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3D-Model of Adult Cardiac Stem Cells Promotes Cardiac Differentiation and Resistance to Oxidative Stress

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ABSTRACT

The regenerative inadequacy of the injured myocardium leads to adverse remodeling, cardiac dysfunction, and heart disease. Stem cell-replacement of damaged myocardium faces major challenges such as inappropriate differentiation, cellular uncoupling, scar formation, and accelerated apoptosis of transplanted cells. These challenges can be met by engineering an in vitro system for delivering stem cells capable of cardiac differentiation, tissue integration, and resistance to oxidative stress. In this study, we describe the formation of three-dimensional (3D) cell aggregates (''cardiospheres'') by putative stem cells isolated from adult dog myocardium using poly-L-ornithine. De novo formation of cardiospheres in growth factor-containing medium occurred over a period of 2–3 weeks, but accelerated to 2–3 days when seeded on poly-L-ornithine. Older cardiospheres developed foci of ''beating'' cells upon co-culture with rat neonatal cardiomyocytes. Cardiospheres contained cells that exhibited characteristics of undifferentiated cells; differentiating cardiomyocytes with organized contractile machinery; and vascular cells capable of forming ''vessel-like'' networks. They exhibited strong resistance to elevated concentrations of hydrogen peroxide in culture and survived subcutaneous injections without undergoing neoplastic transformation up to 3 weeks post-transplantation. These findings suggest that cardiospheres are potentially useful for delivering functional stem cells to the damaged heart. J. Cell. Biochem. 105: 612–623, 2008.

KEY WORDS: CARDIOMYOCYTES; CARDIOSPHERES; DIFFERENTIATION; ENDOTHELIAL CELLS; GROWTH FACTORS; OXIDATIVE STRESS; POLY-L-ORNITHINE; SMOOTH MUSCLE CELLS; SPHEROIDS; STEM CELLS

The progressive loss of cardiomyocytes coupled with the poor regenerative capacity of myocardial tissue following acute ischemia or oxidative stress result in the proliferation of fibroblasts, scarring, decreased cardiac performance, compensatory hypertrophy, and ultimately, lead to heart failure [Claycomb, 1992; Frey and Olson, 2003]. Advances in medicine and technology have led to new strategies in cardiac repair involving transplantation or infusion of neonatal cardiomyocytes [Muller-Ehmsen et al., 2002], skeletal myoblasts [Taylor et al., 1998; Suzuki et al., 2001], embryonic stem cells [Min et al., 2002], endothelial cells and/or progenitors [Condorelli et al., 2001; Murasawa et al., 2005], adult mesenchymal cells [Gojo et al., 2003; Makkar et al., 2005], or bone marrow-derived cells [Orlic et al., 2001; Strauer et al., 2002; Beeres et al., 2006]. Promising results have been obtained in many cases showing improvement in cardiac performance [Kehat et al., 2004], however, studies demonstrating development of a fully functional syncytium between the grafted cells and the recipient myocardium are limited and short-term. Grafted cells often exhibit abnormal action

Abbreviations used: 3D, three-dimensional; FGF-2, fibroblast growth factor-2; FGFR, fibroblast growth factor receptor; cMHC, cardiac myosin heavy chain; cSA, cardiac sarcomeric actinin; CSC, cardiac stem cells; cTnI, cardiac troponin I; CFM, cardiosphere-forming medium; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; ES cells, embryonic stem cells; IGF-1, insulin-like growth factor-1; PLO, poly-L-ornithine; PCNA, proliferating cell nuclear antigen; SCM, stem cell growth medium; SMA, smooth muscle actin; VEGF, vascular endothelial growth factor; vWF, von Willebrand factor.

Additional Supporting Information may be found in the online version of this article.

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potential characteristics when compared with adult cardiomyocytes [Makino et al., 1999; Zhang et al., 2002; Leobon et al., 2003]. Moreover, studies suggest that grafts could induce arrythmias [Smits et al., 2003; Menasche et al., 2003; Perin et al., 2003] and exacerbate conduction abnormalities in the failing heart [Chen et al., 2006]. Other undesirable consequences of transplantation include immunogenic responses [Kofidis et al., 2005], abnormal tissue architecture [Whittaker et al., 2003], neoplastic predisposition, and accelerated cell death [Muller-Ehmsen et al., 2002].

3D cultures of neonatal and adult cardiac myocytes in artificial scaffolds have been engineered to develop functional micro-tissues [Akins et al., 1999; Baar et al., 2004; Kelm et al., 2004] for investigations of the vasculature and perfusion in cultured myocardium [Carrier et al., 2002; Watzka et al., 2004] and for repair of damaged myocardium [Hoerstrup et al., 2000; Yang et al., 2001]. Although the 3D-models containing differentiated cardiomyocytes provide a direct source of contractile tissue to repair damaged myocardium, the development of these systems has been far from adequate. 3D-grafts suffer from limited supply and post-transplantation inflammation, and often fail to mimic the complex structure and behavior of the beating myocardium [Hoerstrup et al., 2000; Yang et al., 2001].

Recent reports of the existence of cardiac stem cells (CSC) in the adult heart have provided new targets and/or tools in cardiac repair. These cells possess unlimited capacity for self-renewal and clonogenic expansion, and exhibit the potential to generate cardiomyocytes, vascular smooth muscle cells, and endothelial cells [Beltrami et al., 2003]. In vitro, adult CSC from human and murine hearts form self-adherent spheroid cultures, termed "cardiospheres" [Messina et al., 2004], resembling 3D-tissue models that could be used for therapy.

This study, therefore, was designed to establish a 3D-cardiac stem cell model capable of differentiating into adult cardiac tissue; and to initiate preliminary investigations of mechanisms regulating the formation of cardiospheres. Furthermore, this study also investigated the response of cardiospheres to oxidative stress and, therefore, evaluated the putative capacity of cardiospheres to promote myocardial regeneration. Clarification of the microenvironmental cues and corresponding molecular mechanisms responsible for the survival and normal differentiation of these sphere-generating cells should assist in defining the processes necessary for regulating heart development and could provide clues for engineering novel stem cell delivery systems for repair of injured myocardium.

MATERIALS AND METHODS

REAGENTS

Recombinant human proteins, including insulin like growth factor-1 (IGF-1), fibroblast growth factor-2 (FGF-2), epidermal growth factor (EGF), vascular endothelial growth factor (VEGF) were purchased from Leinco Technologies (St. Louis, MO); rat collagen type-I and PLO were from Upstate Biotechnology, Inc. (Lake Placid, NY); calcein AM, ethidium homodimer, Hoechst 33342, JC-1 (5,5',6,6'-Tetrachloro-1,1',3,3'tetraethylbenzimidazolylcarbo cyanine iodide), and CellTracker Red were from Molecular Probes (Eugene, OR); MTS and reagents for RT-PCR were from Promega Corp. (Madison, WI); and EGM-2 MV (Microvascular Endothelial Cell Medium-2) with supplements and growth factors were purchased from Cambrex (Walkersville, MD). All other enzymes, biochemical reagents, and PCR primers were from Sigma (St. Louis, MO), unless otherwise indicated.

ISOLATION OF ADULT CARDIAC STEM CELLS

Full thickness left ventricular tissues from adult Mongrel dogs ($n = 5$) were cut into ~1.0 mm cubes and stripped off the endocardial and epicardial layers, thereby preserving the myocardium in place. Myocardial blocks were further diced into smaller pieces, and digested for 1–2 h in basal medium (Dulbecco's modified Eagles medium, 2 mM L-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, 15 mM HEPES buffer) supplemented with 3,500 units/ml collagenase (Type XI-S, C-9697, Sigma), 2.5 mg/ml trypsin, and 250 ng/ml Amphotericin B at 37°C under humidified atmosphere of 95% air, 5% CO_2. Following digestion, tissues were collected, centrifuged, and rinsed with basal medium 2×, then plated onto 6-well plates in growth medium (basal medium containing 10% fetal bovine serum). After 48–72 h in culture, unattached tissues and floating debris were removed and fresh growth medium with 20 ng/ml IGF-1 was added. Numerous blood cells and characteristic dissociated phase bright polygonal-shaped adult cardiomyocytes (AC) rarely attached to the dishes (Fig. 1A) and came off during washes. None of these polygonal cells proliferated when followed up to 8 weeks in culture. On the other hand, small tissue explants attached to the dishes and over the course of 3–4 weeks generated colonies of small phase-bright proliferating cells (putative stem cells). While explants from all five animals generated putative stem cells, only three of the animals produced enough cells that were expanded and used in these experiments. All cultures were maintained at 37°C under a humidified atmosphere of 95% air, 5% CO_2. Confluent cultures were expanded in culture and frozen in 10% DMSO/20% FBS in basal medium until use. Parallel cultures spontaneously developed cardiospheres after 2–3 weeks.

CELL CULTURE AND CARDIOSPHERE FORMATION

Frozen vials of the putative cardiac stem cells (CSC) were plated as monolayers on collagen-coated dishes (5–10 $\mu g/cm^2$) using stem cell growth medium (SCM) consisting of growth medium supplemented with 20 ng/ml FGF-2 and 20 ng/ml IGF-1. Cultures maintained in SCM proliferated and could be passaged at least 7–8 times. In the presence of EGF, cultures formed cardiospheres gradually over a period of 2–3 weeks. To facilitate cardiosphere formation, CSC were plated on a 0.01% poly-L-ornithine (PLO) substratum in cardiosphere-forming medium (CFM) consisting of growth medium supplemented with 20 ng/ml FGF-2 and 20 ng/ml EGF. After 3–5 days in culture, most cardiospheres have detached from the monolayer cultures and floated in the medium. Suspended cardiospheres often attached to tissue culture-treated dishes following transfer in SCM.

To characterize their cellular components, 4–6-day-old floating cardiospheres were collected, dissociated enzymatically, plated on dishes or glass coverslips in SCM or EGM-2 MV (microvascular endothelial cell medium supplemented with FGF-2, EGF, IGF-1,

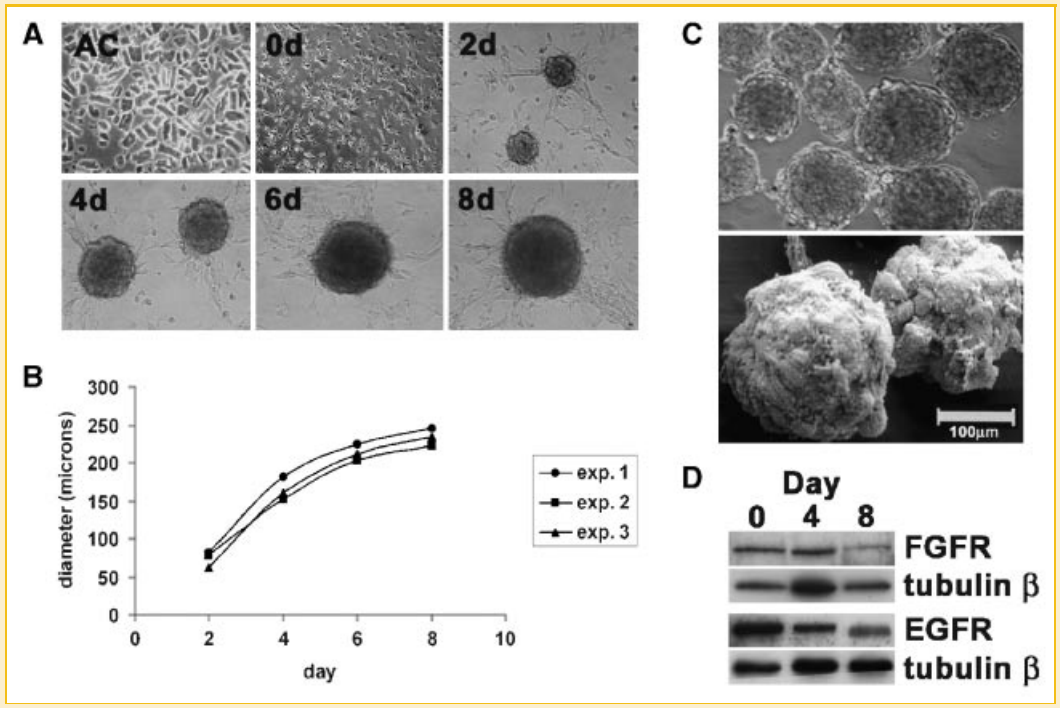

Fig. 1. Cardiosphere formation. A: Phase contrast images of dissociated adult cardiomyocytes (AC) from the dog heart appeared as large phase-bright polygonal structures that remained unattached and did not proliferate up to 8 weeks in culture. CSC cultured on collagen-coated dishes proliferated as monolayer cultures (0d), while cells plated on PLO-coated dishes in sphere-forming medium (SFM) from 2 to 8 days formed spheres with increased duration of culture. B: Growth curve of cardiospheres cultured on PLO in SFM for 2–8 days showed uniform rate of sphere growth. The diameter of 10 random live spheres was determined at each time point in at least three experiments. Student's *t*-test showed significant ($P < 0.005$) size increase from days 2 to 4 indicative of an early growth spurt. C: Phase contrast (upper panel) and SEM (lower panel) images of floating cardiospheres generated following 3–5 days culture on PLO in SFM outlined their smooth profiles. D: Western blot analysis using anti-FGFR (fibroblast growth factor receptor) and anti-EGFR (epidermal growth factor receptor) antibodies on CSC monolayers after collection of detached cardiospheres at 0, 4, 8 days showed decreasing levels of growth factor receptors. Staining for anti-tubulin β was used to normalize samples.

VEGF, hydrocortisone, heparin, ascorbic acid and serum), and processed for immunocytochemistry. All cultures were maintained at 37°C under a humidified atmosphere of 95% air and 5% CO_2.

CHARACTERIZATION OF CSC AND CARDIOSPHERES

Morphological characteristics and expression of cell-specific markers were determined on cardiospheres or cardiosphere-derived cells using Reverse Transcription-Polymerase Chain Reaction (RT-PCR), immunocytochemistry, Western blot analyses, and scanning electron microscopy (SEM). RT-PCR was performed with slight modifications of the procedures reported [Jingjing et al., 2000, 2001]. PCR products for VEGF were amplified using parameters described previously. Primers for amplification of canine VEGF PCR products (5′-ACCCTGGGTGGACATCTTC-3′; positions 237–254) and 5′-GTTCCCGAAACCCTGAGG-3′, positions 791–808) were designed using sequences previously reported to the National Center for Biotechnology Information (NCBI) canine genome sequence database [Jingjing et al., 2000]. PCR for β-actin was run as an internal control to determine adequacy of the cDNA synthesis and absence of contamination from genomic DNA. PCR products were visualized with ethidium bromide on 2% agarose gels.

For immunocytochemistry, cardiospheres or cardiosphere-derived cells were fixed in 2% paraformaldehyde for 20 min and immunostained using antibodies against: stem cells (c-kit, pax6); cardiomyocytes (cardiac myosin heavy chain [cMHC], cardiac troponin I [cTnI], cardiac sarcomeric actinin [cSA]); vascular endothelial cells (von Willebrand factor [vWF]); vascular smooth muscle cells (smooth muscle actin [SMA]); cell division (proliferating cell nuclear antigen [PCNA]); or angiogenic factor (vascular endothelial growth factor [VEGF]). Cells were counterstained with DAPI to determine nuclear localization. Optical sections of stained specimen were captured digitally using the Zeiss LSM 410 confocal microscope as needed. Sphere diameter was measured using the Zeiss LSM 5 software. For Western blot analyses, membranes were probed using antibodies against the FGF-2 receptor (FGFR), EGF receptor (EGFR), cMHC, or cTnI. Membranes were reprobed for β-tubulin to normalize protein loading. The list of antibodies used is shown in Table I.

For SEM, cardiospheres maintained for 3–4 days in CFM were fixed in 2% glutaraldehyde/2% paraformaldehyde for 1 h, washed with phosphate-buffered saline (PBS, pH 7.4), and post-fixed in 1% osmium tetroxide for 1 h. After PBS rinses, samples were dehydrated in a series of graded ethanol, dried for 60 min, and placed on mounting studs. Specimens were sputter coated and viewed under an AMRAY 1000A scanning electron microscope.

To determine their functional capacity, cardiospheres were co-cultured with neonatal cardiomyocytes isolated from 2-day-old rats. Monolayer CSC cultures seeded on PLO using SCM were washed

TABLE I. List of Antibodies

Antibody name	Species	Source	Application(s)	Concentration/ dilution used
Anti-fibroblast growth factor receptor [FGFR]	Rabbit	Santa Cruz Biotechnology (Santa Cruz, CA)	Western blot	0.4 μg/ml
Anti-epidermal growth factor receptor [EGFR]	Rabbit	Santa Cruz Biotechnology (Santa Cruz, CA)	Western blot	0.2 μg/ml
Anti-ckit	Rabbit	Stressgen Biotechnologies Corp. (Victoria BC Canada)	Immunocytochemistry	5 μg/ml
Anti-pax6	Rabbit	Santa Cruz Biotechnology Inc. (Santa Cruz, CA)	Immunocytochemistry	1 μg/ml
Anti-PCNA; Clone PC10	Mouse	NeoMarkers (Fremont, CA)	Immunocytochemistry	1 μg/ml
Anti-sarcomeric actinin [cSA]; Clone EA-53	Mouse	Sigma–Aldrich Corp. (Saint Louis, MO)	Immunocytochemistry	1:400
Anti-cardiac myosin heavy chain [cMHC]; Clone 3–48	Mouse	Abcam Ltd. (Cambridge, UK)	Western blot immunocytochemistry	0.5 μg/ml, 2 μg/ml
Anti-cardiac troponin 1 [cTnI]; Clone 284 (19C7)	Mouse	Chemicon International (Temecula, CA)	Immunocytochemistry	3.3 μg/ml
Anti-Von Willebrand Factor [vWF]	Rabbit	Dako Corp. (Carpinteria, CA)	Immunocytochemistry	1:200
Anti-smooth muscle actin [SMA]; Clone 1A4	Mouse	Sigma–Aldrich Corp. (Saint Louis, MO)	Immunocytochemistry	5 μg/ml
Anti-vascular endothelial growth factor [VEGF]	Rabbit	Santa Cruz Biotechnology (Santa Cruz, CA)	Immunocytochemistry	1 μg/ml

with basal medium, labeled for 30–45 min with 5 μM CellTracker Red, and induced to form cardiospheres with CFM. Labeled floating cardiospheres were collected and transferred at 5–10 spheres/cm^2 onto neonatal rat cardiomyocytes. Phase contrast images and movies were captured digitally with a Nikon 995 CCD camera attached to an inverted microscope daily.

OXIDATIVE STRESS

Cells to be tested as monolayer cultures were plated on 96-well plates at 2.5×10^4 cells/cm^2 in growth medium and allowed to attach overnight. Cells were washed with basal medium and further incubated for at least 24 h in basal medium prior to treatment with hydrogen peroxide (H_2O_2). Cardiospheres were generated by plating cells in 0.01% PLO-coated 6-well plates at 2.5×10^4 cells/cm^2 in CFM. Medium was changed on day 3 and detached spheres were collected on days 4–5. Cardiospheres were rinsed in basal medium, transferred to 96-well plates, and kept for at least 24 h in basal medium prior to H_2O_2 treatment.

Monolayer cultures or cardiospheres were exposed to H_2O_2 0–10 mM in basal medium for 1 h, and incubated further in basal medium for 24 h. Cell viability was determined using the Cell titer 96 MTS assay as described previously [Rosales and Roque, 1997]. Briefly, monolayer cultures or cardiosphere-containing cultures were incubated in 333 μg/ml of MTS and 25 μM phenazine methosulfate, and absorbances read at 490 nm. Absorbance readings were converted to cell numbers based on standard curves derived from the monolayer cultures.

The resistance of cardiospheres to oxidative stress was further determined using fluorescent probes calcein AM and ethidium homodimer to determine tissue viability; or JC-1, to establish integrity of the mitochondrial membranes. Briefly, cardiospheres exposed to 0, 1, or 10 mM H_2O_2 for 1 h and kept in basal medium for 24 h, were incubated for 30 min at 37°C in either a mixture of 1 μM calcein AM and 2 μM ethidium homodimer or in 10 μM JC-1. After incubation, media were aspirated and cardiospheres were washed in growth medium. Cell nuclei were counterstained with 3 μg/ml Hoechst 33342. Reactions were visualized under a Zeiss LSM 410 confocal microscope.

CARDIOSPHERE INJECTIONS

CellTracker Red-prelabeled floating cardiospheres 4–5 days old were used in injection protocols. To determine survival, tissue distribution and integration, or tumorigenicity of transplanted stem cells, five tie2-GFP transgenic mice were anesthetized by Isofluorane inhalation and injected subcutaneously into the external ear with labeled cardiospheres. Mice were allowed a 2–3 week recovery period prior to sacrifice. Unfixed tissues were dissected, viewed, and analyzed using confocal microscopy. Green and red fluorescence were observed using their respective filters. Animal studies were approved by the Institutional Animal Care and Use Committee of the University of North Texas Health Science Center at Fort Worth and was conducted in accordance with *the Guide for the Care and Use of Laboratory Animals* (NIH publication 85-23, revised 1996).

STATISTICAL ANALYSES

All experiments were done at least 3 times. MTS assays were done in triplicate wells at least 3–5 times. Statistical analyses were done using Student's *t*-test.

RESULTS

CARDIAC STEM CELLS FORM CARDIOSPHERES

Fully dissociated adult cardiomyocytes (AC) isolated from left ventricular myocardium of adult dogs appeared as large phase-bright polygonal structures that remained unattached and did not proliferate up to 8 weeks in culture (Fig. 1A, AC). On the other hand, putative cardiac stem cells (CSC) plated on collagen matrix in SCM, containing serum and FGF-2/IGF-1, remained small and grew rapidly in monolayer cultures. To promote sphere formation, CSC were seeded on a PLO substratum and shifted to CFM, containing serum and FGF-2/EGF (Fig. 1A, 0d). Phase contrast microscopy showed the development of small cell clusters on PLO-treated dishes

as early as 24 h of culture. Cellular aggregates appeared to adopt a spheroidal form as they increased in number and size after 2, 4, 6, and 8 days in culture (Fig. 1A, 2d–8d). Moreover, the spheroidal bodies appeared to grow at a uniform rate in the cultures.

To determine the rate of growth of the cardiospheres, the diameters of 10 random unfixed spheres were measured at different time points (2–8 days). Growth curves showed a ~3-fold increase in size of cardiospheres from ~75 μm diameter on day 2 to ~234 μm diameter on day 8 (Fig. 1B). Growth spurt occurred from days 2 to 4 with almost ~2.2-fold increase in the diameter of cardiospheres. The increase in size of cardiospheres slowed down to ~1.3-fold from days 4 to 6, and to ~1.1-fold from days 6 to 8. The remarkable uniformity in sizes at each time point was consistent with the regulated growth of the spheres that most likely resulted from proliferation of CSC in the interior of the spheres.

Many of the cardiospheres detached from the monolayer cultures and became free-floating as early as 72 h into the culture. Phase contrast and SEM images of 3–5-day-old cultures of non-adherent cardiospheres showed smooth surfaces containing outlines of flattened or rounded cells (Fig. 1C). Floating cardiospheres, 3–6 days old, mostly contained solid cores, while larger spheres 10 days and older were often hollow (data not shown).

To determine whether the ability of CSC to form cardiospheres might be related to growth factor response, floating cardiospheres were removed and the residual adherent cultures were subjected to Western blot analyses. Western blots of relatively cardiosphere-free adherent cultures in CFM at 0, 4, 8 days showed decreasing levels of FGFR and EGFR (Fig. 1D), consistent with diminished capacity for growth factor-induced sphere formation in older cultures.

CARDIOSPHERES EXPRESS STEM CELL AND CARDIAC MARKERS

Early cardiospheres, 2–3 days old (Fig. 2A), were strongly immunoreactive for stem cell markers c-kit and pax6 but not for markers of differentiated cardiomyocytes. Intense staining for PCNA was also observed in the spheres, suggestive of the highly proliferative state of the CSC in the early stages. Cardiospheres cultured for 6–8 days (Fig. 2B) were strongly reactive for cardiomyocyte-specific markers—cMHC, cSA, and cTnI; as well as vascular cell markers—vWF and sMA. The most intense staining for markers of differentiated cardiac cells was observed in optical sections taken near the surface of the spheres. On the other hand, ckit-staining appeared strongest in optical sections taken near the equator of the spheres. Whether these staining patterns suggest tissue reorganization within developing cardiospheres remain to be established.

Western blots verified the absence of cMHC or cTnI labeling in the starting monolayer cultures, and accumulation of cMHC or cTnI in cardiospheres with longer incubation periods (Fig. 2C). As with cSA, the expression of cMHC, and cTnI was observed initially in 2-day cultures, albeit at low levels, suggestive of the early differentiation of the CSC into cardiomyocyte progenitors. The lack of expression of differentiation markers for adult cardiomyocytes in immunoblots, immunocytochemistry, and RT-PCR [data not shown] in the starting monolayer cultures argues against contamination with adult cardiomyocytes from the initial isolation.

To determine their functional capacity, cardiospheres were pre-labeled with tracking dyes and plated onto confluent cultures of neonatal rat cardiomyoctes. Monolayer cultures of CSC or cardiospheres by themselves never displayed spontaneous beating in culture that has been widely reported in neonatal rat cardiomyocytes. Although many cardiospheres remained free-floating in the medium, a few CellTracker Red-labeled cardiospheres adhered to the unlabeled neonatal cardiomyocyte feeder layer (Fig. 3). Adherent spheres appeared to integrate with the neonatal cardiomyocytes especially at the edges. Beating cell clusters observed in the cultures were found to colocalize with the CellTracker Red-labeled cells (Supp. 1). The small areas where beating was limited to could be attributed to the smaller number of differentiating cardiomyocytes

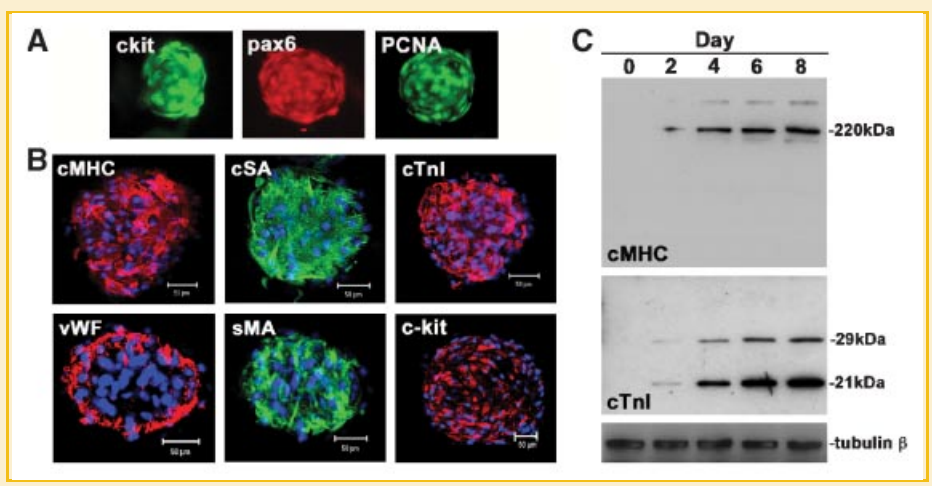

Fig. 2. Cardiosphere phenotype. A: Cardiospheres cultured for 2–3 days on 0.01% PLO in sphere-forming medium (SFM) were strongly reactive for ckit (green), pax6 (red), and PCNA (green). B: Cardiospheres cultured for 6–8 days stained with antibodies against cMHC (red), cSA (green), cTnI (red), vWF (red), sMA (green), and ckit (red). DAPI (blue) was used to counterstain nuclei. C: Western blot analyses of spheres cultured in sphere-forming conditions for 0–8 days showed absence of cMHC or cTnI expression in monolayer cultures at day 0 and increasing levels with longer duration of cultures. The membrane was re-probed with anti-tubulin β to normalize for sample loading. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

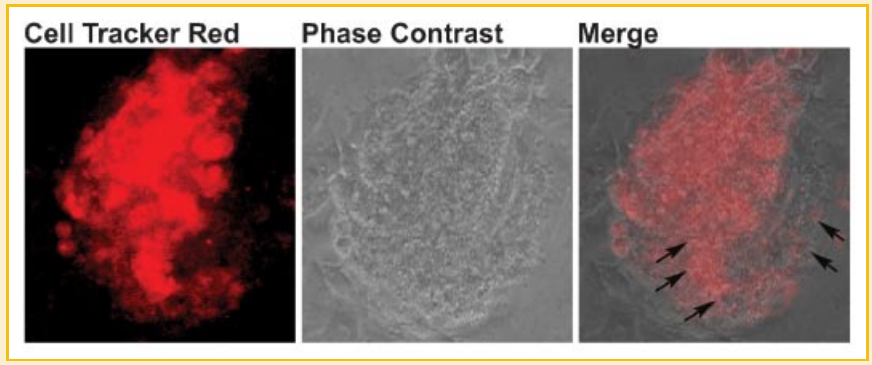

Fig. 3. Co-cultures with neonatal rat cardiomyocytes. Cardiospheres adherent to unlabeled neonatal cardiomyocyte feeder layer could be easily identified by the presence of CellTracker Red. The adherent spheres appeared to integrate with the neonatal cardiomyocytes especially at the edges. Beating cells/clusters often contained the tracking dye (arrows) [see Supplementary Data 1]. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

in the spheres as compared with endothelial and smooth muscle cell types.

CARDIOSPHERE-DERIVED CELLS EXPRESS DIFFERENTIATED CARDIOMYOCYTE AND VASCULAR CELL PHENOTYPES

To further establish cardiac differentiation of CSC, cardiospheres were dissociated enzymatically, cultured in growth medium for 48 h and immunostained for cardiac markers. Cardiosphere-derived cells assumed a variety of morphological phenotypes (Fig. 4A). Most of the cells were small cells bearing few short processes; while others were large and flattened or tubular (Fig. 4A). Cells strongly reactive for cardiomyocyte markers cMHC, cSA, and cTnI were mostly tubular or flattened large cells (Fig. 4B). cMHC and cSA immunostaining showed the intense banding patterns of sarcomeric striations (see inset in Fig. 4B, cMHC) characteristic of organized contractile machinery of cardiac and skeletal muscles. SMA-reactive cells were also flat and large (Fig. 4C). On the other hand, cells reactive for c-kit and vWF were mostly small (Fig. 4C).

CARDIOSPHERES CONTAIN VASCULAR PROGENITOR CELLS

To further determine the potential of cardiospheres to promote angiogenesis, cardiospheres were probed for expression of vascular

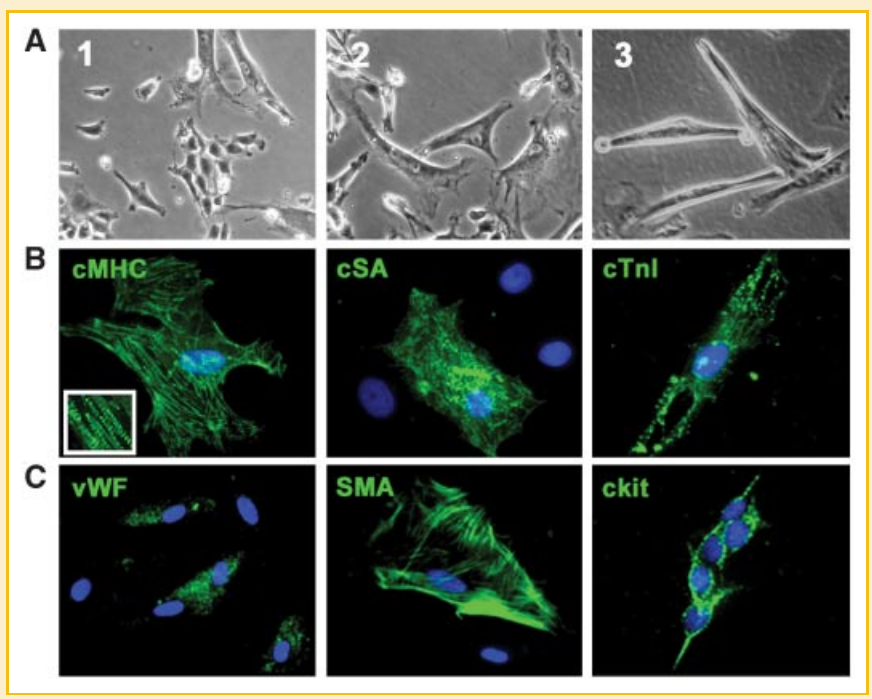

Fig. 4. Cardiosphere-derived cells. A: Cultures derived from dissociated cardiospheres consisted of colonies of small rounded cells bearing few short processes (1) or large cells which were either flattened (2), or tubular (3). B: The large cells were strongly reactive for cardiomyocyte markers cMHC, cSA, and CTnI. cMHC and cSA immunostaining showed intense banding patterns of sarcomeric striations [inset, cMHC]. C: Cells reactive for vWF and c-kit were mostly small, while SMA-positive cells were large and flat. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

cell markers (vWF and SMA) and VEGF, and analyzed under a confocal microscope. Immunocytochemistry showed staining for vWF, SMA, and VEGF in the cardiospheres. Optical sections of cardiospheres showed similar distribution of VEGF labeling to SMA, a marker for smooth muscle cells (Fig. 5A). RT-PCR showed specific and appropriate sizes of different VEGF mRNA species in cardiospheres, as verified by control dog heart (DH) cDNA (Fig. 5B). PCR products of 521, 503, 449 bp compatible with VEGF188, VEGF182, VEGF164 were observed, while the 317bp product for VEGF120 was hardly visible. PCR products of the long VEGF isoforms (VEGF182/VEGF188) appeared stronger than VEGF164, as previously reported in the dog myocardium [Jingjing et al., 2000]. PCR products compatible with human VEGF206 or VEGF145 were not observed.

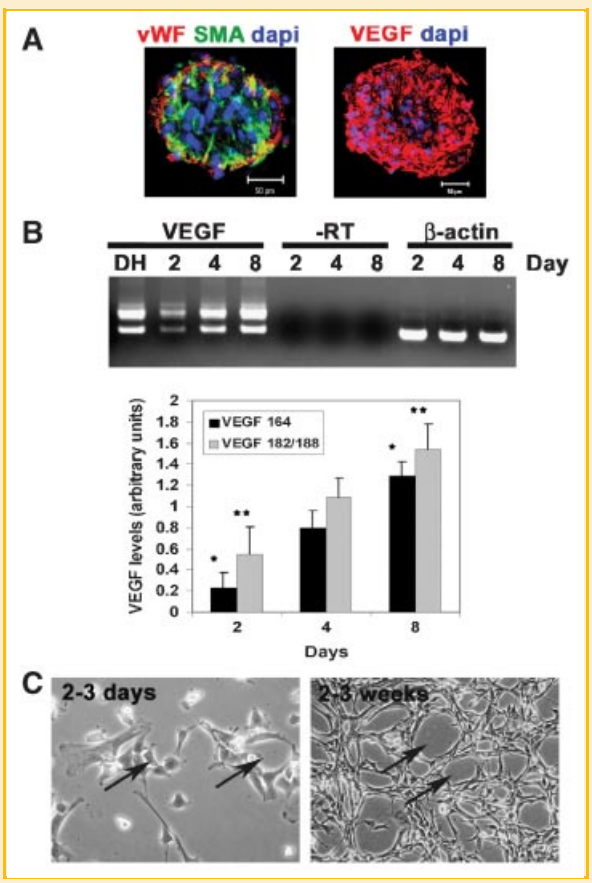

Fig. 5. Vascular cell phenotypes. A: Cardiospheres stained intensely for vWF (red), SMA (green), and VEGF (red). DAPI (blue) was used to counterstain cell nuclei. B: PCR bands were observed at appropriate sizes for VEGF mRNA using dog heart cDNA (DH) as control, unlike in RT control. β actin PCR products were used to normalize for sample loading. Densitometry showed the expression of the different molecular forms of VEGF increased proportionately with longer duration of culture. VEGF levels were significantly higher on day 8 as compared with the levels on day 2 for both VEGF164 (*) or VEGF182/188 (**). Student's *t*-test showed significant differences at $P < 0.005$. C. Small process-bearing cells from dissociated cardiospheres started forming vascular profiles (arrows) as early as 2–3 days, and became spindle-shaped forming numerous networks after 2–3 weeks in microvascular endothelial cell medium. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Omission of the RT during cDNA synthesis resulted in the absence of PCR products. The β-actin products verified the adequacy of the cDNA synthesis and absence of contamination from genomic DNA. Densitometry of reactive bands indicated that the expression of the different molecular forms of VEGF increased proportionately with longer duration of cultures. The intensity of VEGF164 and of VEGF182/VEGF188 PCR products increased significantly by ~5.6- and ~2.8-fold, respectively, from days 2 to 8.

To further characterize cardiospheres, 4–6-day-old spheres were dissociated enzymatically and grown in Microvascular Endothelial Cell Medium-2 (EGM-2 MV). Colonies of small process-bearing cells appeared to initiate the formation of networks, suggestive of vascular profiles, as early as 2–3 days following dissociation. The small cells proliferated, became spindle-shaped, and formed numerous networks after ~2–3 weeks in culture [Fig. 5C]. The behavior of the cardiosphere-derived cells in endothelial cell growth enhancing medium were highly suggestive of their proliferative capacity; while their expression of vWF, SMA, and VEGF, was consistent with their ability to undergo differentiation into vascular cell types with the potential to form vascular networks. Whether this potential will require extraneous induction or endogenous stimulation in cardiac transplant paradigms remains to be determined.

CARDIOSPHERE-FORMATION ATTENUATES OXIDATIVE DAMAGE

Cardiospheres at days 4–5 were treated with various concentrations of H_2O_2 for 1 h and stained with calcein AM/ethidium homodimer or JC-1 24 h later to test for survival or disruption in the mitochondrial membrane potential, respectively. Majority of the cells in cardiospheres were labeled with calcein AM and extruded ethidium homodimer when incubated in 0–1 mM H_2O_2 (Fig. 6A). Few cells survived following exposure to 10 mM H_2O_2 with most calcein AM-labeled cells distributed in the inner layers of cardiospheres and ethidium homodimer-positive cells in the outer layers. Damage to the mitochondrial membranes, as shown by altered JC-labeling, was also observed in cardiospheres treated with 10 mM H_2O_2 but not with 0–1 mM H_2O_2 (Fig. 6B), consistent with the calcein AM/ethidium homodimer staining. To measure the survival of CSC in cardiospheres as compared with those in monolayer cultures, cultures exposed to H_2O_2 were assayed using MTS/PMS (Fig. 6C). The number of surviving cells in monolayer cultures started decreasing immediately following exposure to 1 mm H_2O_2. The number of cells in monolayer cultures decreased ~6.5-fold following exposure to 1–10 mM H_2O_2 as compared with untreated cells or cells treated with 0.1–0.5 mM H_2O_2. In contrast, the number of live cells in cardiospheres remained high following treatment with 0–2 mM H_2O_2. The number of live cells decreased only slightly ~1.5-fold following treatment with 5 mM H_2O_2 when compared with spheres exposed to 0–2 mM H_2O_2 and were mostly dead only following exposure to 10 mM H_2O_2. Since cell counts were based on a standard curve generated using monolayer cultures, it was impossible to compare the exact number of cells between the two cultures. However, the pattern of MTS labeling in the cardiospheres showing that cell death occurred only at higher H_2O_2 concentrations, together with the calcein AM/ethidium homodimer labeling

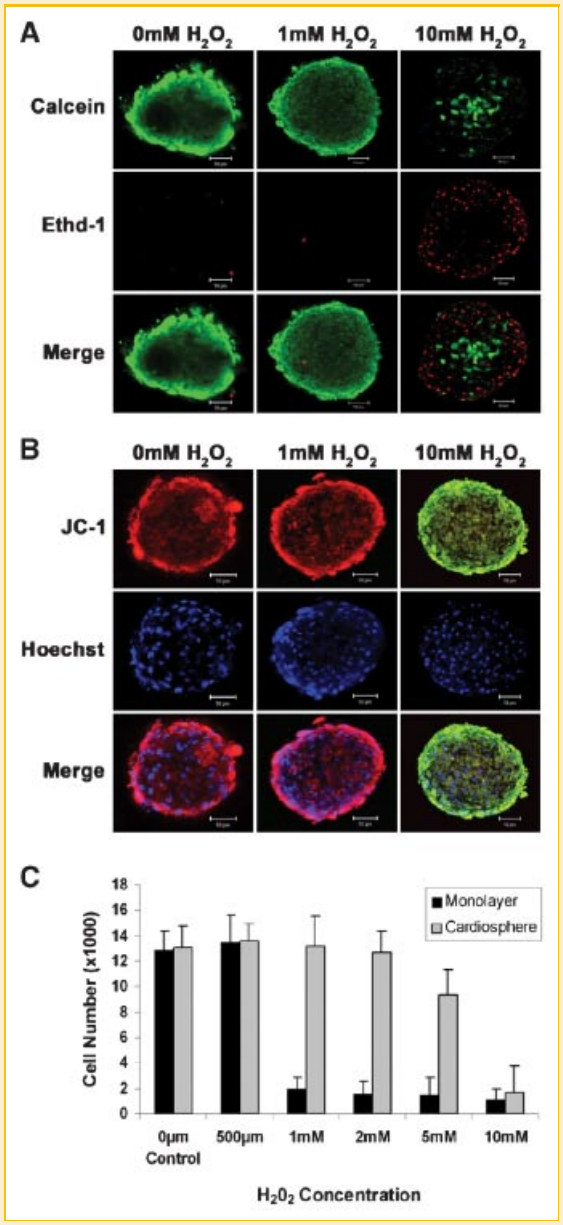

Fig. 6. Response to oxidative stress. A: Cardiospheres, 4–5 days old, treated with 0–5 mM H_2O_2 for 1 h were intensely labeled with calcein AM but were mostly negative for ethidium homodimer (Ethd-1). In spheres exposed to 10 mM H_2O_2, few cells mostly located in the core of the spheres labeled for calcein AM; while most cells in the periphery were intensely reactive for ethidium homodimer (Ethd-1), consistent with cell death. B: Spheres treated with 10 mM H_2O_2 and stained with JC-1 exhibited green fluorescence consistent with increased mitochondrial membrane permeability, unlike those exposed to 0–1 mM H_2O_2 which showed orange-red fluorescence. Spheres were double-labeled with Hoechst 33342 to visualize cell nuclei. C: The number of surviving cells in monolayer cultures decreased significantly following exposure to 1–10 mM H_2O_2 as compared to cells treated with 0 and 0.5 mM H_2O_2. In contrast, the number of live cells in cardiospheres remained high following treatment with 0–2 mM H_2O_2, decreased slightly with 5 mM H_2O_2, and were mostly dead following exposure to 10 mM H_2O_2. Survival assays were performed using MTS and results were reported as cell counts based on a standard curve generated from monolayer cultures. Student's *t*-test showed significant differences at $P < 0.005$. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

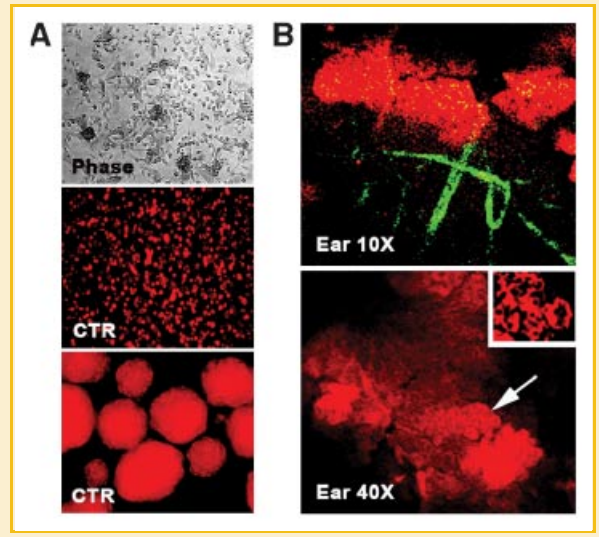

Fig. 7. Cardiosphere injections. A: Monolayer cultures of CSC (Phase) were stained with 5 µM Cell Tracker Red (CTR) and induced to form spheres following culture in SFM for 4–5 days. B: CTR-labeled cardiospheres injected subcutaneously into the external ear of 5 tie2-GFP transgenic mice showed high cellular aggregates (red) adjacent to GFP-expressing vessels (green). Boxed figure shows profiles of selected CTR-labeled cells (arrows) at higher magnifications. Images were captured on the confocal microscope with the red/green (R/G) fluorescence filter. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

and JC-1 staining, were all suggestive of the higher resistance of cardiospheres to oxidative stress.

ANALYSIS OF INJECTED CARDIOSPHERES

Cardiospheres pre-labeled with CellTracker Red (CTR) were tested for viability, tumorigenecity, and the capacity to generate competent transplantation material. Monolayer cultures of CSC maintaining high levels of c-kit expression were stained with 5 µM cell tracker red. Labeled cells formed spheres following culture in SFM for 4–5 days (Fig. 7A). Labeled cardiospheres injected subcutaneously into the external ear of 5 tie2-GFP transgenic mice showed highly cellular aggregates of viable injected cells near GFP-labeled vascular profiles (Fig. 7B). No tumors were observed even after 3 weeks of transplantation, and cells remained localized to areas of injection. GFP-labeled vascular profiles were often seen near the grafts but were never observed to invade the cell aggregates suggesting that the vascular progenitors within the cardiospheres might still require pre-treatment with exogenous angiogenic factors to "prime" the cells to organize into microvessels and integrate with the host vasculature.

DISCUSSION

Traditional cell culture systems consist of monolayer cultures growing in a two dimensional (2D)-architecture. While 2D-culture conditions are generally sufficient for investigations of simple cellular functions and/or activity of epithelial tissues or endothelia, most functional tissues are characterized by complex intercellular

relationships that are best studied only in 3D systems. In cardiovascular research, several approaches have been employed to engineer functional 3D-cell systems from differentiated cardiomyocytes [Akins et al., 1999; Baar et al., 2004; Kelm et al., 2004], that can be used to model cardiac microtissues, and to investigate the potential for generating vascular architecture and perfusion in vitro [Carrier et al., 2002; Watzka et al., 2004]. Simple 3D-cultures have been developed by seeding cells on non-adhesive surfaces and non-tissue culture treated plastics [Keller, 1995; Oudar, 2000] or on highly porous biodegradable polymers using collagen, polyglycolic acid, gelatin, or alginate scaffolds [Dar et al., 2002]. Alternative approaches have utilized hanging-drop cultivation [Keller, 1995; Hescheler et al., 1997; Kelm et al., 2004], rotating bioreactors [Freed and Vunjak-Novakovic, 1997; Timmins et al., 2004; Liu et al., 2006; Guo et al., 2006], and cell sheet engineering utilizing temperature-sensitive substrates [Shimizu et al., 2003].

Studies by Messina et al. [2004] showed adult CSC from human and murine species spontaneously form 3D-spheroids in culture. The 3D-spheroids called ''cardiospheres'' resemble neurospheres formed by neural stem cells and progenitors [Reynolds and Weiss, 1992; Weiss et al., 1996; Suslov et al., 2002; Moeller and Dimitrijevich, 2004] or embryoid bodies composed of differentiating ES cells [Keller, 1995]. To our knowledge, this is the first report describing the formation of cardiospheres by adult stem cells from the dog heart. Moreover, our preliminary data indicated that the formation and growth of cardiospheres could be facilitated and regulated by culturing CSC in surfaces coated with positively charged amino acids such as poly-L-ornithine (PLO) or poly-L-lysine (PLL) in the presence of growth factors FGF-2 and EGF. In the present study, we characterized the growth and differentiation of CSC in PLO/FGF-2/EGF-induced cardiospheres and determined the suitability of cardiospheres as 3D-models of cardiac tissue differentiation and response to oxidative stress.

Our study showed that CSC grown on poly-L-ornithine-coated surfaces in the presence of growth factors FGF-2 and EGF rapidly formed cardiospheres. Cardiospheres grew rapidly but uniformly under these conditions suggesting regulated growth of the spheres. Other positively charged surfaces, such as poly-L-lysine, but not PLO, have been used previously to facilitate the formation of hepatocyte aggregates exhibiting liver-specific functions [Tzanakakis et al., 2001]. PLO, similar to other polycations especially poly-L-lysine, has been used mostly in combination with other molecules for various applications such as non-viral gene delivery [Fimmel et al., 2000; Ramsay and Gumbleton, 2002; Brown et al., 2003], packaging system (in alginate beads) for cellular grafts [Tobias et al., 2001; Calafiore, 2003], or as attachment matrix/scaffold for neuronal and embryonic stem cell cultures [Murashov et al., 2005; Flanagan et al., 2006; Morizane et al., 2006]. Polyamino acids, such as PLO, have also been shown to activate cell signaling pathways [Haber et al., 1991]. Preliminary studies in our laboratory utilizing pharmacological inhibitors or analogues of signaling molecules suggested that activation of transcriptional and post-translational mechanisms might be involved in the regulation of cardiosphere formation (data not shown). Studies are in progress to investigate the role of these PLO-mediated signaling pathways on cardiac tissue differentiation.

Our study also showed that cardiospheres expressed the stem cell markers c-kit and pax6. While CSC are known to express c-kit, to our knowledge, this is the first report of pax6 expression in CSC. Cardiospheres gradually acquired a differentiated phenotype composed predominantly of cardiomyocytes, smooth muscle cells, and vascular endothelial cells. Observations of beating spheres co-cultured with neonatal rat cardiomyocytes also suggest that the cardiospheres possess the ability to functionally and mechanically couple with host myocardium following transplantation.

The growth of cardiospheres slowed down by day 8 till they achieved a maximum diameter of $\sim$300 μM. This probably resulted from cessation of cell division, continued differentiation, or degeneration of core stem cells due to limited diffusion of nutrients or oxygen. Imaging studies of the metabolic environment of 3D-tissues in vitro revealed diminishing gradients of energy in concentric patterns from the surface to the center of the spheres, resulting in a central region of necrosis or apoptosis [Korff and Augustin, 1998; Walenta et al., 1999]. In addition, our preliminary studies demonstrating the susceptibility of cardiospheres to simulated ischemic conditions in vitro (Bartosh et al., in preparation) raise the likelihood that the long term survival of intra-cardiac grafts would require the development of a novel vascular supply to support the nutritional demands of the transplanted tissues. In this study, we now demonstrate size-dependent increase in VEGF transcripts in cardiospheres and differentiation of CSC into vascular endothelial and smooth muscle cells capable of organizing a vascular network. These findings raise the exciting possibility that endothelial and smooth muscle cells in cardiospheres could form primitive vessels capable of linking with the host vessels to promote perfusion and survival of the grafted tissue.

Our study also showed that cardiospheres displayed inherent higher resistance to reactive oxygen species (ROS) as compared to monolayer cultures. This characteristic could allow transplanted cardiospheres to survive the harsh environment in the injured myocardium characterized by inflammatory cytokines, ischemia, and ROS. This characteristic is also consistent with reports showing cell–cell adhesions and interactions in other spheroid cultures can alter and regulate cellular signaling and tissue behavior. For example, in tumor spheroids, integrin and cadherin-mediated cell adhesions regulate the growth and survival of tumor cells [Kantak and Kramer, 1998; Damiano et al., 1999], and perhaps, contribute to increased resistance to chemotherapeutic agents [Kobayashi et al., 1993; St. Croix and Kerbel, 1997]. The integration of endothelial cells into multi-cellular spheroids has also been shown to prevent the rapid cell death response observed in single suspended cultures while promoting increased sensitivity to angiogenic factors [Korff and Augustin, 1998].

Last but not least, our study showed that transplanted cardiospheres survived transplantation conditions and did not form tumors even 3 weeks post-transplantation. Together with their ability to undergo differentiation into various cardiac cell types, as well as the relative resistance to oxidative stress, the lack of tumor-induction in vivo supports cardiospheres as a suitable delivery system of stem cells for tissue regeneration.

In conclusion, we have developed a 3D cell system using poly-L-ornithine seeded with putative cardiac stem cells isolated from the

adult canine heart. To our knowledge, this is the first report describing the establishment of a 3D-model of putative cardiac stem cells from the adult dog heart. The 3D-cultures, called cardiospheres, displayed characteristics of undifferentiated cells, differentiating cardiomyocytes, and/or vascular cells. Moreover, cardiospheres displayed remarkable resistance to oxidative stress and survived transplantation without undergoing neoplastic transformation. Several key characteristics of an intracardiac tissue graft, that is, appropriate differentiation, cellular integration, and resistance to oxidative stress, are required to optimize the beneficial effects of cell-based therapies for cardiac regeneration. Our study shows that cardiospheres display these features. The cell protection seen in cardiospheres in response to oxidative stress makes this 3D-model unique with respect to its putative capacity to survive pro-apoptotic conditions characteristic of the post-ischemic heart when reactive oxygen species are elevated (a time when stem cell transplantation will likely be necessary). The 3D-model can, thus, be used to investigate cardiac and vascular cell differentiation and oxidative stress response as well as provide a novel system for targeted delivery of cardiac precursors to the injury site. Studies are now in progress to establish differentiation, functional integration, and angiogenic response of grafted cardiospheres following transplantation into the dog heart.

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