Comparative Efficacy of Intracoronary Allogeneic Mesenchymal Stem Cells and Cardiosphere-Derived Cells in Swine with Hibernating Myocardium

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Abstract

Rationale—Allogeneic bone marrow-derived mesenchymal stem cells (MSCs) and cardiosphere-derived cells (CDCs) have each entered clinical trials but a direct comparison of these cell types has not been performed in a large animal model of hibernating myocardium.

Objective—Using completely blinded methodology, compare the efficacy of global intracoronary allogeneic MSCs (icMSCs, ~35×10^6) and CDCs (icCDCs, ~35×10^6) vs. vehicle in cyclosporine-immunosuppressed swine with a chronic LAD stenosis (n=26).

Methods and Results—Studies began 3-months after instrumentation when wall-thickening (%WT) was reduced (LAD%WT 38±11% (mean ± SD) vs. 83±26% in remote, p<0.01) and similar among groups. Four-weeks after treatment, LAD%WT increased similarly following icCDCs and icMSCs, while it remained depressed in vehicle-treated controls (icMSCs: 51±13%; icCDCs: 51±17%; vehicle: 34±3%, treatments p<0.05 vs. vehicle). There was no change in myocardial perfusion. Both icMSCs and icCDCs increased LAD myocyte nuclear density (icMSCs: 1601±279 nuclei/mm^2, icCDCs: 1569±294 nuclei/mm^2, vehicle: 973±181 nuclei/mm^2, treatments p<0.05 vs. vehicle) and reduced myocyte diameter (icMSCs: 16.4±1.5 μm, icCDCs: 16.8±1.2 μm, vehicle: 20.2±3.7 μm, treatments p<0.05 vs. vehicle) to the same extent. Similar changes in myocyte nuclear density and diameter were observed in the remote region of cell-treated animals. Cell fate analysis using Y-FISH demonstrated rare cells from sex-mismatched donors.

Conclusions—Allogeneic icMSCs and icCDCs exhibit comparable therapeutic efficacy in a large animal model of hibernating myocardium. Both cell types produced equivalent increases in regional function and stimulated myocyte regeneration in ischemic and remote myocardium. The activation of endogenous myocyte proliferation and regression of myocyte cellular hypertrophy support a common mechanism of cardiac repair.

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DISCLOSURES

Keywords

Cell therapy; ischemic heart disease; hibernating myocardium; cardiac regeneration; allogeneic cell therapy; coronary stenosis

INTRODUCTION

The development of cell-based therapeutic approaches to treat ischemic heart disease has proceeded at a rapid pace since initial clinical trials commenced over a decade ago. Studies have been fueled by optimism that exogenous stem cells could replenish the pool of functional cardiac myocytes that is depleted by acute and chronic injury. There has been an abundance of positive results in preclinical rodent models of myocardial infarction using a variety of cell types with surprisingly few negative studies. Nevertheless, as research has translated to large animal studies and phase I and 2 clinical trials the functional effects of cell-therapy have been variable. Thus, although clinical trials have confirmed the safety of most preclinical cardiac cell-therapies, limited functional improvement has been observed in comparison to animal studies[1–3]. This has been highlighted by a recent meta-analysis of clinical cell therapy studies utilizing individual patient data, which concluded that intracoronary cell therapy did not reduce clinical events nor improve left ventricular (LV) function in patients with recent myocardial infarction[4].

There are several potential explanations for the difficulty in translating preclinical findings to clinical trials. Some are unique to cardiovascular research while others are common to translating preclinical research in other fields of medicine[5]. First, rodents tolerate myocardial infarctions that are much larger than what a human can survive. This amplifies the deleterious effects of LV remodeling in rodent models. At the same time, remodeling in humans is now markedly attenuated by pharmacological therapy and early reperfusion as compared to untreated animal models. In addition, most models employ young animals that do not have the underlying cardiovascular risk factors present in humans with diseases such as hypertension, diabetes and hypercholesterolemia nor the impact of aging. There is also likely publication bias towards positive results with underreporting of negative preclinical studies.

Perhaps a more substantial barrier common to translating preclinical studies in all fields are the way that most are conducted. In comparison to clinical trial methodology, few preclinical studies employ a blinded randomized controlled approach. The team of preclinical investigators and research scientists is rarely blinded to treatment allocation. While interpretation of some aspects are sometimes blinded (e.g. cardiac imaging results), blinding of investigators involved in pathological analysis and other physiological endpoints is rarely employed. A recent meta-analysis of preclinical cardiovascular cell therapy studies found that only 42% of included studies reported blinded assessment of some data and only 11% reported allocation concealment[6]. Thus, many studies may have selection bias related to inclusion/exclusion of experiments and variable observer bias in most of the end-point analyses. These problems have recently been highlighted in the field of cardioprotection research by the NIH-sponsored Consortium for Preclinical Assessment of Cardioprotective

Therapies (CAESAR)[7]. Using rigorous blinded treatment and data analysis comparable to randomized clinical trials, the CAESAR consortium has been unable to show the beneficial effects of two agents that had been widely shown to reduce infarct size in prior small, poorly controlled preclinical studies in multiple species[8, 9]. Thus, the absence of a completely blinded randomized study design may be a significant methodological hurdle to overcome in translation of preclinical research into clinical application.

With this in mind, we determined if a randomized blinded experimental approach could be employed in a single-center preclinical trial comparing two types of cell-therapy vs. vehicle-treated controls. Bone marrow-derived mesenchymal stem cells (MSCs) and cardiosphere-derived cells (CDCs) cultured from explanted heart tissue are promising cell types that have recently entered clinical trials[10–13]. A particularly attractive feature of both cell types is the feasibility of using allogeneic cells, which would permit off-the-shelf availability of cells for treatment and circumvent the need for personalized cell expansion. Both MSCs and CDCs have been shown to improve ventricular function in preclinical studies by our laboratory and others[14–18] but they have not been compared in a head-to-head fashion in a large animal model of myocardial ischemia. Previous studies including our own have been limited by the fact that investigators were not completely blinded to treatment allocation and all functional and pathological analyses. Therefore, the present study addresses these issues by employing a rigorous, clinical trial-based three-way design to directly compare the therapeutic efficacy of intracoronary allogeneic bone marrow-derived mesenchymal stem cells (icMSCs) and intracoronary cardiosphere-derived cells (icCDCs) infused to the entire heart in a swine model of chronic myocardial ischemia. Any potential effects of allogeneic cell rejection were minimized by treating all animals with chronic cyclosporine. The results demonstrate preclinical therapeutic equipoise of allogeneic icMSCs and icCDCs.

METHODS

All procedures and protocols conformed to institutional guidelines for the care and use of animals in research and were approved by the University at Buffalo Institutional Animal Care and Use Committee.

Study design and treatment of data

All personnel involved in data collection and analysis (including echocardiographic imaging and histological assessment of myocyte morphometry and proliferation) were blinded to the treatment status of each animal. Serial data collection and analysis were conducted by an independent biostatistician not involved in the protocols or assays (JAF). Animals were randomized to receive vehicle, icMSCs, or icCDCs in a 1:1:1 ratio. Regional wall thickening of the area supplied by the left anterior descending coronary artery (LAD %WT) 1-month after treatment was selected as the primary endpoint.

Isolation and characterization of MSCs and CDCs

Bone marrow (n=2) and left ventricular tissue (n=3) samples were collected from healthy donor pigs (age 8±3 weeks) for the isolation and cultivation of MSCs and CDCs, as previously described[16, 17, 19] and summarized in the Online Supplement. Cells were cultured

for 4 to 7 passages, at which time they were collected for characterization by flow cytometry and immunohistochemistry or intracoronary infusion to recipient pigs with hibernating myocardium. Flow cytometric and immunohistochemical characterization included assessment of hematopoietic (CD45, cKit), mesenchymal (CD90, CD105) and cardiac (GATA4, Nkx2.5) markers. Prior to administration, both cell types were filtered through a 30 μm pore filter to circumvent administering cell aggregates (MACS pre-separation filters, Miltenyi Biotec) and suspended in heparinized HBSS solution (3000U heparin in 30ml in total) for intracoronary infusion.

Effects of icMSCs and icCDCs on flow and function in hibernating myocardium

Experimental groups and the study timeline are summarized in Supplemental Figure I. Pigs (n=34) were chronically instrumented with a 1.5 mm Delrin occluder on the proximal LAD to produce hibernating myocardium as previously described[20]. Briefly, juvenile pigs were sedated (Telazol 100mg/ml / xylazine 100 mg/ml, 0.022 mg/kg i.m.), intubated and ventilated with a 0.5–2% isoflurane-oxygen mixture. Through a small pericardiotomy, the Delrin occluder was placed on the proximal LAD. Antibiotics (cefazolin, 25 mg/kg and gentamicin, 3 mg/kg i.m.) were given 1-hour before surgery and repeated after closing the chest. Analgesia included an intercostal nerve block (0.5% Marcaine) and intramuscular doses of butorphanol (2.2 mg/kg q6h) and flunixin (1–2 mg/kg q.d.).

Serial physiological studies—Studies began 3-months after instrumentation when stable reductions in LAD wall thickening and perfusion indicative of hibernating myocardium were present[21]. Under propofol sedation (5–10 mg/kg/hr i.v.), a catheter (Millar) was inserted to measure LV pressure and inject microspheres. Regional wall-thickening was assessed with transthoracic echocardiography from a right parasternal approach[16]. All pigs showed anterior dysfunction without dyskinesis. Systolic wall-thickening (WT=ESWT–EDWT; %WT= WT/EDWT×100) was quantified in dysfunctional LAD regions as well as remote, normally-perfused regions of the same heart.

Microsphere perfusion—Microsphere flow was measured at rest and following adenosine vasodilation to characterize hibernating myocardium 3-months after instrumentation and repeated 4 weeks after therapy as previously described[22]. Briefly, we injected 15μm microspheres (~3×10^6) labeled with fluorescing dyes into the left ventricle while a reference arterial sample was withdrawn at 6 ml/min for 90-seconds. At the end of the study, samples were taken from mid-ventricular circumferential rings divided into twelve wedges with each cut into 3 transmural layers. Fluorescent dyes were extracted using standard techniques and quantified at selected excitation wavelengths[23].

Cell administration and follow-up—After baseline physiological measurements, pigs were randomly assigned to receive coded treatments of either vehicle (n=8), ~35×10^6 icMSCs (n=9), or ~35×10^6 icCDCs (n=9) with all investigators and research personnel blinded to treatment allocation. Each treatment was divided into three aliquots and infused into each of the three major coronary artery distributions over 10-minutes at a rate of 1×10^6 cells/minute. All animals were treated with cyclosporine A beginning 3 days before the baseline study and continuing throughout terminal experiments (200 mg/day p.o., Watson

Pharma). Animals returned to the laboratory 4-weeks later for post-treatment physiological studies under propofol anesthesia, after which the heart was arrested with potassium chloride and quickly excised for histopathological analysis.

Myocardial histopathology

Quantitative immunohistochemistry and morphometry were evaluated in all vehicle-, icMSC-, and icCDC-treated animals with hibernating myocardium. Y-FISH analysis was performed in the subset of female animals that received male donor-derived cells to track cell fate, as described below.

Arteriolar and capillary density—Regional tissue sections were incubated with anti-α smooth muscle actin and anti-von Willebrand factor antibodies with appropriate fluorescent-labeled secondary antibodies to identify coronary arterioles and capillaries, respectively. Vessels were counted in at least 10 random fields from the subendocardial and subepicardial layers of each tissue sample (LAD and remote) and expressed per tissue area (mm^2).

Myocyte nuclear density and morphometry—Paraffin-embedded samples from LAD and non-ischemic remote regions were fixed for morphometry as previously described[16, 17, 24]. PAS-stained sections were used to quantify myocyte diameter by counting at least 100 cells from the inner and outer half of the LAD and remote regions. Myocytes were included regardless of size as long as myofilaments could be identified surrounding the nucleus. Myocyte nuclear density was also assessed in PAS-stained sections as previously described[16]. To evaluate whether changes in nuclear density reflected increases in the number of nuclei per myocyte, longitudinal myocytes (n=20 in each histological sample) that had boundaries that were clearly visible (intercalated discs and the lateral borders of the myocyte) were used to quantify the number of nuclei per cell in each heart using previously published methodology[25]. Point-counting of trichrome-stained sections was used to quantify connective tissue[26].

Quantification of myocyte proliferation—Myocardial samples were incubated with anti-Ki67 and anti-phospho-Histone-H3 antibodies to identify myocytes in the growth phase of the cell cycle and undergoing mitosis as previously described[17]. Paraffin-fixed sections (4μm thickness) were incubated with either anti-Ki67 (mouse monoclonal antibody, clone MIB-1, Dako, 1:200) or anti-phospho-histone-H3 (rabbit polyclonal antibody, Upstate Biotech, 1:1000) to detect proliferating cells and anti-cTnI (rabbit polyclonal antibody, Santa Cruz, 1:200) to detect myofilaments. Nuclei were stained with DAPI (Vectasheild).

Fluorescent in situ hybridization (Y-FISH)—Y-FISH (IDLabs, Ontario, Canada) was used to determine the fate of male donor icMSCs (n=4) and icCDCs (n=5) injected into female recipients[17]. Tissue samples were hybridized with FITC-conjugated porcine Y-chromosome probe according to the manufacturer's instructions (IDLabs, Ontario, Canada). Y-FISH staining in male control cardiac tissue was used to correct for the fact that myocyte nuclear diameter was greater than histological slice thickness. The "efficiency" of Y chromosome identification was 37±3% and similar to previously published data[17]. Sections

were also incubated with antibodies to cardiac Troponin I to detect co-localization of Y-FISH in myocytes derived from donor cells.

Statistical analysis

Data are expressed as mean ± standard deviation. Differences among treatment groups were assessed by two-way analysis of variance (ANOVA) with repeated measures and the post-hoc Holm-Sidak test. For all comparisons, $p < 0.05$ was considered significant.

RESULTS

A total of 34 animals were instrumented with a chronic LAD stenosis. Consistent with previous data demonstrating an elevated risk of sudden cardiac death in this model[22], 7 animals died prior to completion of the study. Of these, 5 died prior to the 3-month study and 2 died between the 3- and 4-month studies (1 CDC-treated animal and 1 vehicle-treated animal, >10 days after the intracoronary infusion). One animal was removed from further study at the 3-month time point when angiography demonstrated that there was no stenosis of the LAD and the Delrin occluder had been displaced from the vessel. All remaining animals (n=26) were included in the final data analysis with no exclusions after randomization to treatment.

Allogeneic MSC and CDC cell characterization

Cell culture resulted in comparable total cell injections from each treatment ($32.4 \pm 7.3 \times 10^6$ icMSCs and $35.3 \pm 5.9 \times 10^6$ icCDCs, p=0.40). Flow cytometry confirmed that cell surface marker expression profiles were consistent with previous reports[16, 17, 27]. At the time of infusion, MSCs and CDCs expressed similar frequencies of $CD90^+$ and $CD105^+$. Both were also $CD45^-$ with a small number expressing $cKit^+$ (Figure 1). Despite these similarities, they had markedly different expression of cardiac transcription factors. Both GATA4 and Nkx2.5 were absent in MSCs but ubiquitous in CDCs with nearly all demonstrating that they were cardiac lineage committed at the time of administration.

Comparative physiological effects of icMSCs and icCDCs in swine with hibernating myocardium

There were no differences in hemodynamic variables at rest or during adenosine between treatment groups (Supplemental Table I). Baseline studies 3-months after instrumentation confirmed the presence of viable dysfunctional hibernating myocardium. Regional LAD wall thickening (LAD %WT) was reduced compared with normal remote regions ($38 \pm 11\%$ vs. $83 \pm 26\%$, p<0.01) along with reductions in resting perfusion (LAD 0.63 ± 0.21 vs. 0.80 ± 0.34 ml/min/g in remote, p<0.05). Adenosine-dilated flow was also attenuated (LAD 1.62 ± 0.90 vs. 4.00 ± 2.90 ml/min/g in remote, p<0.001). Indices of LV function and flow were comparable among all treatment groups at baseline.

The effects of icMSCs and icCDCs on regional function are summarized in Figure 2 and Supplemental Table II. One-month after treatment, LAD%WT increased in both icMSC-treated ($50.7 \pm 13.4\%$) and icCDC-treated ($50.9 \pm 16.6\%$) animals vs. vehicle-treated controls ($33.7 \pm 9.2\%$; p<0.05 vs. each cell-therapy). Functional effects of cell therapy were not

limited to the ischemic LAD area, as both cell types prevented the deterioration of remote zone wall thickening that was observed in vehicle-treated animals. As a result, remote zone %WT was significantly higher in animals treated with icMSCs (88.4±26.0%,) and icCDCs (94.3±39.3%) compared with vehicle-treated controls (59.5±18.2%; p<0.05 vs. each cell-therapy) 1-month after treatment. Global LV ejection fraction was minimally reduced at baseline and was not significantly affected by any treatment (Supplemental Table II). Importantly, there were no significant differences in functional improvement comparing icMSCs vs. icCDCs.

Consistent with our previous results[16], there were no significant effects of either cell-therapy on serial measurements of coronary flow at rest or during adenosine vasodilation (Figure 3). Although icMSC- and icCDC-treated animals exhibited a significant increase in capillary density in both LAD and remote regions of the left ventricle (Figure 4), neither treatment promoted arteriogenesis since there was no change in arteriolar density. Thus, the functional improvement afforded by icMSCs and icCDCs was accompanied by capillary angiogenesis without arteriogenesis or increased collateral blood flow.

Comparative effects of icMSCs and icCDCs on cardiomyocyte number and size

Blinded analysis of myocyte nuclear density and diameter are summarized in Figure 5. Nuclear density in vehicle-treated animals was reduced throughout the left ventricle (LAD: 973±181 nuclei/mm^2, Remote: 1,005±88 nuclei/mm^2) compared to previous measurements from normal sham pigs of similar size (~1200–1400 nuclei/mm^2)[17, 26]. icMSC treatment (LAD: 1,601±279 nuclei/mm^2, Remote: 1,387±348 nuclei/mm^2; both p<0.05 vs. vehicle) and icCDC treatment (LAD:1,569±294 nuclei/mm^2, Remote: 1,545±276 nuclei/mm^2; both p<0.05 vs. vehicle) each significantly increased myocyte nuclear density throughout the left ventricle. Increases in nuclear density were not the result of karyokinesis without cytokinesis since the number of nuclei per myocyte was not different among all treatment groups (Vehicle: 4.4±0.3; icMSCs: 4.5±0.6; icCDCs: 4.2±0.3, p-ns). Increases in myocyte nuclear density were also accompanied by reductions in myocyte diameter. Vehicle-treated controls (LAD: 20.2±3.7 μm, Remote: 19.8±1.7 μm) were larger than icMSC-treated (LAD: 16.4±1.5 μm, Remote: 16.9±2.4 μm; both p<0.05 vs. vehicle) and icCDC-treated animals (LAD: 16.8±1.2 μm, Remote: 16.9±1.0 μm; both p<0.05 vs. vehicle). Interestingly, these changes were not associated with anatomic LV hypertrophy, as postmortem LV mass/body mass ratio was not different among groups (Vehicle: 2.5±0.3, icMSCs: 2.3±0.3, icCDCs: 2.3±0.3 g/kg).

Effects of icMSCs and icCDCs on myocyte proliferative markers

Ki67 expression (Vehicle-treated LAD: 573±158 nuclei/10^6 myocyte nuclei; Remote: 540±209 nuclei/10^6 myocyte nuclei) tended to increase throughout the LV after icMSCs (LAD: 1068±585 nuclei/10^6 myocyte nuclei, p=0.11 vs. vehicle; Remote: 994±471 nuclei/10^6 myocyte nuclei, p=0.06 vs. vehicle). After icCDCs, Ki67 expression was greater (LAD: 1866±858 nuclei/10^6 myocyte nuclei, Remote: 1530±588 nuclei/10^6 myocyte nuclei; both p<0.05 vs. vehicle and icMSCs) (Figure 6a). There were similar differences in pHH3 expression (Figure 6b) with icMSC-treated animals being borderline significant (LAD: 156±96 nuclei/10^6 myocyte nuclei, p=0.10 vs. vehicle; Remote: 167±99 nuclei/10^6 myocyte

nuclei, p=0.11 vs. vehicle) vs. vehicle-treated animals (LAD: 73±31 nuclei/10^6 myocyte nuclei, Remote: 57±37 nuclei/10^6 myocyte nuclei). In contrast, the magnitude of pHH3 expression increased significantly after icCDCs (LAD: 302±141 nuclei/10^6 myocyte nuclei, Remote: 362±210 nuclei/10^6 myocyte nuclei; both p<0.05 vs. vehicle and icMSCs).

Engraftment and fate of allogeneic icMSCs and icCDCs

To estimate the number of new myocytes derived from icMSCs and icCDCs, Y^+ cells were assessed in the LAD and remote regions of the hearts of female recipients who had received male donor-derived cells (icMSCs: n=4, icCDCs: n=5, Figure 7a). A similar number of Y^+ cells were observed in icMSC-treated (LAD: 3.1±2.6 Y^+ cells/cm^2; Remote: 0.7±0.5 Y^+ cells/cm^2) vs. icCDC-treated animals (LAD: 1.8±1.4 Y^+ cells/cm^2, p=0.36 vs. icMSC-treated; Remote: 0.3±0.4 Y^+ cells/cm^2, p=0.20 vs. icMSC-treated; Figure 7b). This amounted to only ~1 in 10,000/cm^2 of the new myocytes formed from allogeneic CDCs or MSCs based on our estimates of ~40,000 to 60,000/ cm^2 new myocytes following intracoronary cell therapy (after accounting for myocyte multi-nucleation; Figure 7c). The paucity of Y^+ myocytes despite large increases in total myocyte number provides evidence that both icCDCs and icMSCs stimulated endogenous myocyte proliferation with rare cardiac myocytes derived from injected MSCs or CDCs.

DISCUSSION

There are several important findings from the present study. First, using a blinded, randomized vehicle-controlled study design, we have shown that global infusion of allogeneic icMSCs and icCDCs produces comparable increases in myocardial function and myocyte proliferation. Improvements in function were associated with myocyte proliferation in both ischemic and remote myocardium. Second, while there was no functional arteriogenesis, cell therapy globally increased capillary density and myocyte nuclear density with a reduction in myocyte diameter. This, along with the observation that the number of nuclei per myocyte is unchanged, is consistent with significant myocyte proliferation throughout the left ventricle that is quantitatively similar between the two cell-therapies. Finally, the increase in myocyte nuclear number despite minimal engraftment of allogeneic cells in sex mismatched donor-recipients indicates that new myocytes are formed via endogenous mechanisms rather than direct differentiation of injected cells and is supported by the elevated expression of myocyte proliferative markers. Collectively, our results demonstrate that global intracoronary infusion of allogeneic MSCs and CDCs promotes a similar degree of myocardial repair via the stimulation of endogenous myocyte proliferation.

This is the first study to directly compare allogeneic intracoronary infusion of CDCs and MSCs in a large animal model of chronic myocardial ischemia. A few studies have compared these cell types after direct intramyocardial injection into infarcted tissue. For example, following xenogeneic transplantation of human cells to immunocompromised mice with myocardial infarction, CDCs were shown to be therapeutically superior to bone marrow-derived MSCs, adipose tissue-derived MSCs, and bone marrow-derived mononuclear cells [27]. Xenogeneic cell transfer of human cKit+ cardiac stem cells (CSCs) also promoted greater myocardial repair than human bone marrow-derived MSCs after

injection in mice with permanent coronary artery ligation[28]. In a subsequent study using a porcine reperfused myocardial infarct model, myocardial injection of human CSCs and bone marrow-derived MSCs produced equivalent functional improvement[29]. Interestingly, the combination of human MSCs and CSCs produced greater functional improvement than either cell type alone. While not examined in our study, it is plausible that combining MSCs and CDCs could yield greater functional repair than either cell alone and should be considered in future studies.

While intracoronary infusion of both allogeneic MSCs and CDCs were efficacious, our preclinical results suggest that it may be difficult to demonstrate that either cell type would be superior to the other in a head-to-head clinical trial. The similarity of functional repair afforded by each cell type is perhaps not surprising given several similarities between MSCs and CDCs revealed by in vitro analyses. For example, using a microarray-based approach, Dey and colleagues[30] demonstrated significant overlap in the transcriptional profiles of bone marrow-derived MSCs and CDCs. Consistent with this, we observed nearly identical cell surface antigenic profiles in MSCs and CDCs with high levels of $CD105^+$ and $CD90^+$, a small population of $cKit^+$ cells, and the absence of CD45 expression at the time of infusion. Nevertheless, since Ki67 and pHH3 expression in icCDC-treated animals remained elevated in animals receiving icMSCs at 4-weeks, it is possible that icCDCs could lead to more prolonged stimulation of myocyte proliferation and superior functional improvement if the comparison was performed beyond one-month.

Taken in the context of previous findings, our results add support to the emerging notion that both allogeneic MSCs and CDCs are safe and promote functional improvement. Previous studies have shown that MSCs and CDCs stimulate endogenous myocyte proliferation[15, 31, 32] and subsequent myocardial regeneration despite limited long-term engraftment of injected cells. In the present study, histological analyses indicated that only ~1 of every 10,000 new myocytes was derived from donor-derived MSCs or CDCs, yet cell-treated animals exhibited elevated markers of myocyte proliferation. Taken together, these data are consistent with the idea that exogenous MSCs and CDCs stimulate endogenous myocyte proliferation, resulting in a significant increase in myocyte number. The cellular mechanism by which endogenous myocyte proliferation is stimulated remains unclear and could not be evaluated in our large animal model. Recent data in rats indicate that a potential candidate for the reparative actions of CDCs is the secretion of microRNA-rich exosomes that, in vitro, enhance angiogenesis, promote myocyte proliferation, and decrease apoptosis[33]. There is also evidence supporting an important role of cell-to-cell contact-dependent CDC stimulation of myocyte proliferation that is mediated by β1 integrin signaling[34]. The requirement of cell-to-cell contact to promote myocardial repair extends to MSCs as well. Hatzistergos et al.[15] demonstrated that intramyocardial injection of MSC-conditioned media (containing secreted growth factors) was not sufficient to replicate the beneficial effects of MSC injection in a porcine infarct model. Based on the limited in vivo data at hand, MSCs and CDCs are likely to facilitate endogenous myocyte repair either via cell-to-cell communication or the prolonged release of paracrine factors or exosomes from cells that are retained in the heart.

Our results also demonstrate that icMSCs and icCDCs can promote functional improvement in the absence of changes in myocardial perfusion in the setting of a chronic coronary stenosis. Indeed, the significant improvement in LAD regional wall thickening in icMSC- and icCDC-treated animals was not accompanied by changes in resting or adenosine-vasodilated perfusion. These findings are consistent with previous results in this model[16, 17] and provide further evidence that interventions that stimulate myocyte proliferation can dissociate the myocardial flow-function relationship by improving function independently of perfusion[35]. The failure of either cell type to produce measurable improvements in myocardial blood flow may relate to their inability to significantly increase arteriolar density. At the same time, both cell types stimulated capillary angiogenesis, which has been used by some as an indirect index of improved tissue perfusion. Increases in capillary number did not lead to measurable changes in resting or vasodilated perfusion in the LAD region supplied by the chronic stenosis since capillaries have a negligible contribution to total coronary vascular resistance. Interestingly, however, increases in capillary density matched increases in myocyte nuclear density with the capillary-to-myocyte ratio maintained after icMSCs and icCDCs. This matching has been described by others and may facilitate tissue oxygen exchange. The concomitant regression of cellular hypertrophy would also improve oxygen transport across the sarcolemma via reversion of the so-called "ischemic core" phenomenon. This postulates that increases in myocyte cross-sectional area limit oxygen delivery to the center of the myocyte[36]. Thus, the decrease in myocyte size as well as increases in capillary density could improve oxygen transport at the cellular level.

The majority of cardiac cell therapy studies have attempted to regenerate myocytes in areas of infarction by administering cells down an infarct-related artery using "stop-flow" intracoronary infusion or to infarct border areas via transendocardial injection. However, our laboratory has demonstrated therapeutic success with global intracoronary infusion under continuous flow. This allows cells to be administered throughout the dysfunctional left ventricle, thus promoting beneficial effects in non-ischemic remote areas of the heart. From a practical standpoint, cell infusion without transient coronary occlusion or the use of transendocardial injection devices could be performed easily in any standard catheterization laboratory, thereby facilitating rapid, widespread clinical implementation. This approach is also safe with a variety of cell types, as demonstrated in the present study, as long as cells are infused prior to extended passage, filtering is performed, and an appropriate dose and rate of infusion are selected. Finally, from a clinical perspective, the amount of cyclosporine used (~3 mg/kg/day) is well below that used in organ transplantation. Although previous studies have demonstrated safety and efficacy of allogeneic MSCs and CDCs without immunosuppression[11, 37], it is possible that short-term, low-dose cyclosporine could delay the immune response to allogeneic cells and enhance functional improvement.

Since the swine model of hibernating myocardium used in the present study is characterized by regional dysfunction of viable myocardium in the absence of scar, our results indicate that global icCDCs and icMSCs promote functional repair independent of any potential effects on infarct size. This finding may be particularly relevant in the clinical translation of intracoronary cell therapy, since many patients exhibit large areas of viable, dysfunctional myocardium with residual perfusion due to contemporary reperfusion strategies that successfully limit infarct size and preserved LV EF[38, 39]. Despite improvements in regional

wall thickening, neither cell type produced significant increases in global LV function. This likely reflects the mild impairment in ejection fraction present at baseline as well as the limitations of M-mode echocardiographic assessment. Importantly, recent data from our laboratory demonstrate that global intracoronary CDC delivery can also improve global function when myocardial scar is present and global EF is reduced[40, 41].

The cardiac cell therapy field has grown rapidly over the past decade, but attempts to translate promising preclinical findings to patients have been met with disappointment. Unfortunately, concerns over experimental design and investigator blinding, coupled with publication bias towards positive results, may deserve blame for some of this. A recent review by Rosen and Myerburg[42] contrasted the positive results of numerous unblinded or uncontrolled studies with neutral findings in randomized, blinded, and appropriately controlled trials. These same issues extend to pre-clinical studies conducted in large animal models of heart disease that serve as the pivotal step in the translation of novel cell-based therapeutic approaches[6]. The present study circumvented these limitations by employing an approach where animals were randomized to one of three treatment arms with all data coded and each investigator, research scientist and technician blinded throughout data collection and all analyses. The codified data used a common repository that was only accessible to the investigator overseeing the statistical analysis. Improvements in myocardial function, as well as the quantitative extent of new myocyte formation, were nearly identical between cell types, indicating that MSCs and CDCs exhibit comparable therapeutic potential. Fortunately, these blinded results confirm our previous findings in unblinded studies demonstrating functional repair of hibernating myocardium following global intracoronary infusion of autologous MSCs and CDCs[16, 17].

Methodological considerations

We used a single infusion with a comparable number of cells for each cell type that has previously demonstrated efficacy in our previous studies but differences in therapeutic efficacy could exist at alternative doses[43]. Our study design did not determine the specific component of the heterogeneous CDC population responsible for their effects. Few studies have administered CDCs fractionated using surface markers but one has suggested $CD105^+/CD90^-/cKit^-$ cells to constitute the active fraction[44]. Thus, we may have found enhanced functional improvement if this sub-population of CDCs had been administered. Like nearly all experimental studies, the donors and recipients of MSCs and CDCs in the present study were relatively young, healthy animals. Thus, clinical translation to patients may still be complicated by the fact that the target population is of advanced age with multiple cardiovascular risk factors that could impact therapeutic efficacy. Although there were not significant differences between groups in any measured variable prior to treatment, remote zone wall thickening tended to be higher (p=0.08) in animals randomized to receive icMSCs, which may have influenced our ability to compare the effects of each cell type on this parameter. In contrast to autologous MSCs[16], MSC-treated animals in the present study did not exhibit an increase in LV mass. This could reflect differences between autologous and allogeneic cells or the impact of blinding, randomization, and the use of concurrently-treated animals. Finally, we found different relative increases in Ki67 labeling vs. pHH3 labeling 4-weeks after cell infusion vs. vehicle-treated controls. This could reflect the time

course of these markers prior to tissue harvesting as well as variations in the time over which cells are Ki67 or pHH3 positive with each treatment. Recent studies have demonstrated that physiological stimuli such as revascularization itself can also stimulate cardiac repair and alter these myocyte proliferation indices[45]. Regardless of the proliferative indices measured at a single time point (which reflect a rate of myocyte division at that point in time), the increase in nuclear density and reduction in myocyte size assessed 4-weeks after treatment reflects the cumulative myocyte increase which was the same with icMSCs and icCDCs.

Conclusions

The present study demonstrates therapeutic equipoise of two contemporary cell therapy platforms in a large animal model of chronic ischemia. Global icMSCs and icCDCs each significantly improved regional contractile function and increased myocyte number throughout the LV without affecting myocardial perfusion. These results occurred despite evidence of limited cell engraftment 1-month after treatment, implicating cell therapy-mediated stimulation of endogenous myocyte regeneration as a primary mechanism of repair for both cell types. Our preclinical study demonstrates the feasibility of implementing a rigorous blinded experimental design that is similar to that employed in single center randomized clinical trials. While the results suggest that it may be difficult to identify therapeutic superiority of either cell type over the other in clinical studies, they support their ability to produce myocyte cellular remodeling before global LV dysfunction and heart failure develop.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Nonstandard Abbreviations and Acronyms

MSCs	Mesenchymal Stem Cells
icMSCs	Intracoronary Mesenchymal Stem Cells
CDCs	Cardiosphere-Derived Cells
icCDCs	Intracoronary Cardiosphere-Derived Cells
LAD	Left Anterior Descending Coronary Artery
LADWT	LAD Wall Thickening

LV	Left Ventricular
Y-FISH	Y-chromosome Fluorescent In Situ Hybridization
pHH3	Phosphorylated Histone-H3

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Novelty and Significance

What Is Known?

- Despite extensive preclinical evidence, clinical efficacy of cardiac cell-based therapeutic approaches remains unclear.

- Most patients with coronary artery disease as well as heart failure have relatively preserved left ventricular systolic function as assessed by ejection fraction.

- In ischemic heart disease, significant myocyte loss and compensatory hypertrophy can develop before global LV dysfunction and heart failure.

What New Information Does This Article Contribute?

- Using a blinded, randomized, vehicle-controlled study design, we found that global intracoronary infusion of allogeneic MSCs and CDCs produced comparable improvements in regional function in swine with chronic myocardial ischemia and hibernating myocardium.

- Both icCDCs and icCDCs increased capillary density, myocyte nuclear density and reduced myocyte cellular hypertrophy.

- Few cells were derived from allogeneic cell transplants, clearly implicating activation of endogenous repair mechanisms in icCDCs and icMSCs treated animals.

- The beneficial effects of both cell therapies occur in ischemic as well as remote regions of the heart at a time when reductions in ejection fraction and heart failure are absent.

In this study, using a rigorous, clinical trial-based study design, we demonstrate therapeutic equipoise of two therapies in a large animal model of chronic ischemia associated with myocyte cellular loss and hypertrophy in the absence of infarction. We found that improvements in regional function were accompanied by substantial reverse myocyte remodeling with increases in myocyte number and reductions in myocyte size. The ability of both of these therapies to replace myocytes lost from the stress of chronic repetitive ischemia raises the possibility that treating the entire heart to prevent the development of LV dysfunction and ischemic cardiomyopathy may be feasible. Strategies to prevent the development of heart failure earlier in the course of the disease may potentially have more impact than reversing dysfunction in the advanced failing heart.
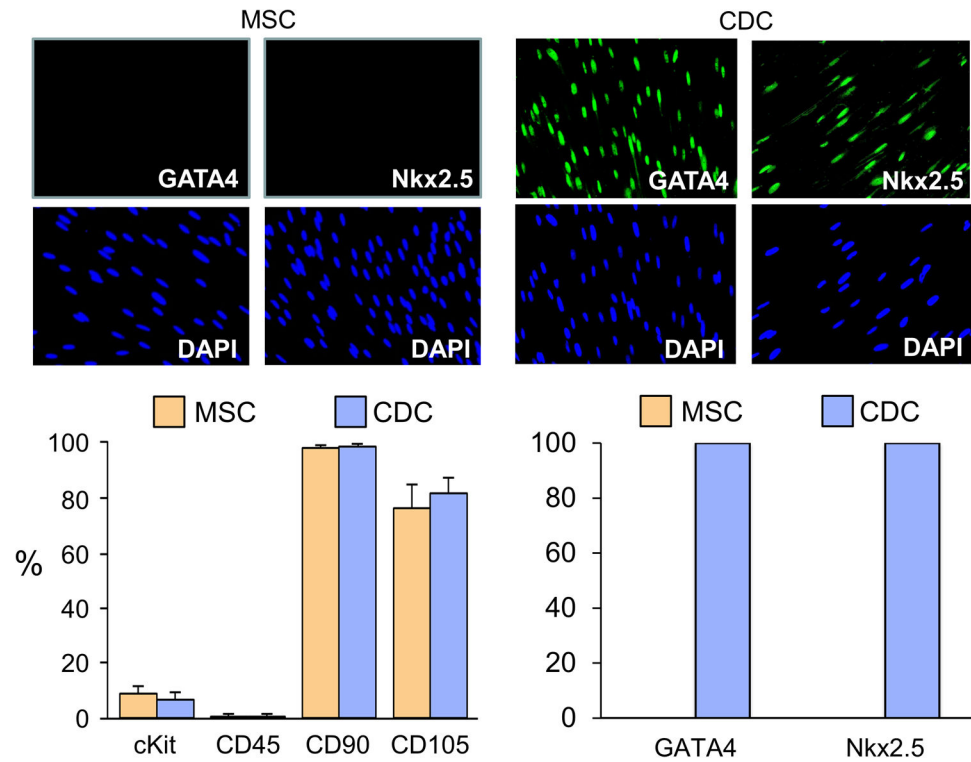
Figure 1. Characterization of Porcine Allogeneic MSCs and CDCs

Summary of MSC and CDC characterization by flow cytometry and immunohistochemistry (n=9 each cell type; corresponding with cell donor and passage at the time of each cell injection). The antigenic profile was similar between cell types, as MSCs and CDCs were each predominately CD90^{+}, CD105^{+} and CD45^{−}, with a small number of cKit^{+} cells (lower left figure). In contrast, CDCs exhibited uniform expression of the cardiac transcription factors GATA4 and Nkx2.5, demonstrating their commitment to a cardiac lineage, while MSCs did not express GATA4 or Nkx2.5 (top image and lower right figure).
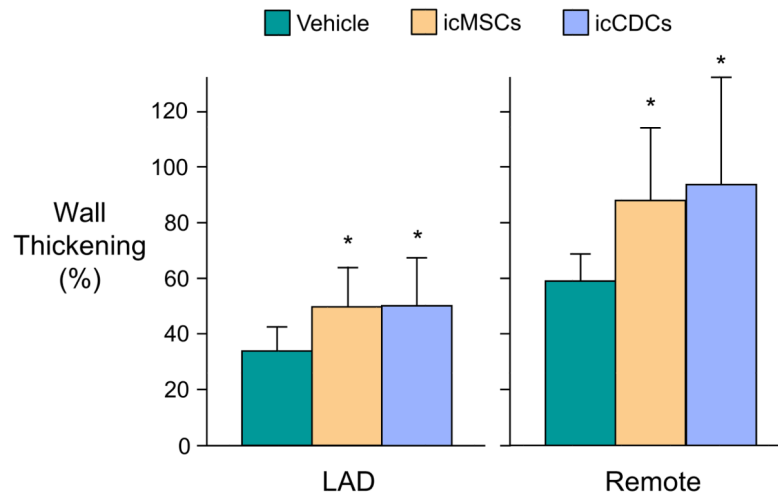
Figure 2. Global icMSCs and icCDCs Elicit Comparable Improvements in Regional Wall Thickening Throughout the Left Ventricle

One-month after treatment, icMSC- and icCDC-treated animals exhibited significantly greater wall thickening in the ischemic LAD and non-ischemic remote regions of the LV compared vs. vehicle-treated controls. *p<0.05 vs. Vehicle.
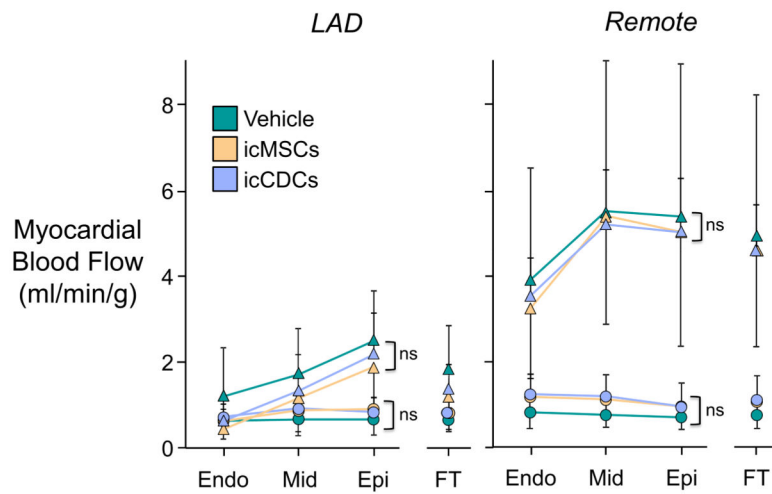
Figure 3. Myocardial Perfusion at Rest and During Adenosine Vasodilation is Not Affected by Global icMSCs or icCDCs

Swine with hibernating myocardium exhibited a significant reduction in myocardial blood flow at rest (circles) and during adenosine vasodilation (triangles) in the ischemic LAD region (left panel) compared with non-ischemic remote myocardium (right panel). There was no significant change in animals treated with icMSCs or icCDCs. Endo: subendocardium; Mid: midmyocardium; Epi: subepicardium; FT: full-thickness
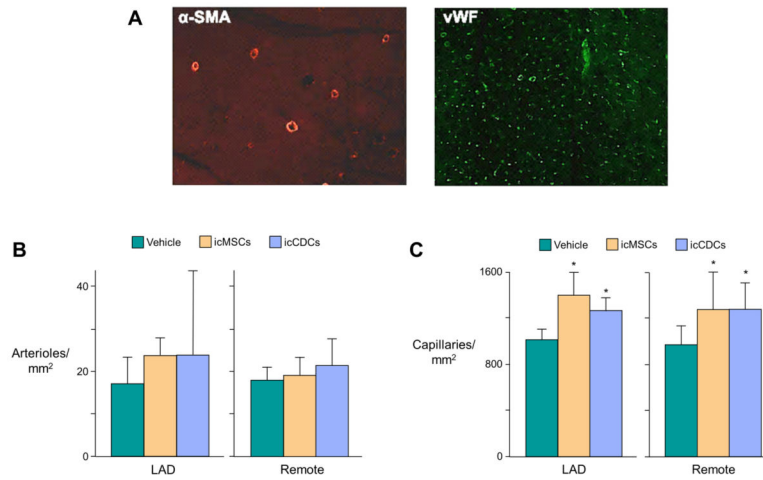
Figure 4. Global icMSCs and icCDCs Promote Capillary Angiogenesis but Do Not Affect Arteriolar Density

(A) Representative images of α-smooth muscle actin-positive arterioles (left panel) and von-Willebrand factor-positive capillaries (right panel). **(B)** Arteriolar density was not significantly different among icMSC, icCDC, or vehicle-treated animals. **(C)** Global intracoronary cell infusion increased capillary density throughout the LV compared with vehicle-treated controls. There was no difference between icMSCs and icCDCs. *p<0.05 vs. Vehicle
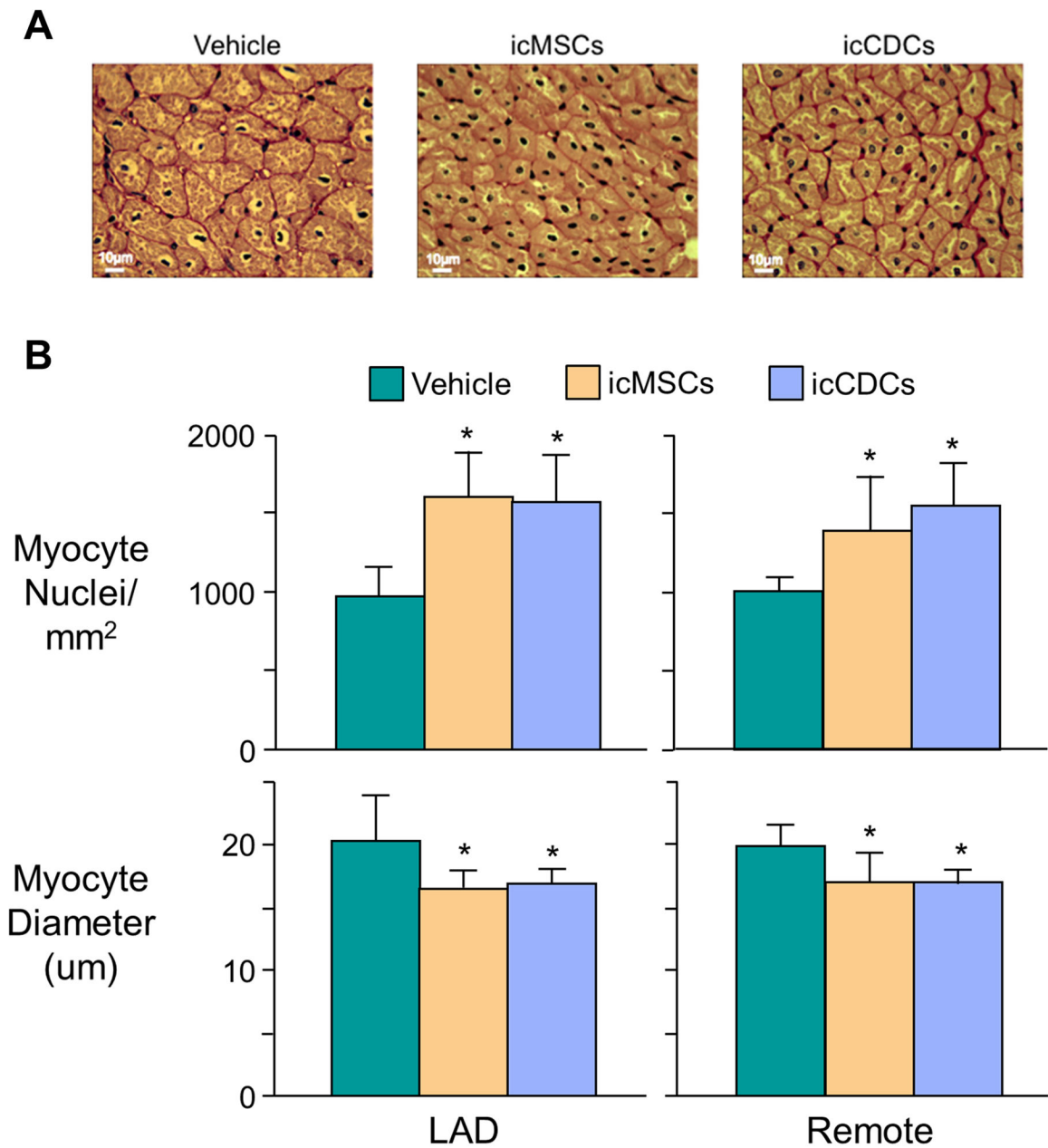
Figure 5. Global icMSCs and icCDCs Increase Myocyte Nuclear Density and Reduce Myocyte Diameter in LAD and Remote Myocardium

(A) PAS-stained sections from the ischemic LAD region. (B) Both icMSCs and icCDCs produced a significant increase in myocyte nuclear density in the ischemic LAD and normally-perfused remote regions of the left ventricle. These changes were accompanied with reductions in myocyte diameter (bottom panel). *p<0.05 vs. Vehicle.
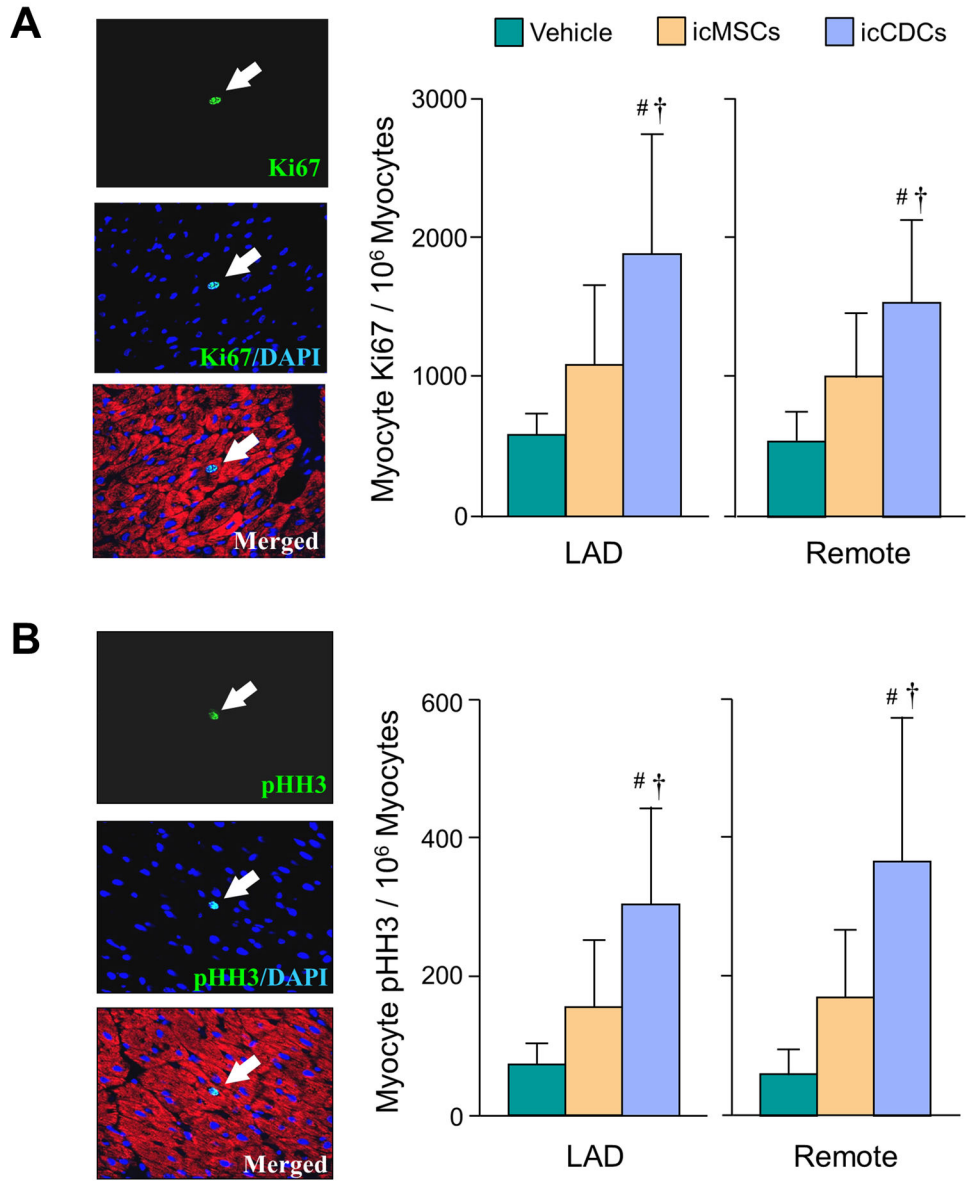
Figure 6. Myocyte Proliferative Indices
(A) Myocytes in the growth phase of the cell cycle one-month after treatment were quantified with Ki67 (arrow). Ki67-positive myocytes were higher after icCDC treatment as compared to icMSCs and Vehicle-controls. **(B)** Mitotic myocytes were quantified with phospho-histone H3 (pHH3, arrow). Mitotic myocytes were higher after icCDCs vs icMSCs and Vehicle-controls. *p<0.05 vs. Vehicle; †p<0.05 vs. icMSCs.
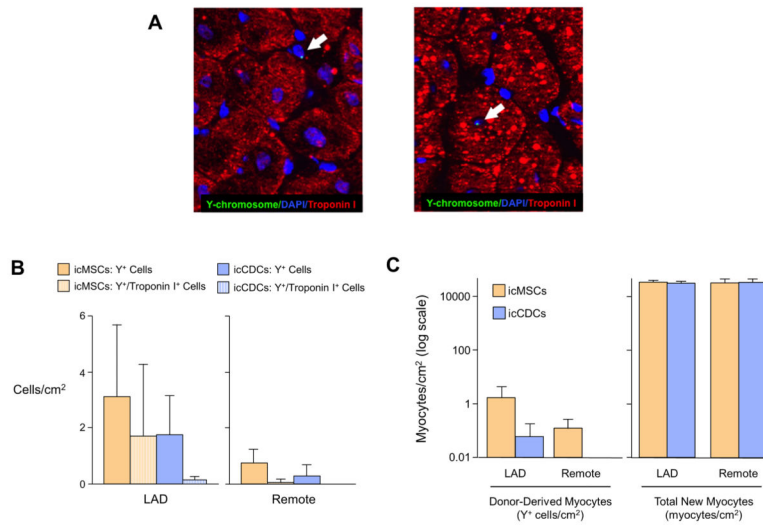
Figure 7. The Majority of New Myocytes Derived from Global icMSCs and icCDCs Arise From Endogenous Sources

(A) Y-chromosomes were detected by Y-FISH. Y-FISH positive cells were primarily found in the interstitial space (left panel) with rare examples of Y$^+$ cardiac myocytes (right panel). **(B)** The frequency of Y$^+$ cells was low but similar in icMSC-treated and icCDC-treated animals. Rare Y$^+$ cells also expressed Troponin I (hatched bars), indicative of cardiomyocyte differentiation. **(C)** The number of new myocytes differentiating from icMSCs and icCDCs was very small in comparison to the estimated increase in myocytes based on increases in myocyte nuclear density, implicating endogenous recipient cells as the primary source of new myocyte formation.