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PRE-CLINICAL RESEARCH

Intramyocardial Injection of Autologous Cardiospheres or Cardiosphere-Derived Cells Preserves Function and Minimizes Adverse Ventricular Remodeling in Pigs With Heart Failure Post-Myocardial Infarction

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Objectives The purpose of this study was to test the safety and efficacy of direct injection of cardiosphere-derived cells (CDCs) and their 3-dimensional precursors, cardiospheres, for cellular cardiomyoplasty in a mini-pig model of heart failure after myocardial infarction.

Background Intracoronary administration of CDCs has been demonstrated to reduce infarct size and improve hemodynamic indexes in the mini-pig model, but intramyocardial injection of CDCs or cardiospheres has not been assessed in large animals.

Methods Autologous cardiospheres or CDCs grown from endomyocardial biopsies were injected through thoracotomy 4 weeks after anteroseptal myocardial infarction. Engraftment optimization with luciferase-labeled CDCs guided the choice of cell dose (0.5 million cells/site) and target tissue (20 peri-infarct sites). Pigs were randomly allocated to placebo (n = 11), cardiospheres (n = 8), or CDCs (n = 10). Functional data were acquired before injection and again 8 weeks later, after which organs were harvested for histopathology.

Results Beyond the immediate perioperative period, all animals survived to protocol completion. Ejection fraction was equivalent at baseline, but at 8 weeks was higher than placebo in both of the cell-treated groups (placebo vs. CDC, p = 0.01; placebo vs. cardiospheres, p = 0.01). Echocardiographic and hemodynamic indexes of efficacy improved disproportionately with cardiospheres; likewise, adverse remodeling was more attenuated with cardiospheres than with CDCs. Provocative electrophysiologic testing showed no differences among groups, and no tumors were found.

Conclusions Dosage-optimized direct injection of cardiospheres or CDCs is safe and effective in preserving ventricular function in porcine ischemic cardiomyopathy. Although CDCs and cardiospheres have equivalent effects on left ventricular ejection fraction, cardiospheres are superior in improving hemodynamics and regional function, and in attenuating ventricular remodeling. (J Am Coll Cardiol 2011;57:455–65) © 2011 by the American College of Cardiology Foundation

Cardiac failure secondary to myocardial infarction (MI) is a major public health problem. Left ventricular (LV) dysfunction after MI is an important predictor of subsequent outcome (1), and although therapy has improved markedly in the past 30 years, the mortality and morbidity of ischemic cardiomyopathy remain unacceptably high (2). Consequently, cell therapy is under active investigation as a potential therapeutic modality after MI. An effective cell therapy would offer patients a regenerative treatment option in addition to the currently available pharmacological and device options. Autologous sources of cells are of particular interest because immunologic rejection is avoided. Intracoronary infusion of autologous bone marrow–derived mononuclear cells has been evaluated in phase II clinical trials (3,4), but routine clinical use has not gained regulatory approval.

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Abbreviations and Acronyms

ANOVA = analysis of variance

CDC = cardiosphere-derived cell

E_{max} = end-systolic elastance

IV = intravenous

LV = left ventricular

LVEF = left ventricular ejection fraction

MI = myocardial infarction

Cardiosphere-derived cells (CDCs) are a heterogeneous mix of cells expanded from cardiac tissue, with formation of self-assembling spherical clusters of heart-derived cells (i.e., cardiospheres) as an intermediate processing step (5,6). CDCs are clonogenic and exhibit multilineage potential (7), thus fulfilling key criteria for cardiac stem cells, and they can be readily and reliably expanded from tiny specimens of heart muscle: Smith et al. (6) found that ~20 mg human heart samples yielded ~1.5 million CDCs on average within 45 days. Because endogenous regeneration of cardiomyocytes at a rate of about 1% to 2% per year has been demonstrated in humans (8), and because CDCs are pre-programmed to differentiate into cardiac lineages, they represent a logical cell candidate for therapeutic testing.

Direct injection of CDCs in small animal models has been shown to improve cardiac function in the setting of experimental MI (6,9,10), but data from large-animal models are lacking. Coronary infusion of CDCs in pigs reduces relative infarct size and improves hemodynamic indexes, but LV ejection fraction (LVEF) is not affected (11). Cardiospheres (50 to 200 μm in diameter) would be expected to embolize at the arteriolar level, and thus are not well suited for intracoronary administration. Nevertheless, direct head-to-head comparison in a small-animal model has revealed superior benefits of cardiospheres relative to CDCs when both are delivered by intramyocardial injection (12). Thus, 2 questions remain to be addressed in a clinically relevant large animal model: 1) Is direct injection superior to intracoronary delivery? 2) Are cardiospheres more effective than CDCs?

Here, we tested the safety and efficacy of intramyocardial injection of cardiospheres and of CDCs in a mini-pig model of ischemic cardiomyopathy. We first sought to quantify and optimize the engraftment of CDCs when injected directly into myocardium, and then to assess the safety and efficacy of the optimized dose of CDCs (and, by comparison, cardiospheres) in improving cardiac function.

Methods

Study design. The current studies were performed in 2 parts. Study 1 (Fig. 1A) consisted of open-label experiments to quantify engraftment 24 h after intramyocardial CDC injection in the porcine MI model. The engraftment data were used to inform the dosage and target tissue of injection of CDCs for study 2, which was a pivotal placebo-controlled, blinded randomized study of safety and efficacy of either cardiosphere or CDC direct intramyocardial injection (Fig. 1B). Animals were randomly allocated to receive either placebo or 10 million cells in cardiosphere or CDC form, administered as 20 injections of 0.5 million per site. The dose of 10 million cells was chosen on the basis of the engraftment data from study 1, which had demonstrated the highest percentage engraftment of cells occurred when a smaller number was injected at each site in the peri-infarct border zone. Cells or placebo were injected under direct visualization by open-chest surgery performed 4 weeks after MI. The pigs were then followed for 8 more weeks, to assess safety and efficacy.

Creation of MI and acquisition of ventricular biopsy specimens. General anesthesia was induced in adult female Yucatan mini-pigs by intramuscular ketamine 20 mg/kg, acepromazine 0.25 mg/kg, and atropine 0.05 mg/kg, followed by 10 ml intravenous (IV) thiopental. Endotracheal intubation was then performed, and anesthesia maintained by ventilation with 1% to 2% isoflurane. Under sterile

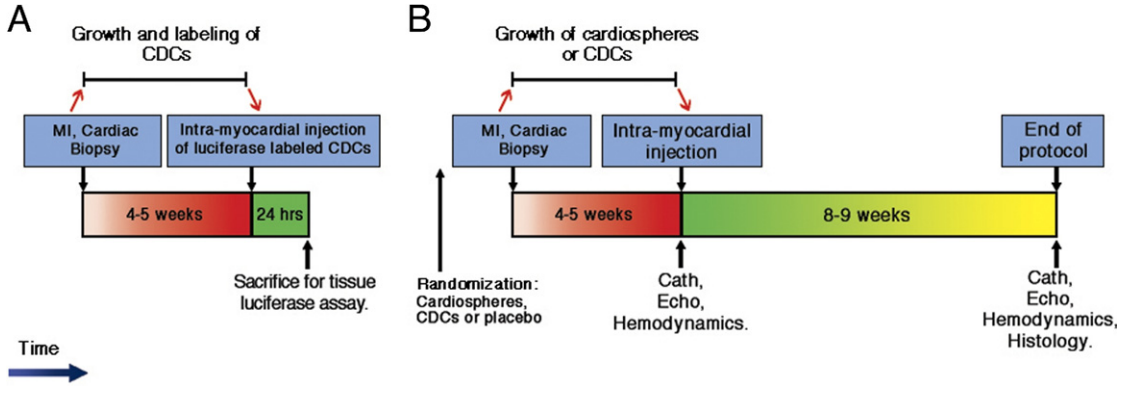

Figure 1 Study Protocols

(A) 24-h engraftment study design (n = 7). **(B)** Pivotal randomized, blinded, placebo-controlled study design (placebo n = 11, cardiospheres n = 8, cardiosphere-derived cells [CDCs] n = 10). Cath = catheterization; echo = echocardiography; MI = myocardial infarction.

conditions, the mini-pigs were subjected to an anteroseptal MI by inflation of an angioplasty balloon in the mid-left anterior descending artery to cause coronary occlusion for 2.5 h. Catheters were inserted through the left carotid artery. Ventricular tachycardia and/or ventricular fibrillation requiring external cardioversion were very common, despite administration of 50 mg IV amiodarone, and additional 50 mg boluses of IV amiodarone to a maximum of 200 mg if frequent ventricular premature beats were seen. There was an overall mortality of 34% associated with creation of the MI in these studies.

After reperfusion, during the same episode of general anesthesia, 4 to 6 right ventricular biopsies were obtained using a standard clinical cardiac bioptome introduced through the right internal jugular vein. The biopsies were immediately placed into ice-cold cardioplegia solution (Ca^{++} and Mg^{++}-free PBS with 5% dextrose, mannitol 68.6 mmol/l, KCl 1.6 mmol/l, $NaHCO_3$ 3.1 mmol/l, and heparin), and cardiospheres or CDCs were grown from these biopsy samples.

Growth of cardiospheres and CDCs. The CDCs were cultured according to published protocols (5,6). Briefly, cardiac biopsy specimens (10 to 40 mg) were minced, and subjected to collagenase IV digestion. These explants were plated onto fibronectin-coated plastic plates with cardiac explant medium (Iscove's Modified Dulbecco's Medium [Invitrogen, Carlsbad, California], 20% FBS, 1% penicillin-streptomycin, 1% L-glutamine, 0.1 mM 2-mercaptoethanol). A monolayer of adherent cells grew out from the biopsy, which was harvested after 1 to 2 weeks. The harvested outgrowth was replated onto poly-D-lysine–coated wells. Under these conditions, within 3 to 5 days, the majority of the cells gave rise to free-floating clusters of cells, termed cardiospheres. In a third phase, the adherent cells were discarded, and the floating cardiospheres were collected and plated once again onto fibronectin-coated cellware. The cardiospheres adhered and flattened to form a monolayer of cells referred to as CDCs, which were passaged as they became confluent.

So-called "secondary cardiospheres" were used in the in vivo experiments, meaning that an equivalent number of CDCs (10 million) were harvested and counted, then plated back into poly-D-lysine–coated wells where they formed cardiospheres for a second time, which were injected into the animals. The exact number of cells may have actually been higher than 10 million (due to further proliferation after counting), or lower than 10 million (due to adherence of some cells to the plastic), but this estimation was used to compare "equivalent" numbers of cells in the cardiosphere and CDC groups.

Labeling and detection of injected CDCs for engraftment studies. Cultured cells were transduced at the outgrowth stage with a lentiviral vector encoding the firefly luciferase gene, and further processed to create CDCs. Seven animals that had undergone the MI and right ventricle biopsy protocols received intramyocardial injection of 0.5 million, 2.0 million, or 10 million CDCs per injection site in intrainfarct, peri-infarct (borderzone), or remote normal ventricular locations. The animals were sacrificed 24 h later for assessment of cell engraftment, as described (11).

A standard curve was constructed by measuring the luciferase signal from known numbers of transduced CDCs spiked into homogenized cardiac tissue. A standard curve specific for each batch of cells needed to be constructed because of variation of transduction efficiency with each batch. The injected cardiac tissue was then homogenized and analyzed for luciferase signal, which was converted to cell number by reference to the standard curve. Off-target expression of luciferase in lungs, liver, spleen, and kidneys was quantified in a similar manner. This is a validated method with high sensitivity and reproducibility (11,13,14).

Pivotal study. INTRAMYOCARDIAL INJECTION OF EXPERIMENTAL TREATMENT BY OPEN CHEST SURGERY. Thirty-three pigs had general anesthesia induced a second time, 4 weeks after MI, with the same drugs. The LVEF was measured by contrast ventriculography, with the image intensifier in the 60° right anterior oblique position. If the LVEF was ≥55%, then the pig was deemed to have had too small of an MI and was excluded from the study. That applied to 1 animal. Intramyocardial direct injection was performed by open-chest surgery under sterile conditions. Sternotomy was performed and the pericardium opened to expose the heart. Twenty intramyocardial injections of either cardiospheres (0.5 million cells suspended in 0.1 ml per injection), CDCs (0.5 million cells suspended in 0.1 ml per injection), or placebo (0.1 ml of phosphate buffered saline alone) were performed into the beating heart, using a 1-ml tuberculin syringe and 26-G needle. The injections were spaced around the perimeter of the macroscopically visible infarct scar, approximately 1 cm from the gross border. With the exception of 3 procedure-related perioperative complications (1 episode of catheter-related ventricular tachycardia, 1 punctured lung, 1 cardiogenic shock), all animals survived after surgery and completed the protocol; thus, post-procedure mortality was nil. This resulted in 29 pigs in the final analysis: n = 11 allocated to receive placebo (vehicle alone), n = 8 allocated to receive cardiosphere injections, and n = 10 allocated to receive CDC injections.

Assessments of LV structure and function. Contrast left ventriculography was performed using a pigtail catheter, with injection of 30 ml of iodinated contrast (Omnipaque, GE Healthcare, Princeton, New Jersey) at 15 ml/s. We found that transthoracic echocardiography was inadequate to image the cardiac apex; instead, epicardial echocardiography (Aspen, Acuson Corp., Mountain View, California) was performed while the chest was open, by placement of a 5-MHz ultrasound probe into a sterile sleeve, and direct placement of the probe on the epicardial surface of the heart. The LV chamber size and ejection fraction were calculated by tracing the endocardial border during systole

and diastole in apical 4-chamber and apical 2-chamber views. Measurements of the LV septal wall were made in the apical 4-chamber view. Basal septal measurements were made adjacent to the mitral valve leaflet insertion. Apical septal measurements were made at the junction of the apical one-third and basal two-thirds of the septum, at the peri-infarct position. These data were used to compare thickness and systolic thickening of the septum. Impedance catheter measurements (Millar Instruments, Houston, Texas) were used to acquire simultaneous LV pressure and volume measurements, allowing derivation of multiple indexes of systolic and diastolic LV function.

Terminal evaluation. The terminal evaluation in the pivotal protocol further included an electrophysiologic study with provocative testing to examine inducibility of ventricular tachycardia, followed by euthanasia and histopathology of heart, lung, liver, spleen, kidneys, and brain.

Genetic labeling and later detection of injected CDCs. Please refer to the Online Appendix for these methods and results.

Statistical analysis. Data are presented in tables as mean ± SD, and in graphs as mean ± SEM. Statistical analyses were performed in SPSS version 17 for Windows (SPSS Inc., Chicago, Illinois). For comparison of the 3 treatment groups, first Levene's test for equality of variances was performed. If the null hypothesis was accepted, the variances were assumed to be similar, and the 3 groups were compared by 1-way analysis of variance (ANOVA). Only if the ANOVA was found to be significant were post-hoc comparisons between groups performed by the method of least-significant difference. If Levene's test had a p value <0.05, a difference in variances between the groups was assumed and the nonparametric Kruskal-Wallis test was used, followed by post-hoc comparison by Dunn's test. This nonparametric comparison only needed to be used for 3 variables: final end-systolic elastance (E_{max}), LVEF by echocardiography at baseline, and delta heart rate. All p values <0.05 were deemed significant for ANOVA, Kruskal-Wallis, and post-hoc comparisons.

Results

Creation of the model. Five of 12 animals died during creation of MI in the engraftment studies, and 15 of 47 animals died during creation of MI in the pivotal study, giving an overall mortality rate of 34% associated with creation of MI in the 2 studies. With the exception of 3 acute procedure-related perioperative complications (1 episode of catheter-related ventricular tachycardia, 1 punctured lung, 1 cardiogenic shock), all animals that received intramyocardial injections survived to complete the study protocol.

Engraftment studies. Autologous, luciferase-labeled CDCs (0.5 million, 2 million, or 10 million per site) were injected, either into the area of infarction, the peri-infarct border zone, or remote normal myocardium. Each dose was tested in 2 to 7 animals, with individual animals receiving multiple anatomically marked injections of different dosages to minimize animal use. Twenty-four hours later, the animals were sacrificed and the hearts (and other organs) were removed to assess short-term engraftment. Myocardial tissue samples were taken from the injection target areas, and from off-target organs (lungs, liver, spleen, kidneys). Within the heart, injection into the scarred infarcted area yielded no surviving cells at any injected dosage (Fig. 2). In contrast, 8% to 9% of injected CDCs survive at 24 h after injection into remote normal myocardium, regardless of dosage. In the peri-infarct border zone, engraftment was inversely related to the number of cells injected, being ~8%, 1.8%, and 0.1% of the 0.5 million, 2 million, and 10 million doses, respectively.

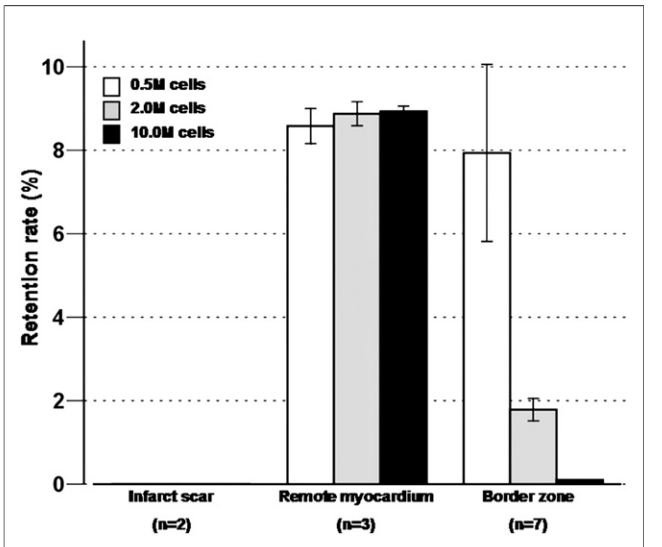

Figure 2 Engraftment of CDCs 24 h After Intramyocardial Injection

When injected into the infarct scar, no cardiosphere-derived cells (CDCs) are detectable 24 h later. When injected into remote normal myocardium, approximately 8% to 9% of each dose of injected cells remains in situ and viable. When injected into the peri-infarct border zone, the proportion of cells surviving is inversely proportional to the number of cells injected; 0.5 million **(open bars)**, 2.0 million **(gray bars)**, and 10.0 million **(solid bars)** indicate the number of cells injected into myocardium at each site.

On the basis of these results, we chose the lowest dose of 0.5 million cells per site for the pivotal studies. The results of the engraftment studies are consistent with previous similar studies, none of which have described engraftment greater than 10% at 24 h (14). It is possible that a dose even lower than 0.5 million cells per site would result in higher percentage engraftment, but in this series the lowest dose tested was 0.5 million per site. In addition, given that cardiac stem cells have little long-term survival advantage in normal myocardium (15), and that they seem not to engraft at all when injected directly into an area of myocardial infarction, we targeted the peri-infarct zone in the pivotal study. It is also known that engraftment is very low at 8 weeks relative to acute engraftment (9,16), so luciferase was not used in the long term studies; instead, lacZ

Table 1 Echocardiographic Indexes

	Placebo (n = 9)	CDC (n = 9)	CSph (n = 5)	p Values			
				ANOVA	Placebo vs. CDC	Placebo vs. CSph	CDC vs. CSph
Ejection fraction, %							
Baseline	43 ± 7	44 ± 12	43 ± 5	0.98*	—	—	—
Final	40 ± 7	47 ± 5	44 ± 5	0.07	—	—	—
Treatment effect (delta)	−3 ± 11	+3 ± 10	+1 ± 5	0.39	—	—	—
Systolic volume, ml							
Baseline	29.5 ± 4.8	24.7 ± 5.0	31.4 ± 3.9	0.04	0.04	0.49	0.02
Final	40.5 ± 11.8	34.7 ± 7.2	31.8 ± 5.6	0.21	—	—	—
Treatment effect (delta)	+10.9 ± 13.2	+10.0 ± 6.2	+0.4 ± 5.4	0.13	—	—	—
Diastolic volume, ml							
Baseline	52.0 ± 9.4	44.2 ± 5.3	55.6 ± 9.8	0.04	0.054	0.44	0.02
Final	66.1 ± 12.9	65.0 ± 10.7	56.2 ± 7.8	0.27	—	—	—
Treatment effect (delta)	+14.0 ± 15.0	+20.8 ± 10.1	+0.7 ± 8.5	0.02	0.25	0.06	<0.01

*Baseline ejection fraction did not exhibit homogeneity of variance between groups, so the Kruskal-Wallis test was performed instead of analysis of variance (ANOVA).
CDC = cardiosphere-derived cells; CSph = cardiospheres.

labeling was used in a subset of the CDC-treated animals in the longer pivotal study.

Quantification of off-target expression at 24 h revealed no measurable cells in liver, spleen, or kidney, but 0.9% of injected CDCs could be detected in the lungs (see Supplementary Table 1). This is ~10-fold lower than the engraftment seen in the target myocardium. These data are consistent with recent findings that many directly injected cells wash away through coronary veins before they are able to "stick" in the myocardium (9,17); the percentage retained in the heart can be increased by iron-loading cells and applying an apical magnet (9), an approach that we did not use here but which merits further investigation.

Pivotal placebo-controlled study. On the basis of the 24 h engraftment data, a dosage of 0.5 million CDCs (or an equivalent cell number of cardiospheres) per site was selected, and direct intramyocardial injections performed in 20 peri-infarct sites, giving a total cell dosage of 10 million CDCs. Numerically coded loaded syringes were provided to the surgeon, who was blinded as to group assignment (as were all other investigators) until completion of all protocols and data analysis. It is possible that the surgeon may have been able to recognize whether he was injecting a cell suspension or vehicle alone because of the turbidity of cell suspensions. However, surgeons did not communicate any suspicions to the rest of the team, and they did not analyze any of the data.

Figure 3 shows the LVEF data derived from contrast ventriculography. The LVEF at baseline was equivalent in the 3 groups. Figure 3B also demonstrates that, 8 weeks after injection, the LVEF was significantly higher than placebo in both of the cell-treated groups (ANOVA $p = 0.02$; placebo vs. CDCs $p = 0.01$, placebo vs. cardiosphere $p = 0.01$), whereas there was no significant difference in final LVEF between the CDC group and the cardiosphere-treated group. Figure 3C shows that the treatment effect (final minus baseline LVEF) was significantly higher than placebo in both of the cell-treated groups (ANOVA $p = 0.002$; placebo vs. CDC $p = 0.001$, placebo vs. cardiosphere $p = 0.01$). Echocardiographic measurement of LVEF yielded qualitatively similar differences in final LVEF and delta LVEF measurements, although the differences between the groups were not statistically significant by this modality (Table 1). Echocardiographic measurement did, however, demonstrate progressive ventricular dilation in placebo and CDC groups, which was attenuated in the cardiosphere-treated animals (Fig. 4B, Table 1). Baseline systolic and diastolic LV volume measurements were randomly lower in the CDC-treated animals (Table 1).

In addition, echocardiography revealed that final measurements of LV septal wall thickness were increased in both of the cell-injected groups relative to placebo (Fig. 5B). The thickening fraction of the apical septum was also increased in cardiosphere-injected pigs (Fig. 5C), adding to the evidence that cardiospheres have an efficacy advantage over CDCs. These salutary changes indicate improved morphology and function in the infarct region with autologous CDC or cardiosphere injections.

Table 2 and Figure 4A outline the results of LV pressure-volume loop analysis. Most measurements were made at steady state. However, one important measurement, E_{max}, was derived, by definition, as the slope of the end-systolic pressure-volume relationship from the family of loops produced during balloon occlusion of the inferior vena cava (18,19) (Fig. 4A). The E_{max} is a rigorous load-independent measure of contractility. Final E_{max} in the cardiosphere group was higher than in placebo-treated pigs, indicating improved LV contractility in these animals (placebo 1.03 ± 0.29 mm Hg/ml, CDC 1.66 ± 0.45 mm Hg/ml, cardiosphere 3.16 ± 1.32 mm Hg/ml; Kruskal-Wallis $p = 0.003$; placebo vs. CDC p = NS, placebo vs. cardiosphere $p = 0.03$, cardiosphere vs. CDC p = not significant). The E_{max} in the CDC group tended to increase but was not significantly higher than in placebo-treated animals.

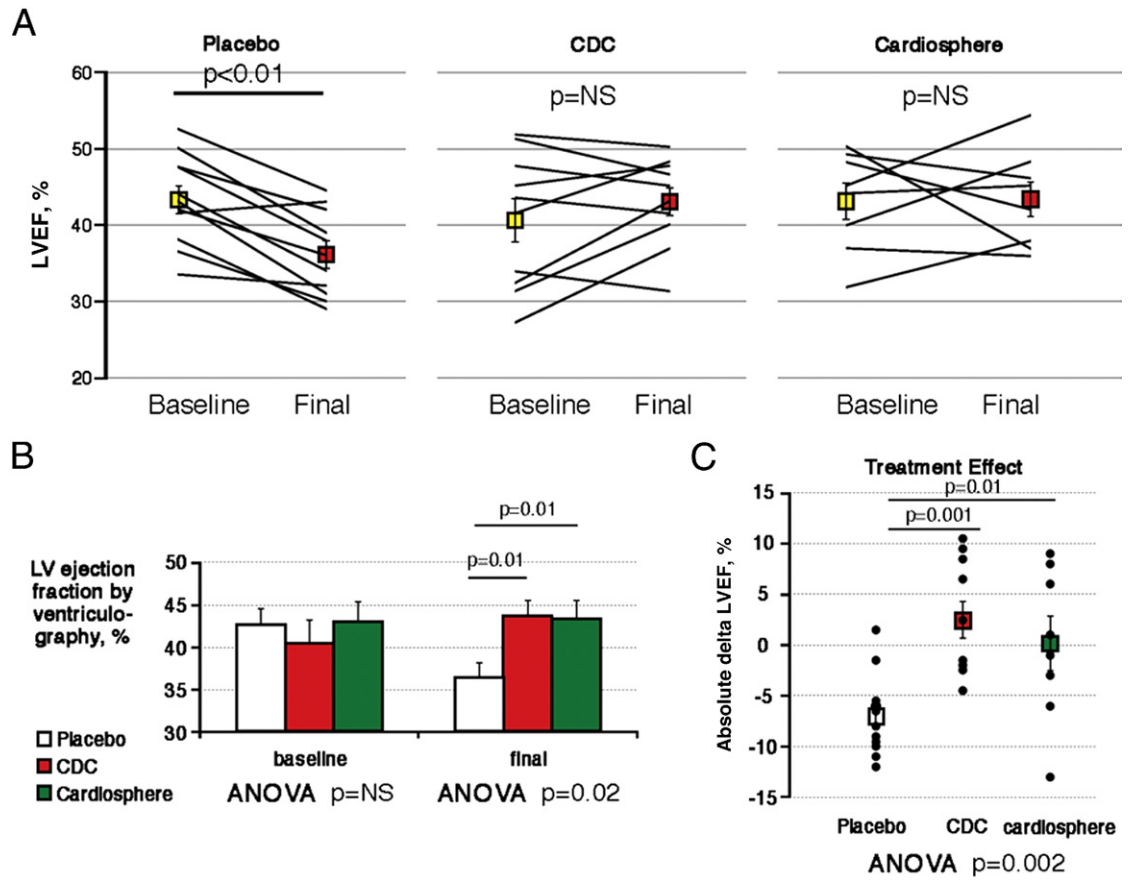

Figure 3 **Intramyocardial Injection of Cardiospheres or CDCs Results in Significant Improvement of LVEF Relative to Placebo**

(A) Left ventricular ejection fraction (LVEF) by ventriculography is shown for each animal at baseline and final measurements **(lines)**, with mean shown as **squares with error bars**. The LVEF in the placebo group deteriorated significantly ($p < 0.01$) whereas neither of the cell-treated groups deteriorated (p = not significant [NS]). CDC = cardiosphere-derived cell. **(B)** The LVEF did not differ between placebo group **(open bars)**, cardiosphere group **(red bars)**, or CDC-treated group **(green bars)** at baseline (analysis of variance [ANOVA] $p = 0.63$); however, by the conclusion of the study, both cell-treated groups had a significantly higher LVEF compared with the placebo group (ANOVA $p = 0.02$; placebo vs. CDCs $p = 0.01$, placebo vs. cardiosphere $p = 0.01$). The 2 cell-treated groups were not statistically different from each other at the final measurement. **(C)** The treatment effect (final minus baseline LVEF) was significantly different between groups (ANOVA $p = 0.002$), with both cell-treated groups significantly different from placebo. Each **solid circle** represents 1 animal; the **squares with error bars** represent the mean treatment effect for each group.

Steady-state hemodynamics showed few differences (Table 2) except for a greater fall in LV end-diastolic pressure in the cardiosphere-treated group (Fig. 4C). Taken together with the lesser increase of end-diastolic volume in this group, cardiosphere-injected animals experience disproportionate benefit with regard to attenuation of adverse ventricular remodeling relative to the other 2 groups (CDCs or placebo).

Indicators of safety. Ventricular tachycardia was readily inducible by application of programmed extrastimuli in all animals before sacrifice, consistent with previous reports (11,20). However, there were no deaths (sudden or otherwise) in either group after the immediate periprocedural period.

Post-mortem examination, with gross analysis as well as histology of heart, brain, kidney, lung, liver, and spleen (Table 3), detected no tumors 8 weeks after intramyocardial injection of CDCs or cardiospheres.

Long-term CDC engraftment. Fluorescence immunohistochemistry in the 2 animals with $lacZ^+$ CDCs, revealed the presence of labeled cells 8 weeks after injection. Supplementary Figure 1 shows 2 examples of islands of cardiomyocytes with lacZ-positive nuclei in the peri-infarct zone, 1 from each animal that received intramyocardial genetically labeled CDCs. This finding establishes that a proportion of injected autologous CDCs, or their progeny, which will also be labeled by this integrating vector, persists for 8 weeks within the border zone of infarcted myocardium. However, the current methodology does not clarify whether the $lacZ^+$ cardiomyocytes have occurred through differentiation of the injected CDCs, or by fusion of the injected CDCs with resident cardiomyocytes. We did not perform lacZ labeling in any of the cardiosphere-injected animals, but we have found that human cardiospheres do engraft better

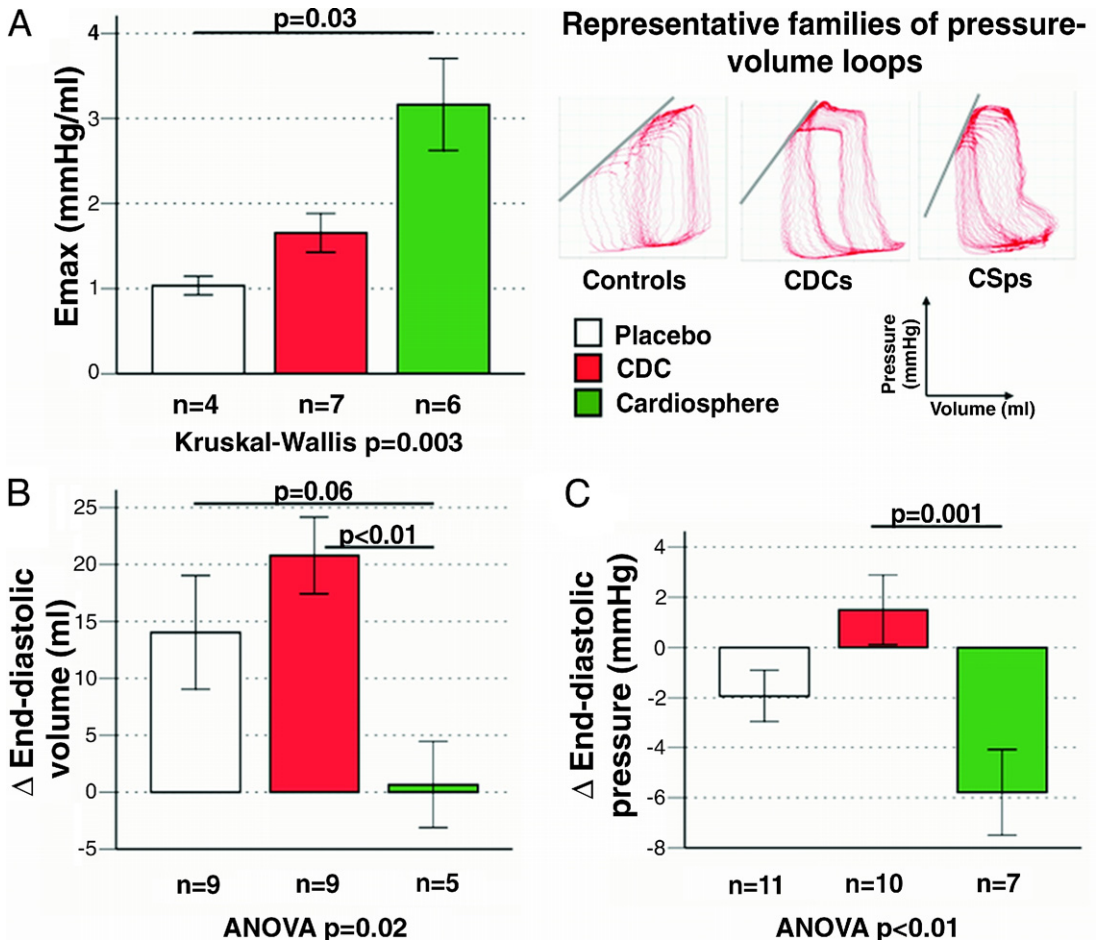

Figure 4 **Multiple Indexes Reveal That Cardiosphere Administration Is Superior to CDC Administration**

(A) The end-systolic elastance (E_{max}) was significantly higher in the cardiosphere group **(green bars)** than in the placebo-injected pigs **(white bars)**. The E_{max} in the cardiosphere-derived cell (CDC) group **(red bars)** was not significantly higher than that of placebo-treated animals. (Levene's test $p < 0.05$; Kruskal-Wallis $p = 0.003$; placebo vs. CDC p = NS; placebo vs. cardiosphere $p = 0.03$). Representative families of PV loops are shown **(right)** for placebo, CDC, and cardiosphere-treated animals. **(B)** Delta diastolic volume (final end-diastolic volume [EDV] minus baseline EDV) is significantly lower in the cardiosphere-treated group **(green bar)** than in the CDC-treated group **(red bar)**, with a trend to being lower than in the placebo group **(white bar)** also (analysis of variance [ANOVA] $p = 0.02$). **(C)** Delta end-diastolic pressure (EDP) measurements demonstrated a significantly higher fall in EDP in cardiosphere-injected animals compared with CDC-treated animals (ANOVA $p < 0.01$; cardiospheres vs. CDCs $p = 0.001$). This finding indicates that the ventricles of cardiosphere-injected hearts **(green bar)** were coping better with the infarction than were the CDC group **(red bar)** and placebo group **(white bar)**.

than human CDCs when injected into SCID mouse hearts (12). In addition, these labeling studies do not inform the relative contributions of direct regeneration versus indirect (paracrine) effects on the efficacy end points, although we have found that both mechanisms play a role in post-MI mice injected with human CDCs (16).

Discussion

The present study is notable for the rational selection process for the dose and location of cell injections to be tested; the randomized, blinded, and placebo-controlled design; and the head-to-head comparison of 2 promising heart-derived cell products that can be readily grown from percutaneous biopsies. The data reveal that direct surgical injection of autologous cardiospheres or CDCs effectively halts the deterioration in LVEF after a large myocardial infarction, compared to a 7% absolute reduction in LVEF over 8 weeks of observation in placebo-treated animals. Although cell treatment did not increase the LVEF relative to baseline, the deterioration of the placebo group meant that the final LVEF was significantly better in either of the cell-injected groups than in the placebo-injected group. If differences of this magnitude were translated to the clinical arena, this may well be a clinically meaningful benefit. For comparison, a meta-analysis of the effect of intracoronary infusion of bone marrow–derived mononuclear cells indicated a mean

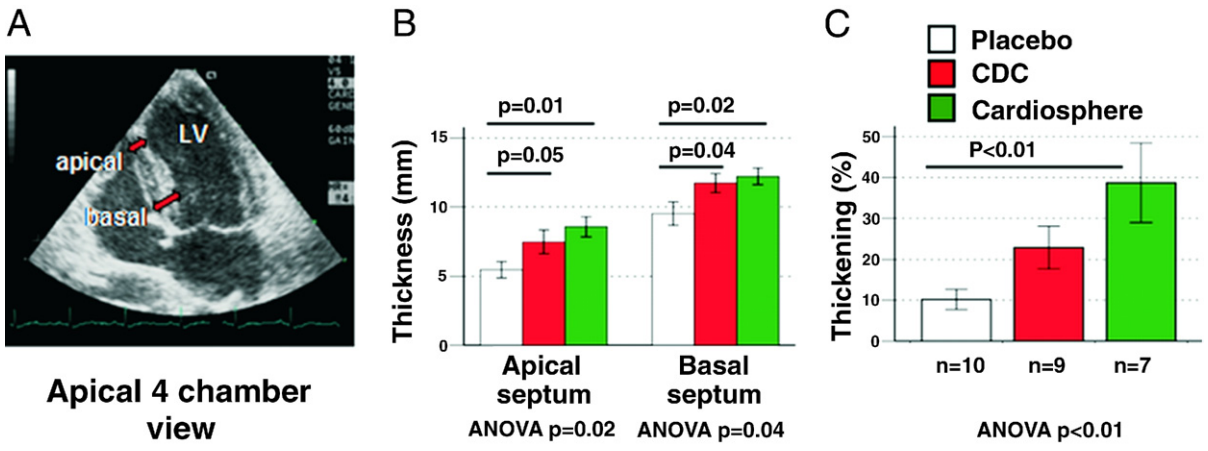

Figure 5 **Echocardiography Reveals Improved Regional Contractility in Cardiosphere-Injected Pigs Relative to Sham-Injected Controls**

(A) A representative apical 4-chamber epicardial echocardiography image. ANOVA = analysis of variance; LV = left ventricle. **(B)** Final measurements of septal thickness are increased in cardiosphere-derived cell (CDC)-injected pigs **(red bars)** and cardiosphere-injected pigs **(green bars)** relative to sham-injected controls **(white bars)**. **(C)** Apical (peri-infarct) septal thickening is increased in cardiosphere-injected pigs **(green bar)** relative to sham-injected controls **(white bar)** (ANOVA $p < 0.01$; placebo vs. cardiosphere group $p < 0.01$). The **red bar** indicates CDC-injected pigs.

absolute augmentation of LVEF of only 3.7% relative to controls (4).

Cardiospheres, but not CDCs, also increased end-systolic elastance and attenuated the ventricular dilation associated with myocardial infarction.

The engraftment data indicate that: 1) no cells survive when injected into the infarct scar; 2) short-term engraftment is ~8% regardless of injected cell dose in remote normal myocardium; and 3) in the infarct border zone, "less is more": the percent survival at 24 h decreases progressively from ~8% to <1% as dosage escalates. This is the first description that proportional engraftment of injected cells is improved by injection of lower cell doses at each injection site. One interpretation of these results is that survival in the border zone may be limited by the tenuously perfused, substrate-limited, peri-infarct environment. In this scenario, while the absolute number of injected cells able to survive remains about the same, percentage survival of injected cells is greater with injection of lower numbers of cells per site. This discovery has potential implications for trials of intramyocardial administration of many other cell types, not only CDCs. In practical terms, we chose not to inject into normal myocardium, as it is unclear whether cells thus injected would track into and influence remote sites of chronic injury, and we avoided injection into the scar, given the finding of no engraftment. We thus chose to inject the peri-infarct area, and to use the lowest tested dose, namely, 0.5 million cells per site, in each of 20 sites (a number chosen as a practical limit to achieve good coverage of the border zone, rather than empirically).

The preservation of global LVEF in cell-treated animals, compared to the deterioration in the placebo group, was not observed with intracoronary administration of CDCs in the same porcine model of MI (11). Thus, intramyocardial injection may represent a more effective route of administration than the intracoronary route (11). We additionally find that cardiospheres are superior to CDCs in terms of hemodynamic benefit as well as in attenuation of adverse remodeling. Direct expansion of resident cardiac stem cells from human specimens was originally described by Messina et al. (5), who collected cardiac-derived cells and subcultured them as 3-dimensional cell aggregates, which they dubbed cardiospheres. This technique was patterned from the neurosphere experience (21). The Marbán laboratory adapted and miniaturized the cardiosphere technique for utility with percutaneous endomyocardial biopsies as the tissue of origin, plating cardiospheres in monolayer culture to yield therapeutically relevant numbers of CDCs (6). However, the relative utility of cardiospheres versus CDCs was not entirely clear before the present study. Intriguing early reports demonstrated that injection of small numbers of cardiospheres effectively doubles LV fractional shortening after MI (5), as opposed to large numbers of monolayer-cultured CDCs, providing a 64% increase in the LVEF (6). Further, cardiosphere culture increased the expression of c-kit, a stem cell marker, from 10% to 30% after 6 days of culture (5). Subsequent transition from cardiosphere to monolayer culture results in decreased c-kit expression (6). These results suggest that cardiosphere culture might enhance the "stemness" of cardiac-derived cells, and that the implantation of car-

Table 2 Pressure-Volume Loop-Derived Indexes

	Placebo (n = 11)	CDC (n = 10)	Cardiospheres (n = 7)	ANOVA p Value
Heart rate, beats/min				
Baseline	121 ± 12	119 ± 12	117 ± 17	0.72
Final	112 ± 17	109 ± 13	117 ± 19	0.65
Treatment effect (delta)	−9 ± 16	−9 ± 12	−1 ± 36	0.72*
P_{max}, mm Hg				
Baseline	97.0 ± 11.5	86.9 ± 6.9	93.8 ± 14.6	0.13
Final	87.1 ± 11.4	88.4 ± 10.4	86.2 ± 8.1	0.90
Treatment effect (delta)	−9.9 ± 12.9	+1.5 ± 11.2	−7.9 ± 16.0	0.14
LVEDP, mm Hg				
Baseline	14.6 ± 3.1	14.5 ± 5.1	18.0 ± 5.3	0.20
Final	12.6 ± 3.7	16.0 ± 6.2	12.1 ± 4.1	0.19
Treatment effect (delta)	−1.9 ± 3.4	+1.5 ± 4.4	−5.8 ± 1.7	0.01†
dP/dt max				
Baseline	1,967 ± 370	1,770 ± 278	1,784 ± 693	0.56
Final	1,589 ± 446	1,430 ± 370	1,422 ± 333	0.58
Treatment effect (delta)	−378 ± 462	−340 ± 280	−382 ± 876	0.93
dP/dt min				
Baseline	−1,984 ± 480	−1,584 ± 346	−1,784 ± 394	0.11
Final	−1,516 ± 291	−1,527 ± 357	−1,546 ± 444	0.99
Treatment effect (delta)	468 ± 600	56 ± 501	247 ± 520	0.25
Tau, ms				
Baseline	39.84 ± 4.87	38.93 ± 5.51	41.28 ± 7.21	0.70
Final	41.25 ± 7.21	40.92 ± 4.57	41.88 ± 3.82	0.94
Delta	+1.41 ± 8.39	+1.98 ± 6.12	−0.03 ± 5.42	0.84

*The delta heart rate variable did not exhibit homogeneity of variance between groups, so the Kruskal-Wallis test was performed instead of analysis of variance (ANOVA). †For further details about post-hoc comparisons of delta LVEDP among the 3 groups, please refer to Figure 4C.

dP/dt max = maximum rate of rise of left ventricular pressure; dP/dt min = the minimum rate of rise of left ventricular pressure; LVEDP = left ventricular end-diastolic pressure; P_{max} = maximum pressure generated by left ventricle during cardiac cycle; tau = measure of left ventricular relaxation; other abbreviations as in Table 1.

diospheres may disproportionately boost myocardial function relative to monolayer-cultured cells (12). Such speculation gains credence from the present findings that cardiospheres are superior to CDCs in terms of hemodynamics and ventricular remodeling.

Two recent reports have challenged the stem cell characteristics and cardiomyogenic potential of cardiospheres (22,23), and another has shown no demonstrable functional benefit and an absence of viable cardiac stem cells 8 weeks after injection (24). Methodological variations can explain the negative findings in these reports (7,25). Experiments in our laboratory over the past 6 years (flow cytometry, genetic lineage tracing, clonal cell expansion, and protocol optimization experiments) have characterized CDCs as a natural mixture of progenitor and support cells expanded from myocardial biopsy specimens, with validated, reproducible growth characteristics, clonogenicity and multilineage potential (6,7). The functional benefit of cells derived from cardiac biopsy specimens is also quite reproducible (6,9,10,12), despite low long-term engraftment rates of injected cells (9,14,16). The exact mechanism of functional benefit appears to involve an important, if not dominant, contribution of indirect effects to boost angiogenesis and cardiomyogenesis (16).

Open-chest surgery would be unlikely to gain clinical acceptance unless adjunctive to clinically indicated surgery; however, less invasive methods of intramyocardial administration, such as transendocardial catheter-mediated delivery, may be more acceptable if this route were also shown to be efficacious, as has been shown with mesenchymal stem cells (26).

The field is very cognizant of the potential for dangerous side effects of cardiac cell therapy, such as teratoma related to embryonic stem cell (27) or induced pluripotent stem cell (28) administration, or ventricular arrhythmia related to skeletal myoblast administration (29). In the context of these safety concerns, the absence of tumor, the equivalence of ventricular tachycardia inducibility in the control and treatment groups of the current study, and the zero post-treatment mortality in the present study are reassuring. With regard to translation, a phase I-II clinical study of CDCs—the CADUCEUS (CArdiosphere-Derived aUtologous stem CElls to reverse ventricUlar dySfunction) study—is under way (30). That study utilizes CDCs delivered through the intracoronary route. The present findings motivate future clinical studies involving direct intramyocardial injection of either CDCs or cardiospheres—with intriguing results hinting that the latter may be preferable.

Table 3 Absence of Tumor Formation Related to Intramyocardial CDC or Cardiosphere Injection

	Tumor Present in These Organs?						
	Heart	Lungs	Liver	Spleen	Kidneys	Brain	Other Findings?
Placebo animals							
1	No	No	No	No	No	No	—
2	No	No	No	No	No	No	—
3	No	No	No	No	No	No	—
4	No	No	No	No	No	No	Several foreign body granulomas in MI scar
5	No	No	No	No	No	No	Several foreign body granulomas in MI scar
6	No	No	No	No	No	No	Posterolateral myocardial infarction similar in age to the main anteroseptal infarct
7	No	No	No	No	No	No	—
8	No	No	No	No	No	No	—
9	No	No	No	No	No	No	—
10	No	No	No	No	No	No	Several large areas of mummified myocytes with the MI, heavily calcified
11	No	No	No	No	No	No	Granulomas within the MI
CDC-treated animals							
1	No	No	No	No	No	No	Multiple lung abscesses
2	No	No	No	No	No	No	—
3	No	No	No	No	No	No	Foreign body reaction within myocardium
4	No	No	No	No	No	*	—
5	No	No	No	No	No	No	—
6	No	No	No	No	No	No	—
7	No	No	No	No	No	No	—
8	No	No	No	No	No	No	—
9	No	No	No	No	No	No	—
10	No	No	No	No	No	No	—
Cardiosphere-treated animals							
1	No	No	No	No	No	No	Several small granulomas with calcification within the MI
2	No	No	No	No	No	No	Several small granulomas with calcification within the MI
3	No	No	No	No	No	No	Several small granulomas with calcification within the MI
4	No	No	No	No	No	No	—
5	No	No	No	No	No	No	Chronic pyelonephritis of 1 kidney; retained gauze in epicardial location
6	No	No	No	No	No	No	—
7	No	No	No	No	No	No	Granulomas with calcification with the MI
8	No	No	No	No	No	No	—

*Not submitted for histology.
MI = myocardial infarction; other abbreviations as in Table 1.

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Key Words: animal model ■ cardiosphere-derived cell ■ heart failure ■ myocardial infarction ■ stem cell.

APPENDIX

For supplementary Methods, Results, figure, and table, please see the online version of this article.