**, 226, 2000.
48. Chizzonite, R.A., and Zak, R. Regulation of myosin isoenzyme composition in fetal and neonatal rat ventricle by endogenous thyroid hormones. *J Biol Chem* **259**, 12628, 1984.

49. Dillmann, W.H., Rohrer, D., Popovich, B., and Barrieux, A. Thyroid hormone induced changes in cardiac proteins and mRNAs. *Horm Metab Res Suppl* **17**, 26, 1987.
50. Andries, L.J., Sys, S.U., and Brutsaert, D.L. Morphoregulatory interactions of endocardial endothelium and extracellular material in the heart. *Herz* **20**, 135, 1995.
51. Yamazaki, T., Komuro, I., Kudoh, S., Zou, Y., Shiojima, I., Hiroi, Y., Mizuno, T., Maemura, K., Kurihara, H., Aikawa, R., Takano, H., and Yazaki, Y. Endothelin-1 is involved in mechanical stress-induced cardiomyocyte hypertrophy. *J Biol Chem* **271**, 3221–8, 1996.
52. Swynghedauw, B. Developmental and functional adaptation of contractile proteins in cardiac and skeletal muscles. *Physiol Rev* **66**, 710, 1986.
53. Sadoshima, J., and Izumo, S. Mechanical stretch rapidly activates multiple signal transduction pathways in cardiac myocytes: potential involvement of an autocrine/paracrine mechanism. *EMBO J* **12**, 1681, 1993.
54. Dzau, V.J. The role of mechanical and humoral factors in growth regulation of vascular smooth muscle and cardiac myocytes. *Curr Opin Nephrol Hypertens* **2**, 27, 1993.
55. Goodwin, T.J., Prewett, T.L., Wolf, D.A., and Spaulding, G.F. Reduced shear stress: a major component in the ability of mammalian tissues to form three-dimensional assemblies in simulated microgravity. *J Cell Biochem* **51**, 301, 1993.
56. Hammond, T.G., and Hammond, J.M. Optimized suspension culture: the rotating-wall vessel. *Am J Physiol* **281**, F12, 2001.
57. Csiszar, A., Labinskyy, N., Smith, K.E., Rivera, A., Bakker, E.N., Jo, H., Gardner, J., Orosz, Z., and Ungvari, Z. Down-regulation of bone morphogenetic protein 4 expression in coronary arterial endothelial cells: role of shear stress and the cAMP/protein kinase A pathway. *Arterioscler Thromb Vasc Biol* **27**, 776, 2007.
58. Bartman, T., Walsh, E.C., Wen, K.K., McKane, M., Ren, J., Alexander, J., Rubenstein, P.A., and Stainier, D.Y. Early myocardial function affects endocardial cushion development in zebrafish. *PLoS Biol* **2**, E129, 2004.
59. Dennis, G., Jr., Sherman, B.T., Hosack, D.A., Yang, J., Gao, W., Lane, H.C., and Lempicki, R.A. DAVID: database for annotation, visualization, and integrated discovery. *Genome Biol* **4**, P3, 2003.
60. Huang da, W., Sherman, B.T., and Lempicki, R.A. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. *Nat Prot* **4**, 44, 2009.
61. Pandey, K.N. Biology of natriuretic peptides and their receptors. *Peptides* **26**, 901, 2005.
62. McGrath, M.F., and de Bold, A.J. Determinants of natriuretic peptide gene expression. *Peptides* **26**, 933, 2005.
63. Arber, S., Hunter, J.J., Ross, J., Jr., Hongo, M., Sansig, G., Borg, J., Perriard, J.C., Chien, K.R., and Caroni, P. MLP-deficient mice exhibit a disruption of cardiac cytoarchitectural organization, dilated cardiomyopathy, and heart failure. *Cell* **88**, 393, 1997.
64. Xin, M., Small, E.M., van Rooij, E., Qi, X., Richardson, J.A., Srivastava, D., Nakagawa, O., and Olson, E.N. Essential roles of the bHLH transcription factor Hrt2 in repression of atrial gene expression and maintenance of postnatal cardiac function. *Proc Natl Acad Sci USA* **104**, 7975, 2007.
65. Koibuchi, N., and Chin, M.T. CHF1/Hey2 plays a pivotal role in left ventricular maturation through suppression of ectopic atrial gene expression. *Circ Res* **100**, 850, 2007.
66. Mikhailov, A.T., and Torrado, M. The enigmatic role of the ankyrin repeat domain 1 gene in heart development and disease. *Int J Dev Biol* **52**, 811, 2008.
67. Haddad, F., Qin, A.X., Bodell, P.W., Jiang, W., Giger, J.M., and Baldwin, K.M. Intergenic transcription and developmental regulation of cardiac myosin heavy chain genes. *Am J Physiol* **257** (5 Pt 1), C913, 1989.

Address correspondence to:

Robert E. Akins, Jr., Ph.D.

Nemours Biomedical Research Center

A.I. duPont Hospital for Children

ARB 283, 1600 Rockland Road

Wilmington, DE 19803

E-mail: rakins@nemours.org

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