A Strong Regenerative Ability of Cardiac Stem Cells Derived From Neonatal Hearts

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Background—Human cardiac stem cells (CSCs) promote myocardial regeneration in adult ischemic myocardium. The regenerative capacity of CSCs in very young patients with nonischemic congenital heart defects has not been explored. We hypothesized that isolated neonatal-derived CSCs may have a higher regenerative ability than adult-derived CSCs and might address the structural deficiencies of congenital heart disease.

Methods and Results—Human specimens were obtained during routine cardiac surgical procedures from right atrial appendage tissue discarded from 2 age groups: neonates and adults patients. We developed a reproducible isolation method that generated cardiosphere-derived cells (CDCs), regardless of starting tissue weight or age. Neonatal-derived CDCs demonstrated increased number of cardiac progenitor cells expressing c-kit$^+$, flk-1, and Islet-1 by flow cytometry and immunofluorescence. When transplanted into infarcted myocardium, neonatal-derived CDCs had a significantly higher ability to preserve myocardial function, prevent adverse remodeling, and enhance blood vessel preservation and/or formation when compared with adult-derived CDCs. Last, neonatal-derived CDCs were more cardiomyogenic than adult-derived CDCs when cocultured with neonatal cardiomyocytes and displayed enhanced angiogenic function compared with adult-derived CDCs.

Conclusions—Neonatal-derived CDCs have a strong regenerative ability when compared with adult-derived CDCs that may depend on angiogenic cytokines and an increase prevalence of stem cells. This has important implications in the potential use of CDCs in future clinical trials. **(*Circulation*. 2012;126[suppl 1]:S46–S53.)**

Key Words: myocardial infarction ■ cardiac stem cells ■ stem cell differentiation ■ paracrine effect ■ cell therapy ■ neovascularization

The identification and application of resident cardiac stem cells for regeneration of damaged myocardium challenges the previous dogma of a terminally differentiated, nonrepairable heart. Recent human phase I clinical trials in adult myocardial ischemic patients have demonstrated the beneficial regenerative abilities of 2 different resident cardiac stem cells: c-kit$^+$ cardiac stem cells and cardiosphere-derived cells (CDCs).[1,2] C-kit$^+$ cells, which express the surface receptor tyrosine kinase, are self-renewing, clonogenic, and multipotent, with the ability to differentiate into cardiomyocytes, smooth muscles, and endothelial cells.[3,4] In the Cardiac Stem Cells in Patients with Ischemic Cardiomyopathy (SCIPIO) trial, c-kit$^+$ cell treatment in adult heart failure patients improved left ventricle function and reduced infarct size 12 months after treatment. On the other hand, CDCs contain a heterogeneous pool of differentiated and undifferentiated cells, which include c-kit$^+$ cells.[5] CDCs similarly differentiate into all three cardiac lineage cells as seen in animal cardiomyopathy models.[6–8] In the cardiosphere-derived autologous stem cells to reverse ventricular dysfunction (CADUCEUS) trial, treatment with CDCs in adult ischemic patients decreased infarct size and increased myocardial mass. However, there was little effect on left ventricular ejection fraction. Even though these results are clinically promising, the number of patients in both treatment arms was small. Thus, larger, more appropriately powered studies are needed to verify the efficacy of these studies.

Whereas adults with cardiac failure often have ischemic cardiomyopathy, pediatric cardiac heart failure is more varied and includes a spectrum of cardiomyopathies, congenital cardiac diseases, and arrhythmias.[9] The etiologies of heart failure in children may extend beyond myocardial ischemia to include volume or pressure overload to the systemic ventricle that may include both ventricles.[9] Similar to adults, the prevalence of heart failure in children is on the rise during the last decade and the prognosis for patients who are admitted to

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the hospital with heart failure remains poor.[10] Neonates with heart failure are the most medically and surgically challenging and have the highest congenital mortality rates.[11] Current medical therapies or devices do not address the fundamental loss or deconditioning of cardiac tissue in pediatric heart failure patients. To address this issue, resident CSC therapy may be a strategy that regenerates the damaged myocardium by generating viable myocardium or by improving the myocardial environment through secreted growth factors. Cardiac cellular therapy may be tested either as a stand-alone cellular injection into the systemic ventricle or as an adjunct to the surgical reconstruction repair that may be planned for these patients, such as the Norwood or superior cavopulmonary connection for hypoplastic left heart syndrome (HLHS) patients.

Previously, we have shown that c-kit$^+$ CSCs are most abundant in the neonatal stage and subsequently decrease in prevalence with advancing age in congenital heart patients.[7] We further demonstrated that human CDCs from congenital heart patients have cardiac regenerative abilities. Not addressed, however, was whether the regenerative abilities of CDCs derived from neonates differed from CDCs derived from adults. Given the recent results of CADUCEUS trial, the mechanism(s) of the regenerative capacity of human CDCs is unclear, and further studies are required to identify the characteristics of CDCs that may lead to an improvement of left ventricular function. For instance, it is unclear whether age of the heart may play a role in the function of the CDCs. Thus, we hypothesized that neonatal-derived CDCs would demonstrate an augmented regenerative capacity when compared with adult-derived CDCs because of a more abundant cardiac stem cell pool in neonatal hearts. In this study, CDCs were isolated from human neonates and adults and were examined for stem cell and cardiac lineage markers. Additionally, the regenerative capacity of the CDCs was tested in an immunodeficient rodent model of myocardial infarction. The overall goal of this work was to provide essential and practical data required for using neonatal-derived CDCs in future clinical trials.

Methods

Acquisition of Human Tissue Samples and Cell Culture

This study was approved by the Institutional Review Committee at Children's Memorial Hospital and Northwestern University Hospital. After parental or patient consent was given, specimens (70±80 mg) from the right atrial (RA) appendage were obtained from neonate (n=43) and adult patients (n=13) during routine cardiac surgeries. The neonatal patients varied in diagnosis (Online Data Supplement Table I). All tissue samples were processed for immunostaining and harvesting of human cardiac progenitor cells (CPCs) via cardiosphere development. Human CDCs were generated through the use of the protocol described by Smith et al,[7] with modifications (see expanded Methods in the Online Data Supplement).

Flow Cytometry Analysis of CDCs

CDCs at P1 were evaluated by flow cytometry using a Becton-Dickinson FACS caliber (San Jose, CA), with 10 000 events collected. Cells were incubated with fluorochrome-conjugated primary antibodies against c-kit$^+$, flk1, NKX2-5, Ki67$^+$, Sca1$^+$, cardiac troponin T (cTnT), CD105, and antibodies against hematopoietic lineage surface marker CD34 and CD45. Isotype controls were run for each immunosubtype.

Infarct Model

Myocardial infarction was induced by permanent ligation of the left anterior descending (LAD) coronary artery in immunodeficient male rats (weight, 250–300 g). The heart was exposed via a left thoracotomy, and the proximal LAD was ligated. Afterward, 1 million neonate or adult CDCs or cardiac fibroblasts suspended in 250 to 400 μL of vehicle were injected at multiple sites. Rats with induced infarction and without cell injection or rats injected with cardiac fibroblasts served as controls for the study.

Echocardiography

Transthoracic echocardiograms were performed on rats, using a VisualSonics Vevo 770 ultrasound unit (VisualSonics, Toronto, Canada). Baseline echocardiograms were acquired at 1 day before myocardial infarct surgery. Echocardiographic examinations were also performed at 7 days after myocardial infarction, with additional echocardiograms acquired at 4 weeks after myocardial infarction. Two-dimensional and M-mode echocardiography were used to assess fractional area change (FAC). Images were obtained from the parasternal long axis and the parasternal short axis at the midpapillary level.

Myocardial Histology

Hearts were excised under anesthesia, perfused with 4% paraformaldehyde, cryo-protected, and embedded. Sections were cut to 7 μm, using a commercial cryostat, and used for isolectin B4 (Invitrogen; Carlsbad, CA), cardiac troponin T (Santa Cruz Biotechnology; Santa Cruz, CA), and anti-human nuclei (Millipore; Billerica, MA) staining. Sections were counterstained with nuclear DAPI (4′,6-diamidino-2-phenylindole) stain (Sigma; St Louis, MO). To calculate infarct size, at least 4 Masson trichrome–stained sections at various levels along the long axis were analyzed for collagen deposition. The midline technique for infarct size determination was used as described previously.[12]

Real-Time RT-PCR

RNA was isolated from cardiac fibroblast and CDCs using a commercial RNeasy kit (Qiagen; Valencia, CA). Real-time PCR was run using a total of 5-ng template cDNA for each sample. Each sample was run in duplicate, using ABI FAST SYBR green supermix (Applied Biosystems; Foster City, CA) for multiple genes, including angiogenin (ANG), vascular endothelial growth factor (VEGF), fibroblast growth factor-1 (FGF-1), fibroblast growth factor-2 (FGF-1), platelet-derived growth factor-B (PDGF-B), insulin-like growth factor (IGF-1), and ribosomal protein 13A (RPL13A). Relative RNA abundance was calculated using the following equation: $2^{-\Delta\Delta cT}$.

Assessment of CDC Differentiation Potential

A coculture system using neonatal cardiomyocytes and either neonate or adult CDCs was used to determine cardiomyogenic potential. Briefly, neonatal rats were euthanized and cardiomyocytes were isolated, using a commercial kit (Worthington Biomedical Corp, Lakewood, NJ). Cells were seeded at a density of 50 000 cells per well of an 8-well chamber slide; after 24 hours, 5000 neonate or adult CDCs were added for an additional 14 days. Differentiation of CDCs toward cardiac lineages was assessed using fluorescence microscopy by staining cells for cTnT, human mitochondria (Millipore; Billerica, MA) and a nuclear DAPI (4′,6-diamidino-2-phenylindole) stain (Sigma; St Louis, MO).

Statistical Analysis and Interpretation

A Kruskal-Wallis test with a Dunn post hoc test was used for the interpretation of infarct size and PCR data sets. A Wilcoxon rank rum test was used for histological and flow cytometry data sets. Data are presented with median and interquartile range (25th, 75th). A

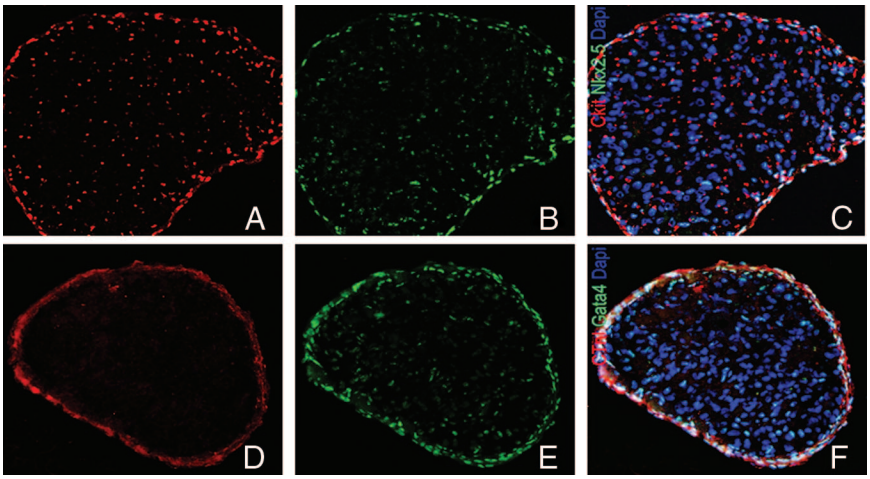

Figure 1. Generation and characterization of cardiospheres derived from neonatal patients and adult patients. Intact cardiospheres were sectioned and stained for c-kit$^+$ (**A**), NKX2-5 (**B**), serial staining with c-kit+ and NKX2-5 (**C**), GATA4 (**D**), cTNI (**E**), and serial staining with GATA4 and cTNI (**F**) (nuclei are shown in blue; magnification ×10). Generally, there was no difference in expression levels and localization between neonatal and adult cardiospheres.

probability value of <0.05 indicated statistical significance. A 2-way ANOVA with a Bonferroni post hoc test was used to interpret ejection fraction data.

Results

Characterization of CDCs Derived From Neonate and Adult Cardiac Biopsies

CSCs were isolated and expanded from RA tissue, using a modified version of the cardiosphere method. RA heart samples were taken from neonates (n=43) and adults (n=13) and ranged in weight size from 25 to 200 mg. Immunostaining of intact cardiospheres identified cells that expressed c-kit$^+$, NKX2-5, c-kit$^+$, and TnI (Figure 1A through 1H). The c-kit$^+$ cells were located in the inner and outer core of the cardiopshere. The cardiosphere expression pattern for GATA-4, Islet-1 (ISL1) and TnI was similarly varied throughout the cardiosphere. We saw no difference in the expression of these markers in cardiospheres derived from neonates or adults.

From these cardiospheres, CDCs could be expanded beyond 30 days and yielded greater than 2×10^6 cells. Even neonatal right atrial samples weighing between 20 to 40 mg generated similar cell numbers using the current CDC modifications. CDCs were examined for their expression of stemness markers and cardiac lineage commitment markers to determine whether the expanded CDCs remained in an uncommitted state or differentiated state. Neonatal-derived CDCs displayed statistical significantly higher levels of c-kit$^+$ and flk-1 compared with adults [c-kit$^+$, n=4 in each group: 21.1% (11.6, 35.04) versus 3.2% (0.3, 11.2), P=0.04; flk-1, n=3 in each group: 25.9% (17.4, 35.2) versus 8% (1.7, 13.5); P=0.04; Figure 2A]. Because the Islet-1 (ISL1) antibody was not conducive for flow cytometric analysis, ISL1 expression was determined by quantitative immunofluorescence, which showed a statistically significant increase of ISL1 cells when compared with adults [36.2% (34.3, 39.3) versus 3.2% (0, 6.5); P=0.0001; n=3; Figure 2B]. Differences did not reach statistical significance in other lineage and commitment markers: Sca-1, CD105, CD31, Ki67, and NKX2-5 (Online Data Supplement Table II). These results indicated that the neonatal-derived CDCs had a higher prevalence of cardiac progenitor cells in comparison to adult-derived CDCs.

CDCs Derived From Neonates Display Augmented Regenerative Potential

To determine whether the increased number of cells expressing c-kit$^+$, flk-1, and ISL1 in neonatal-derived CDCs correlated with improved regenerative potential, 2 sets of experiments were performed. First, CDCs cocultured with rat neonatal cardiomyocytes were used to quantify cardiomyogenic potential. After 2 weeks of coculturing, there was a 3-fold increase in cTnT-expressing cardiomyocytes derived from neonatal-derived CDCs compared with adults [Figure 3C; neonate 2.8% (2.7, 4.8) versus adult 0.8% (0, 1.8) cells per total; P=0.04]. Positive cells were generally found surrounding dense neonatal cardiomyocyte clusters (Figure 3A and 3B). These results indicated the robust cardiomyogenic potential of the neonatal-derived CDCs in vitro that may contribute to their eventual functionality in vivo.

The second experiment involved an observer blinded study to determine the regenerative potential of transplanted CDCs within acutely infarcted myocardium of immunodeficient rats. Soon after the left anterior descending (LAD) artery was ligated, CDCs, human fibroblast cells, or Iscove modified Dulbecco medium (IMDM; control) were injected into the peri-infarct or infarct regions of the left ventricle. The baseline left ventricular ejection fraction calculated by fractional area change (FAC), were similar among all rats receiving CDCs, cardiac fibroblasts, or IMDM (Figure 4). Echocardiography performed at 7 days revealed a higher FAC in hearts receiving neonatal-derived CDCs than in those receiving adult-derived CDCs, cardiac fibroblasts, or controls (neonate, n=4: 56.4±1.4% versus adult, n=4: 39.5±3.6%, P<0.001; cardiac fibroblast, n=4: 32.9±4.6%, P<0.01 and control; n=5: 30.6±3.1%, P<0.01). Differences among adult-derived CDCs, cardiac fibroblasts or control treated hearts did not reach statistical significance, but there was a trend for more functional recovery in the adult-derived CDCs. The improved left ventricular function was maintained from day 7 to 28 and represented the sustained effect of the neonatal-derived CDCs (neonate, n=4: 53.9±5.1% versus

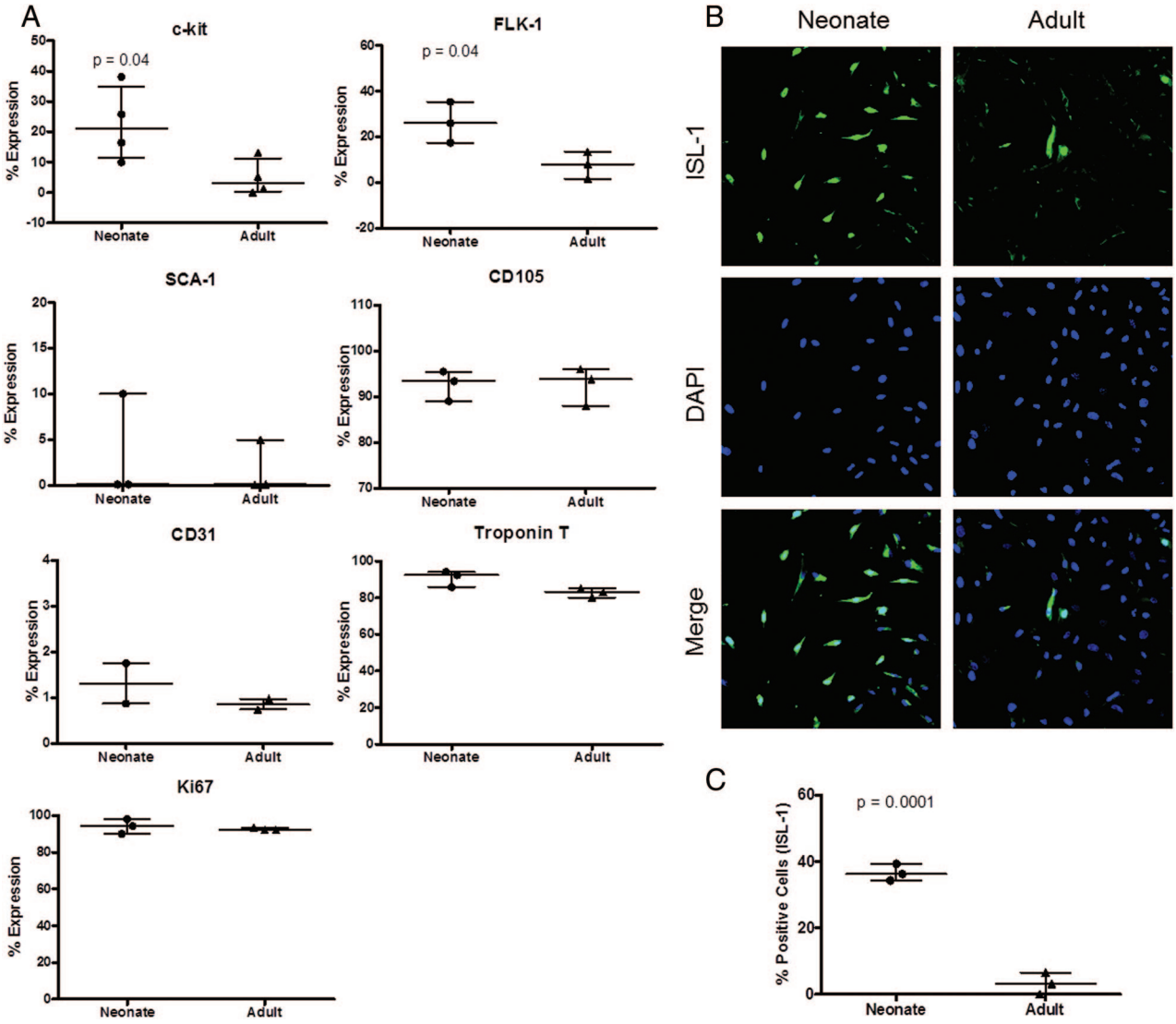

Figure 2. Phenotypic characterization of cardiosphere-derived cells (CDCs) isolated from neonatal and adult cardiac biopsies. **A**, CDCs derived from neonatal cardiac biopsies display significantly upregulation expression of c-kit and flk-1 by flow cytometry compared with adult CDCs [c-kit$^+$, n=4: 21.1% (11.6, 35.04) versus 3.2% (0.3, 11.2); flk-1, n=3: 25.9% (17.4, 35.2) versus 8% (1.7, 13.5), P=0.04 for both markers]. Other phenotypic markers, including SCA-1, CD105, CD31, Ki67, and cTNT, showed no change. **B** and **C**, ISL1 expression was determined by immunofluorescence between neonatal and adult CDCs. Neonatal-derived CDCs showed augmented nuclear localized ISL1 expression compared with adult-derived CDCs [36.2% (34.3, 39.3) versus 3.2% (0, 6.5), P=0.0001, n=3].

adult, n=4: 35.8±3.4%, P<0.05; cardiac fibroblast, n=5: 29.9±4%, P<0.05 and control, n=5: 30±1.9%, P<0.05).

We further examined the impact of CDCs on the infarcted myocardium by examining myocardial sections via histology analysis after 28 days of transplantation. By tracking the CDCs with a human nuclear marker, we observed modest engraftment [Figure 5C; neonate, n=4: 3.0 (1.2, 6.6) versus adult, n=4: 1.6 (0.7, 2.2) CDCs per total per field, P=0.2] of CDCs in the peri-infarct and infarct regions (Figure 5A and 5B). Immunophenotypic characterization revealed that the engrafted neonatal-derived CDCs stained positive for the cTnT and demonstrated structural maturation. Although cardiac differentiation occurred infrequently, these results suggested that part of the regenerative potential of neonatal-derived CDCs may be contributed to the differentiation of these cells to working cardiomyocytes (Figure 5D). We did not find any adult-derived CDCs that differentiated into mature cardiomyocytes staining positive for cTnT.

To further assess the relative roles of direct differentiation, we measured bioluminescence in mice that were injected with luciferase-labeled CDCs. This method was able to actively track the luciferase-labeled CDCs during various time intervals after injection to provide a qualitative measure of transplant cell survival. After 1 week, the luciferase signal had dropped by approximately 30% of that on day 1, and by 3 weeks, little to no detectable signal was determined (Online Data Supplement Figure I). Similar results have been previously reported using this same luciferase methodology but using different patient population derived CDCs.[13,14] Even though this method is nonquantitative and prone to limitations by tissue attenuation and reporter gene silencing, the data suggest that CDCs are most likely noncontributory after 3 to 4 weeks after transplantation.

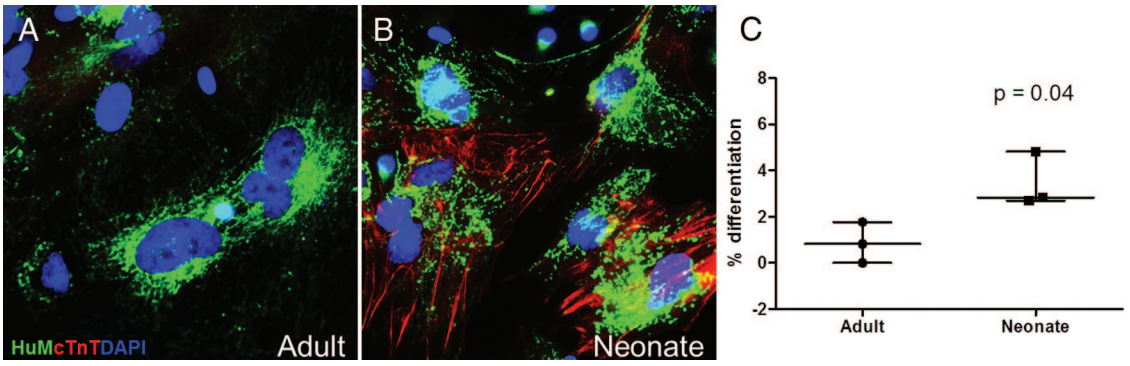

Figure 3. The efficacy of cardiosphere-derived cell (CDC) differentiation into cardiomyocytes in vitro. Adult (**A**) and neonatal-derived (**B**) CDCs were cocultured with rat neonatal cardiomyocytes for 14 days to induce cardiomyogenesis. Sections were stained with a human specific mitochondria (green) and cTNT (red) to depict CDC differentiation into mature cardiomyocytes (nuclei are blue; magnification ×40). **C**, Neonatal CDCs demonstrated an increased capacity for differentiation compared with adult CDCs [neonatal, 2.8% (2.7, 4.8) versus adult, 0.8% (0, 1.8) cells per total, $P=0.04$, n=3], as evidenced by an increased number of structurally competent cardiac cells expressing human mitochondria.

Additionally, we quantified regeneration by staining sections with Masson trichrome to determine the extent of infarct expansion after LAD ligation. A typical Masson trichrome staining pattern in hearts transplanted with control, cardiac fibroblast, or CDCs is shown in Figure 4B. Positive red-stained regions (viable tissue) within the predominately blue-stained (fibrous tissue) infarct zone are evident in all hearts transplanted. A trend for reduced infarct expansion was noticed with the neonatal-derived CDCs in comparison to the adult-derived CDCs and cardiac fibroblasts. Additionally, neonatal-derived CDCs transplanted hearts had a smaller infarct size within the infarct zone than hearts treated with control media [control, n=4: 45.2% (41.1, 47.3), cardiac fibroblasts, n=3: 40% (35.6, 47.1), neonatal, n=4: 25.2% (13.5, 32.3), adult, n=4: 33.6% (30.4, 41.5), $P<0.01$ control versus neonate]. Taken together, these data indicated that neonatal-derived CDCs resulted in enhanced functional recovery and more favorable remodeling in the infarcted myocardium than adult-derived CDCs.

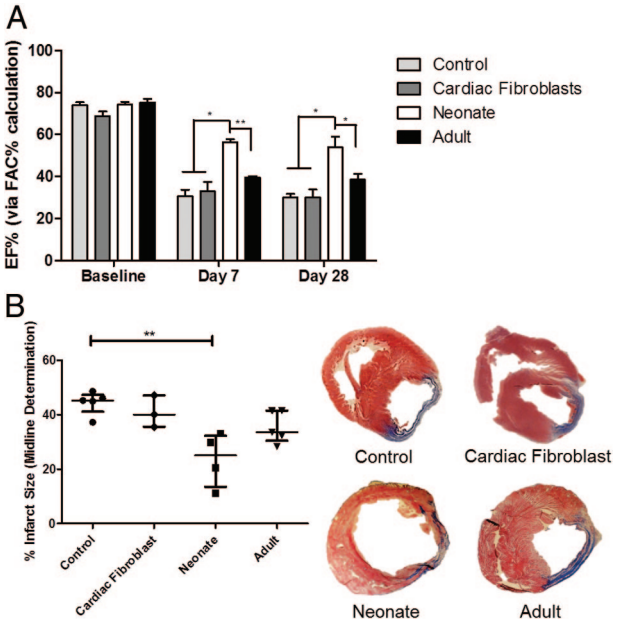

Figure 4. Neonatal cardiosphere-derived cells (CDCs) preserved cardiac function after myocardial infarction in immunodeficient rats that were treated with either control, cardiac fibroblast, neonatal CDCs, or adult CDCs. Echocardiographic analysis was performed before and after surgery at day 7 and day 28. **A**, Left ventricle blood pool fractional area change in diastole (FAC, ejection fraction) was significantly improved in infarcted hearts treated with neonatal CDCs compared with control, cardiac fibroblast, and adult CDC groups at both day 7 and day 28. Baseline echocardiograms showed no presurgical variation among the 4 groups (day 7: neonate, n=4: 56.4±1.4% versus adult, n=4: 39.5±3.6%; cardiac fibroblast, n=4: 32.9±4.6% and control, n=5: 30.6±3.1%; $^{*}P<0.001$, $^{**}P<0.01$; day 28: neonate, n=4: 53.9±5.1% versus adult, n=4: 35.8±3.4%; cardiac fibroblast, n=5: 29.9±4% and control, n=5: 30±1.9%; $^{*}P<0.05$). **B**, Infarct size was improved after treatment with neonate CDCs compared with control 4 weeks after infarction [control, n=4: 45.2% (41.1, 47.3); cardiac fibroblasts, n=3: 40% (35.6, 47.1); neonatal, n=4: 25.2% (13.5, 32.3); adult, n=4: 33.6% (30.4, 41.5); $^{**}P<0.01$, control versus neonate]. There was no significant prevention of infarct expansion using adult CDCs as a therapeutic platform.

Differential Secretion of Angiogenic Factors May Account for Improved Regenerative Potential

To determine if neonatal-derived CDCs secreted different angiogenic factors in comparison to adult-derived CDCs and cardiac fibroblasts, cells were cultured under hypoxic conditions to simulate ischemia and RT-PCR was performed to assess the secretion profiles. ANG [cardiac fibroblasts: 0.04 (0.03, 0.05), neonatal: 1.04 (0.8, 1.16), adult: 0.36 (0.17, 0.48), $^{*}P<0.04$ and $^{**}P<0.01$) and VEGF (cardiac fibroblasts: 0.1 (0.1, 0.12), neonatal: 0.81 (0.61, 1.22), adult: 0.29 (0.19, 0.39), $^{*}P<0.05$ and $^{**}P<0.01$] showed statistical differences that suggest augmented secretion of proangiogenic factors in neonatal-derived CDCs (Figure 6A and 6B, n≥3 for all). IGF-1 and FGF-1 showed no change (Figure 6C through 6F). FGF-2 in neonatal-derived CDCs was only enhanced compared with cardiac fibroblasts, but differences did not reach statistical significance compared with adult-derived CDCs [cardiac fibroblasts: 0.13 (0.11, 0.17), neonatal: 0.92 (0.34, 2.74), adult: 0.31 (0.24, 0.86), $P<0.05$]. To confirm these results in vivo, immunofluorescence for blood vessel preservation and/or formation in infarcted hearts treated with neonatal-derived CDCs or adult-derived CDCs was performed using isolectin B4. Hearts treated with neonatal-derived CDCs displayed higher levels of blood

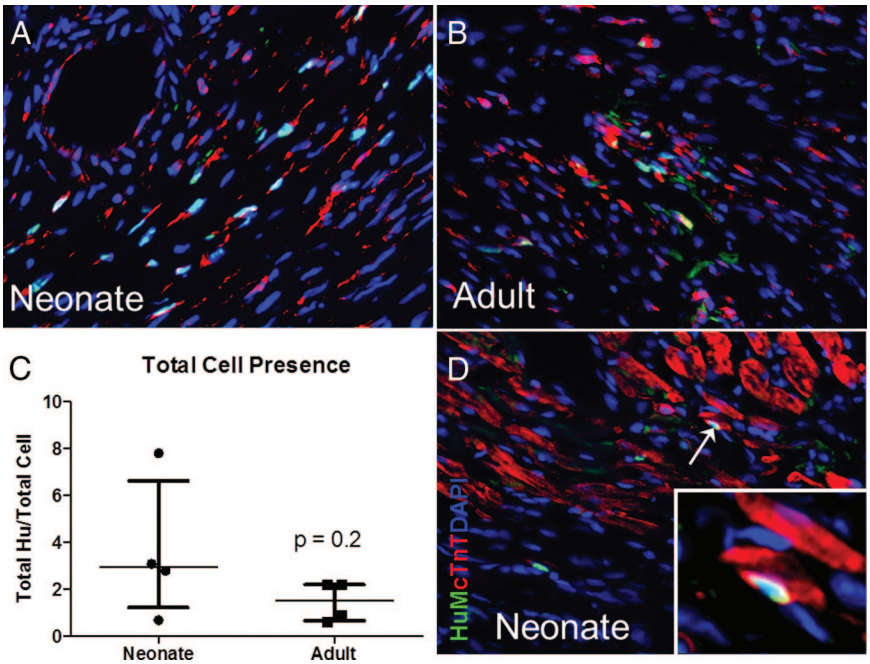

Figure 5. The efficacy of cardiosphere-derived cell (CDC) differentiation to cardiomyocytes in vivo. **A**, Neonate, or **B**, adult CDC engraftment at 4 weeks after transplantation in an immunodeficient rat model of myocardial infarction. CDCs were tracked using human antinuclei (green) and costained with cTnT (red) to detect differentiation into cardiomyocytes. **C**, Generally there was a trend for increased engraftment/survival of neonatal CDCs compared with adult CDCs, however. **D**, Only CDCs derived from neonates demonstrated a tendency to differentiate into a typical cardiomyocytes phenotype as demonstrated by broad cTNT expression and formation of striations. We did not find any adult CDCs with this phenotype (magnification ×20).

vessel preservation and/or formation [neonatal: 8.5 (6.5, 10.5) versus adult: 4.5 (4, 5.8), $P=0.04$, n=4; Figure 6G]. Blood vessels were generally found in the peri-infarct wall, though fewer blood vessels were also found spanning the infarct within the lateral wall (Figure 6H and 6I).

Discussion

Our results suggest that neonatal-derived CDCs can be reproducibly isolated and expanded from as little as 20 mg of myocardial tissue, which can be harvested during routine pediatric cardiac surgery. Neonatal-derived CDCs has a higher prevalence of different cardiac stem cells in comparison to adult-derived CDCs. Additionally, the transplanted neonatal-derived CDCs can significantly improve cardiac function in comparison to adult-derived CDCs when tested in immunodeficient in a rodent model of myocardial infarction. The cardiac regeneration observed with neonatal-derived CDCs may be due to differentiation into functional cardiomyocytes or may be due to increased blood vessel preservation and/or formation resulting from secreted angiogenic factors. These novel findings of human CDCs support the strong regenerative ability of neonatal-derived CDCs, a potentially new therapy for congenital heart failure patients. Equally important is the observation that all CDCs may not be equivalent in their functional ability and may need to be optimized, depending on patient age.

The heterogeneity of CDCs may be particularly favorable for cardiovascular applications because they contain uncommitted stem cells normally residing within the heart.[5] In our study, neonatal-derived CDCs displayed an increased prevalence of c-kit$^+$, flk-1, and ISL1 stem cells when compared with adult-derived CDCs. C-kit$^+$- and ISL1-expressing stem cells have been extensively characterized in the heart, but it is still unclear whether these cells represent distinct categories of undifferentiated cells with unique functional activities.[3,15] The increased prevalence of cardiac stem cells observed in our study may be explained by our previous work that demonstrated higher number of stem cells in neonates.[7] These types of stem cells have been shown to replenish the population of cardiomyocytes and cardiac vascular cells that die during a heart's lifetime.[16] Furthermore, neonatal-derived CDCs have the unique ability to secrete variable growth factors such as VEGF and angiogenin. Both of these factors are highly proangiogenic and may be involved in restricting adverse remodeling and cardiomyopathic progression via blood vessel preservation and/or formation. The more proangiogenic secretion profile of neonatal-derived CDCs compared with adult-derived CDCs may be due to differences in CDC phenotypes and stem cell numbers. Understanding the molecular basis of this ability may have important implications on how to further improve the activity of adult-derived CDCs. Overall, these neonatal-derived CDCs are highly regenerative.

Implications for Pediatric Heart Failure

To date, there has been no direct comparison between the regenerative abilities of neonatal and adult derived-human stem cells. Recently, rodent cardiac c-kit$^+$ cells derived from neonates have shown an increased cardiomyogenic potential over that of adult-derived c-kit$^+$ cells, but the underlying molecular basis at this time is unknown.[17] Additionally, 2 relevant clinical trials offer insight into cell-based therapy in human adult ischemic patients. The SCIPIO trial demonstrated that the delivery of a pure population of c-kit$^+$, lineage-negative cardiac stem cells to adult patients with myocardial infarction augment ejection fraction (12.3%) and decreased infarct size (30%) over 12 months. The CADUCEUS trial reported their phase I clinical results in ischemic adult patients, using CDCs, and demonstrated decreased scar formation by 30% to 70% and increased left ventricular mass. Unfortunately, this finding did not result in any statistically significant change in ejection fraction with the treatment of

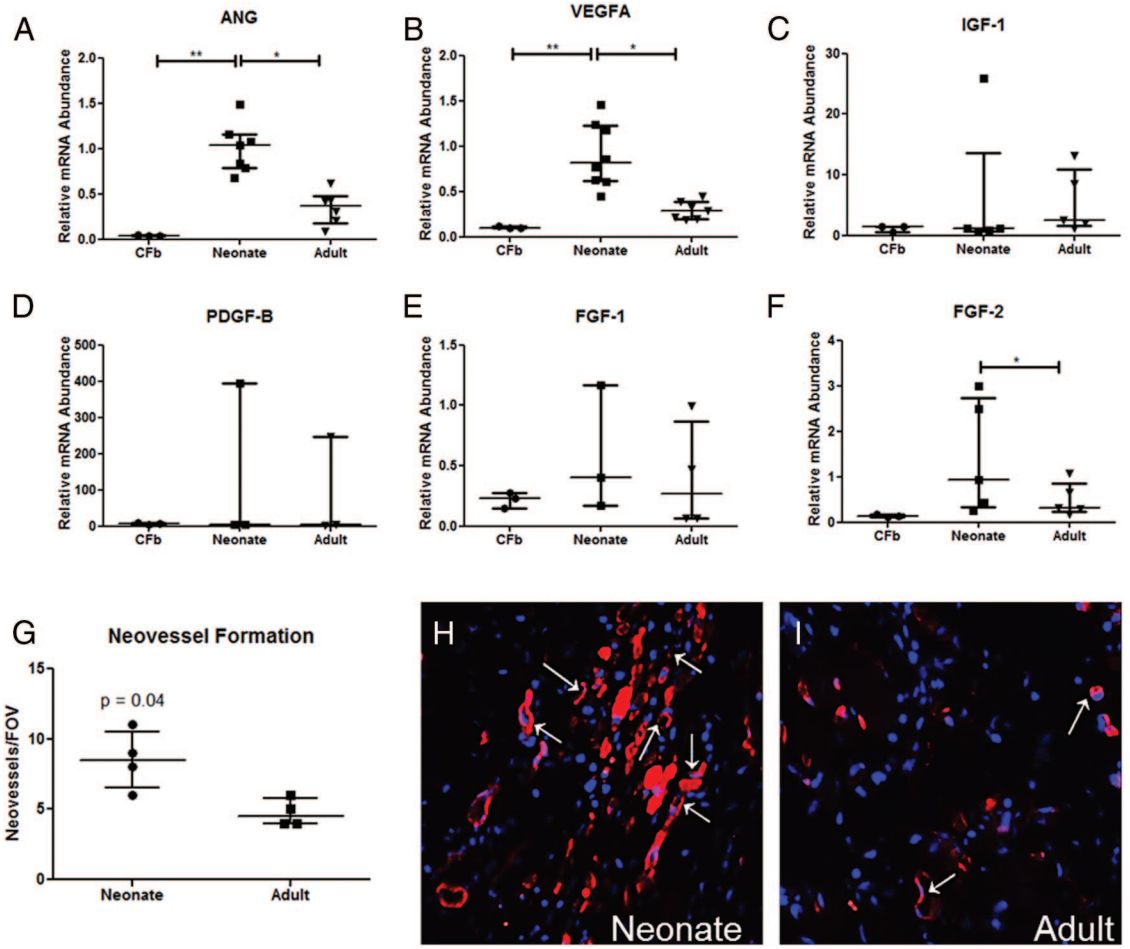

Figure 6. Enhanced angiogenic potential of cardiosphere-derived cells (CDCs) derived from neonates. To determine whether CDCs derived for neonatal patients demonstrate an enhanced angiogenic secretion profile, expression levels of several trophic factors were analyzed using RT-PCR. CDCs derived from neonatal cardiac biopsies display upregulated expression of angiogenin (ANG) (**A**) and vascular endothelial growth factor (VEGF) (**B**) by RT-PCR compared with adult CDCs and cardiac fibroblasts (CFb) [ANG-CFb: 0.04 (0.03, 0.05); neonatal: 1.04 (0.8, 1.16); adult: 0.36 (0.17, 0.48); $^{*}P<0.04$ and $^{**}P<0.01$; and VEGF-CFb: 0.1 (0.1, 0.12); neonatal: 0.81 (0.61, 1.22); adult: 0.29 (0.19, 0.39); $^{*}P<0.05$ and $^{**}P<0.01$]. mRNA expression levels for other factors, including insulin-like growth factor 1 (IGF-1) (**C**), platelet-derived growth factor-B (PDGF-B) (**D**), and fibroblast growth factor (FGF)-1 (**E**) show no change. **F**, FGF-2 in neonatal CDCs was only enhanced compared with cardiac fibroblasts, but differences between neonate and adult CDCs did not reach statistical significance [CFb: 0.13 (0.11, 0.17); neonatal: 0.92 (0.34, 2.74); adult: 0.31 (0.24, 0.86); $^{*}P<0.05$]. **G**, To determine if the augmentation of proangiogenic factors translated to increase blood vessel preservation and/or formation, immuoflouresence was performed using Isolectin B4 (red; magnification ×20). Infarcted hearts treated with neonatal CDCs (**H**) displayed a significantly higher level of blood vessel preservation and/or formation compared with adult CDCs (**I**) [neonatal: 8.5 (6.5, 10.5) versus adult: 4.5 (4, 5.8); $P=0.04$, n=4].

adult-derived CDCs. The results of these 2 trials suggest that c-kit^{+} cells may play an important role in the augmentation of cardiac function, which may explain the differences that we noticed with neonatal-derived CDCs in which there was a higher prevalence of cardiac stem cells, with c-kit^{+} cells being one of them. Our results also suggest that all derived CDCs are not equivalent in their functional ability to improve left ventricular ejection fraction. One explanation is that neonatal-derived CDCs release more VEGF and angiogenin, which triggers more blood vessel formation and/or preservation when compared with adult-derived CDCs.[18,19] Last, neonatal-derived CDCs may stimulate regeneration at multiple levels, which include preventing apoptosis,[18] secreting trophic factors,[6] and stimulating the endogenous cardiac stem cell pool.[20,21] Despite the lack of more direct cardiomyocyte differentiation, CDCs may improve regeneration at multiple levels that need further exploration.

The goal of cellular therapy for pediatric heart failure patients is to regenerate lost or dysfunctional myocardium. In contrast to adults, pediatric heart failure etiologies include a variety of conditions that encompass ischemia, cardiomyopathies, and congenital lesions.[9] Since neonates are the most medically and surgically challenging and carry the highest surgical mortality rates, we have focused on generating clinical protocols that have focused mainly on HLHS patients. The physiology of HLHS is dominated by the absence of an anatomic left ventricle such that the morphological right ventricle provides the entire cardiac output, which ultimately may lead to right ventricular dysfunction.[11] The etiology of right ventricular dysfunction is multifactorial, including pressure and volume overload, intrinsic right ventricle muscle dysfunction, and myocardial ischemia. The unique ability of neonatal-derived CDCs to function at multiple

levels, including generating myocardium and releasing angiogenic factors, may make these cells ideal to treat HLHS patients. Additionally, CDCs can be frozen for future use allowing repeat injections when cardiac dysfunction may arise later in time.

Conclusion

The strong regenerative ability of neonatal-derived CDCs warrants further investigation as a treatment for pediatric heart failure patients. Given the ease and expandability of these cells from right atrial tissue, the use of neonatal-derived CDCs may provide an attractive cellular-based therapy, either as a stand-alone intervention or as an adjunct to surgical repair for congenital heart patients.

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Disclosures

None.

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A Strong Regenerative Ability of Cardiac Stem Cells Derived From Neonatal Hearts

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SUPPLEMENTARY MATERIAL

Expanded Materials and Methods

Acquisition of Human Tissue Samples and Cell Culture

This study was approved by the Institutional Review Committee at Children's Memorial Hospital. After parental consent was given, specimens (70±80 mg; mean, 40 mg) from the right atrial (RA) appendage were obtained from neonatal patients (n=43) and adult patients (n=11) during routine congenital cardiac surgeries all tissue samples were processed for immunostaining and harvesting human cardiac progenitor cells (hCPCs) via cardiosphere development.

Human cardiosphere derived cells (CDCs) were generated through the use of the protocol described by Smith et al with modifications. Briefly, RA tissue was minced and mildly digested with 0.05% trypsin/EDTA and collagenase type II (Worthington Biomedical Corp, Lakewood, NJ). Explants were plated on fibronectin in Iscove modified Dulbecco medium (IMDM) with 20% FBS. At 2 to 3 weeks after plating, phase-bright cells were removed and plated at low density (1.5 to 3×104 cells per 1 mL) in cardiosphere-growing medium (CGM). Cardiospheres were removed and plated on fibronectin in human cardiac stem cell expansion medium (HCSCEM; Celprogen, San Pedro, CA). Phase-bright cells were harvested from individual explant cultures every 3 to 5 days up to 4 consecutive times. hCDCs were then expanded on fibronectin-coated plates.

Flow Cytometry Analysis of CDCs

CDCs at P4 were evaluated by flow cytometry using a Becton-Dickinson FACS caliber (San jose, CA) with 10,000 events collected. Cells were incubated with fluorochrome-conjugated primary antibodies against c-kit, FLK1, nkx2.5, ki67, Sca1, Troponin T, CD105 and antibodies

against hematopoietic lineage surface marker CD34 and Cd45. Isotype controls were run for each immuno-subtype.

Animal Handling

Male athymic nude rats obtained from Taconic (New York, NY) were allowed to acclimate to housing conditions for at least one week before use. Rats used for experiments were between 200-350g. All animals received care in compliance with federal and institutional guidelines with approval from the Institutional Animal Care and Use Committee.

Infarct Model and Patch Application

Myocardial infarction (MI) was induced by permanent ligation of the left anterior descending (LAD) coronary artery in athymic nude male rats (250-300 g). Rats were anesthetized with 5% isoflurane in pure oxygen. Afterwards, rats were weighed and intubated for mechanical ventilation. After endotracheal intubation and initiation of ventilation, isoflurane was reduced to the amount required to prevent the pedal reflex (1.5-2%). The heart was exposed via a left thoracotomy, and the proximal LAD was ligated using 6-0 silk suture. Ten min after ligation, 1 million neonate or adult-derived CDCs or cardiac fibroblasts suspended in 250-400 μL IMDM were injected at multiple sites. Rats with induced infarction without cell injection rats injected with cardiac fibroblasts served as controls for the study. Buprenorphine (0.1 mg/kg) was injected subcutaneously after surgery (and as necessary), and rats were allowed to recover under close supervision.

Echocardiography

Transthoracic echocardiograms were performed on rats using a VisualSonics Vevo 770 ultrasound unit (VisualSonics, Toronto, Canada). The VisualSonics RMV 716 Scanhead with center frequency 17.5 MHz, frequency band 11.5–23.5 MHz, and focal length 17.5 mm was used for echo acquisition in rats. Baseline echocardiograms were acquired at 7 days post-MI with additional echocardiograms acquired at 4 weeks post-MI. The baseline post-MI echocardiograms allowed determination of the extent and location of infarction. Animals with a baseline ejection fraction above 50% were excluded from the study. The animals were maintained lightly anesthetized during the procedure with 1.5% isoflurane delivered through a face mask. The animals were kept warm on a heating pad, and the body temperature was continuously monitored using a rectal thermometer, maintaining it at between 35 and 37°C by adjusting the distance of a heating lamp. Under these conditions, the animal's heart rate could be maintained between 300-400 beats per minute. Two-dimensional and M-mode echocardiography were used to assess diastolic function and fractional area change. Images were obtained from the parasternal long axis, the parasternal short axis at the mid-papillary level and four chamber view.

Myocardial Histology

After the hemodynamic studies, hearts were excised under anesthesia, perfused with 4% paraformaldehyde and then cryo-protected by immersion in 30% sucrose for 48-96 hours. Isopentane cooled in liquid nitrogen was used to freeze hearts immersed in optimal cutting temperature (OCT) medium. Sections were cut to 7 μm using a commercial cyrostat and used for isolectin B4, cardiac troponin T and anti-human nuclei staining. Briefly, frozen heart sections were air dried, and OCT was removed by rinsing slides in PBS. Isolectin B4 (Invitrogen; Carlsbad, CA) diluted 1:250 in Tris buffered saline with 0.1% Tween-20 was added to sections

for 30 min at 37^{o}C. Sections were washed in PBS and counterstained with DAPI for 5 min. Sections were rinsed in dH_2O and mounted with an antifade aqueous mounting medium (Vector Labs; Burlingame, CA). Vessel density was calculated as the number of vessels (Since Isolectin B4 can cross-react with macrophages, we only counted structures with a clear lumen and not associated with intact myocardium) per field of view (FOV). At least six FOVs were taken for each frozen section along the anterior, lateral and posterior portions of the LV. Additional markers were used to determine engraftment of cardiac differentiation. Tissue sections, cells were stained for human nuclei (Millipore; Billerica, MA) and cardiac troponin T (cTnT, Santa Cruz Biotechnology; Santa Cruz, CA; diluted in 0.3% BSA in PBS) followed by staining with appropriate fluorescent conjugated secondary antibodies (diluted in 5% donkey serum) and a nuclear DAPI (4',6-diamidino-2-phenylindole) stain (Sigma; St. Louis, MO). Cells were mounted using an antifade mounting medium and viewed under a flourescence microscope (Nikon; Melville, NY). To calculate infarct size, at least four Masson's Trichrome stained sections at various levels along the long axis were analyzed for collagen deposition. The midline technique for infarct size determination was used as described previously (Takagawa *et al.*, 2007). Briefly, the LV midline was drawn at the center of the anterior (lateral) wall along the length of the infarct. This circumference was divided by the total midline circumference of the heart to determine infarct size.

Real Time RT-PCR

RNA was isolated from cardiac fibroblast and CDCs using a commercial RNeasy kit (Qiagen; Valencia, CA). RNA concentration and purity were measured using a spectrophotometer. Afterwards, 500ng of RNA was converted into cDNA using an Applied

Biosystems cDNA synthesis kit (Applied Biosystems, Foster City, CA). The reaction mixture was incubated for 5 min at 25oC, 30 min at 42oC and lastly, 5 min at 85oC. Real Time PCR was run using a total of 5 ng template cDNA for each sample. For each run, a negative control (water only, no template) was analyzed simultaneously, and each sample was run in duplicate using ABI FAST SYBR green supermix (Applied Biosystems; Foster City, CA) for multiple genes including: ANG, VEGF, FGF-1, FGF-1, PDGF-B, IGF-1 and ribosomal protein 13A (RPL13A). Primer assays for each primer set were obtained from Qiagen. The fast PCR protocol consisted of an initial denaturing step at 95oC for 20 s. Next, samples were run at 94oC (denaturation) for 3 s, 60oC (annealing) for 30 s for 40 cycles. Relative RNA abundance was calculated using the following equation: $2^{-\Delta\Delta cT}$, where the first delta represents threshold subtraction ("delta 1") from the endogenous control and the second delta represents the division of "delta 1" by an internal control.

Assessment of CDC Differentiation Potential

A co-culture system using neonatal cardiomyocytes and either neonate or adult CDCs was used to determine cardiomyogenic potential. Briefly, day 1-2 rat neonates were sacrifices and hearts obtained for cardiomyocyte isolation. Hearts were digested over 48 hours using a commercial kit (Worthington Biomedical Corp, Lakewood, NJ) and cells were seeded at a density of 50,000 cells per well in an eight well chamber slide. After 24 hours, 5,000 neonate or adult CDCs were added and the co-culture was allowed to incubate at 37oC in 5% CO2 for 14 days. Differentiation of CDCs toward cardiac lineages was assessed using fluorescence microscopy. Cells were fixed using 4% paraformaldehyde (PFA) for 15 min and permeabilized using 0.1% Triton X-100. Cells were blocked with 5% donkey serum. Next, cells were stained

for cTnT (diluted in 0.3% BSA in PBS) followed by staining with appropriate fluorescent conjugated secondary antibodies (diluted in 5% goat serum) and a nuclear DAPI (4',6-diamidino-2-phenylindole) stain (Sigma; St. Louis, MO). Cells were mounted using an antifade mounting medium and viewed under a fluorescence microscope (Nikon; Melville, NY).

Supplementary Figure Legend

Figure S1. Loss of CDC engraftment after 4 weeks. One million CDCs labeled with Luciferase were injected into a rodent model of MI. Cells were tracked over 28 days using a Xenogen optical imaging system. Cell presence and density decreased throughout the duration of the study. No signal was detected after 28 days

Supplementary Table 1: Patient Diagnosis and Number for Cardiosphere Generation

Neonatal Diagnosis	Number of the Patients
Transposition of the Great Arteries	10
Truncus Arteriosus	7
Coarctation of the Aorta	7
Hypoplastic Left Heart Syndrome	5
Total Anomalous Venous Connection	5
Tetralogy of Fallot	3
Pulmonary Atresia	4
Interrupted aortic arch	1

Supplementary Table 2: Summary of Phenotypic Characterization of CDCs

Marker	Neonate	Adult
c-kit	++	+
Flk-1	++	+
ISL-1	++	+
Sca-1	+	+
Nkx2.5	++++	++++
Ki67	++++	++++
CD31	-	-
CD34	-	-
CD105	++++	++++

Supplementary Figure S1