Mesenchymal stem cells (MSCs) can repair a variety of tissues after injury\(^1\) and are currently being used in clinical trials to treat patients with cardiovascular disease.\(^2\) Nevertheless, their precise mechanism of action and the role of myocyte regeneration versus angiogenesis are controversial. Most preclinical investigation has focused on animal models of myocardial infarction in which intravenous, intramyocardial, and intracoronary administration of MSCs has been demonstrated to reduce infarct size and improve left ventricular (LV) function in both acute and chronic infarction.\(^3–13\) Although early studies demonstrated the ability of autologous and allogeneic MSCs to differentiate into vessels,\(^5,14\) smooth muscle,\(^14\) and cardiac muscle,\(^15,16\) the quantitative extent of MSC differentiation into a cardiac cellular phenotype has been small and inconsistent with measured reductions in infarct volume.\(^5,8,11\) Recent investigations have hypothesized that MSCs effect cardiac repair through a paracrine mechanism that stimulates proliferation of endogenous myocytes, but in vivo studies quantifying the magnitude of myocyte proliferation are limited. Most analyses focus on the small rim of border zone tissue between normal and infarcted myocardium rather than larger remote regions or areas at risk of ischemia.\(^5,9,17–22\)

Recently, we demonstrated that pravastatin can mobilize cKit\(^+\) and CD133\(^+\) bone marrow progenitor cells (BMPCs).\(^23\) The BMPCs localized in the heart and were accompanied by improved myocardial function in swine, with hibernating myocardium devoid of infarction in the absence of an

**Rationale:** Mesenchymal stem cells (MSCs) improve function after infarction, but their mechanism of action remains unclear, and the importance of reduced scar volume, cardiomyocyte proliferation, and perfusion is uncertain.

**Objective:** The present study was conducted to test the hypothesis that MSCs mobilize bone marrow progenitor cells and improve function by stimulating myocyte proliferation in collateral-dependent hibernating myocardium.

**Methods and Results:** Swine with chronic hibernating myocardium received autologous intracoronary MSCs (icMSCs; \(\approx 44 \times 10^6\) cells, \(n=10\)) 4 months after instrumentation and were studied up to 6 weeks later. Physiological and immunohistochemical findings were compared with untreated hibernating animals (\(n=7\)), sham-normal animals (\(n=5\)), and icMSC–treated sham-normal animals (\(n=6\)). In hibernating myocardium, icMSCs increased function (percent wall thickening of the left anterior descending coronary artery \(24 \pm 4\% – 43 \pm 5\%\), \(P<0.05\)), although left anterior descending coronary artery flow reserve (adenosine/rest) remained critically impaired (1.2 \pm 0.1 versus 1.2 \pm 0.1). Circulating cKit\(^+\) and CD133\(^+\) bone marrow progenitor cells increased transiently after icMSC administration, with a corresponding increase in myocardial cKit\(^+\)/CD133\(^+\) and cKit\(^+\)/CD133\(^–\) bone marrow progenitor cells (total cKit\(^+\) \(223 \pm 49–4415 \pm 866/10^6\) cardiomyocytes, \(P<0.05\)). In hibernating hearts, icMSCs increased Ki67\(^+\) cardiomyocytes (\(410 \pm 83-2460 \pm 610/10^6\) nuclei, \(P<0.05\)) and phospho-histone H3–positive cardiomyocytes (\(9 \pm 5-116/12/10^6\) nuclei, \(P<0.05\)). Myocyte nuclear number (from 75 \(336 \pm 5037\) to 114 \(424 \pm 9564\) nuclei/mm\(^3\), \(P<0.01\)) and left ventricular mass (from 2.5 \pm 0.1 to 2.8 \(\pm 0.1\) kg, \(P<0.05\)) increased, yet myocytes were smaller (14.5 \pm 0.4 versus 16.5 \pm 0.4 \(\mu m\), \(P<0.05\)), which supports endogenous cardiac myocyte proliferation. In sham-normal animals, icMSCs increased myocardial bone marrow progenitor cells with no effect on myocyte proliferation or regional function.

**Conclusions:** Our results indicate that icMSCs improve function in hibernating myocardium independent of coronary flow or reduced scar volume. This arises from stimulation of myocyte proliferation with increases in cKit\(^+\)/CD133\(^+\) bone marrow progenitor cells and cKit\(^+\)/CD133\(^–\) resident stem cells, which increase myocyte number and reduce cellular hypertrophy. (Circ Res. 2011;109:00-00.)

**Key Words:** mesenchymal stem cells ■ hibernating myocardium ■ bone marrow progenitor cells
increase in myocardial perfusion. Although BMPC mobilization had no effect on myocyte proliferation in the normal heart, it increased the frequency of myocytes in the proliferative phase of the cell cycle in diseased hearts and increased myocyte nuclear density with small myocytes that suggested that endogenous myocyte proliferation was stimulated. Likewise, studies with skeletal muscle injection of porcine MSCs into Syrian hamsters and MSCs that overexpressed insulin-like growth factor-1 and Akt in infarcted animals support the notion that paracrine factors can mobilize BMPCs and potentially facilitate cardiac repair.

On the basis of these observations, the known secretion of a variety of growth factors from MSCs, and the demonstrated ability of selected growth factors to mobilize BMPCs, we tested the hypothesis that MSCs stimulate myocyte proliferation through a similar mechanism. To more definitively ascertain the effects of MSCs on angiogenesis and myocyte regeneration independent of infarct volume, we used an in vivo model of hibernating myocardium. In this setting, chronic dysfunction arises from repetitive ischemia in collateral-dependent myocardium, and regional myocyte loss with compensatory cellular hypertrophy develops in the absence of infarction. Thus, changes in regional perfusion and increases in regional myocyte number can be used to assess the regenerative effects of cell-based therapy and exclude preservation of myocytes that arise from inhibition of cell death. We used autologous MSCs expanded ex vivo to circumvent tissue incompatibility with xenogenic or unmatched allogenic cells. Our results in a model devoid of infarction indicate that intracoronary administration of MSCs (icMSCs) can improve function in hibernating myocardium independent of increased perfusion. icMSCs stimulate myocytes to reenter the cell cycle in the diseased but not the normal heart and effect substantial myocyte proliferation and mobilization of endogenous BMPCs.

Methods

Procedures and protocols conformed to institutional guidelines for the care and use of animals in research. Detailed experimental and histological protocols (available in the Online Data Supplement at http://circres.ahajournals.org) have been described previously.

Effects of icMSCs on Flow and Function in Hibernating Myocardium (n = 17)

Pigs were chronically instrumented with a 1.5-mm Delrin stenosis on the proximal left anterior descending coronary artery (LAD) as described previously (online Data Supplement). We performed 2 baseline physiological studies 3 and 4 months after instrumentation to establish that stable reductions in LAD wall thickening and resting LAD perfusion were present before icMSC injection (Online Figure I). Under propofol sedation, a Millar catheter was inserted into the LV for microsphere injection. Regional wall thickening was assessed with echocardiography. Microsphere flow was assessed at rest and after adenosine vasodilation. At the 4-month study, animals received icMSCs (44 × 10⁶, n = 10) and were studied 2 weeks (n = 6) or 6 weeks (n = 4) later. In 4 animals, MSCs were transfected with AdvEGFP to determine myocardial differentiation. After physiological measurements, the heart was excised for flow and histological analyses. Results were compared with untreated animals with hibernating myocardium (n = 7).

Mobilization of BMPCs With icMSCs (n = 6)

We assessed the ability of icMSCs to mobilize BMPCs (cKit⁺ and CD133⁺ mononuclear cells) in normal swine as detailed in the online Data Supplement. Baseline measurements in peripheral blood (30 mL) were compared with those 3 days and 2 weeks after administration of icMSCs. Data from fluorescent activated cell sorter analysis are expressed as CD133⁺ or cKit⁺ cells per 1 million mononuclear cells.

Myocardial Histopathology

Histopathology in animals that received MSCs was compared with untreated animals. We assessed the temporal evolution of myocardial proliferation by evaluating animals studied at 2 and 6 weeks after icMSC administration compared with untreated animals with hibernating myocardium. We also compared the effects of icMSCs in sham-normal animals (n = 6) to that in untreated sham-normal animals (n = 5). To determine whether increases in BMPCs were caused by microembolization, we evaluated myocardial progenitor cell levels in normal swine (n = 6) in which MSCs were infused into the distal aorta. The histopathologic and immunohistochemical techniques have been presented previously and are discussed in the online Data Supplement.

Myocyte Nuclear Density and Morphometry

Samples from hibernating LAD and normal remote regions were fixed and paraffin embedded. Trichrome-stained sections were used to quantify connective tissue. Periodic acid-Schiff–stained sections were used to quantify myocyte diameter, nuclear length, and regional myocyte nuclear density.

Quantitative Analysis of Myocyte Proliferation and Myocardial cKit⁺ and CD133⁺ Cells

To identify myocyte proliferation, anti-Ki67 and anti-phospho-histone H3 staining were performed. BMPCs (cKit⁺/CD133⁺) were quantified by confocal immunofluoresence (online Data Supplement). Preliminary studies demonstrated that tissue CD133⁺ and cKit⁺ cells were CD45⁻. These were co-stained with GATA-4, Nkx2.5, and cardiac troponin I antibodies to determine the extent to which each population differentiated into a cardiac lineage.

Statistical Analysis

Data are expressed as mean ± SE. Differences between groups were assessed by 2-way ANOVA and the post hoc Holm-Sidak test. Temporal physiological changes between initial and final studies were assessed by paired t tests with each animal used as its own control. For all comparisons, P < 0.05 was considered significant.

Results

All animals were in good health at the time of study. Baseline studies performed at 4 months confirmed dysfunctional hibernating myocardium (n = 17). Regional LAD wall thickening (LAD%WT) was reduced compared with normal remote regions (24% ± 2% versus 64% ± 4%, P < 0.05), with reductions in resting perfusion (LAD 0.72 ± 0.06 versus 0.94 ± 0.05 mL/min/g in remote regions, P < 0.01). Flow during adenosine was severely attenuated (LAD 1.22 ± 0.22 versus 4.47 ± 0.25 mL/min/g in remote regions, P < 0.001). Although survival in this animal model is limited by sudden...
administration, regional LAD wall thickening increased significantly, which indicates the absence of functional collateral development. Whereas coronary flow reserve remained critically impaired, which increased after iCMSC administration. Increases in LAD%WT were significant in animals studied 2 weeks (n=6) and 6 weeks (n=4) after iCMSC administration.

**Effects of iCMSCs on Flow and Function**

Serial physiological studies before iCMSCs confirmed a response to adenosine. There was no spontaneous improvement in flow or function in untreated animals (n=7). After iCMSC administration, regional LAD wall thickening increased significantly, whereas coronary flow reserve remained critically impaired, which indicates the absence of functional collateral development. Increases in LAD%WT were significant in animals studied 2 weeks (n=6) and 6 weeks (n=4) after iCMSC administration.

Death, it was similar in each group, with 10 of 13 animals that received iCMSCs surviving (77% survival rate) versus 7 of 10 untreated swine (70% survival rate). No sham-normal pigs that received MSCs died.

TTC staining did not demonstrate infarction. Increases in LAD interstitial connective tissue were similar in pigs that received iCMSCs versus those that were untreated (LAD 8.6±0.9% versus 6.6±1.4%, P=NS; remote 5.2±0.5% versus 4.1±0.8%, P=NS). When iCMSCs were transfected with Ad-VEGF, EGFP+ cardiac cells were too rare to quantify, and staining was restricted to endothelium and smooth muscle (data not shown). We did not identify any EGFP+ cardiac myocytes.

**Increased BMPCs After MSCs**

Circulating mononuclear cells were not altered after iCMSC administration (from 4395±842 cells/μL to 3881±788 cells/μL after iCMSC administration, P=NS), but selected BMPC populations were transiently mobilized. Three days after iCMSC administration, circulating cKit+ and CD133+ cells increased 4-fold and remained significantly elevated at 2 weeks (Online Figure III). Corresponding increases in tissue cKit+/CD45− (Figure 2) and CD133+/CD45− (Figure 3) cells occurred in hibernating and sham-normal myocardium. After 6 weeks, CD133+ cells returned to control levels, but cKit+ cells remained elevated. Cointaining with GATA-4 and Nkx2.5 (Figure 4) demonstrated that a small percentage (<5%) of myocardial cKit+ and CD133+ cells were differentiating into a vascular or cardiac muscle lineage, and these increased after iCMSC administration. Although there was no troponin I detected in cKit+ cells in untreated hibernating myocardium, cointaining after iCMSC administration was present in 5.6±1.9% of the cKit+ cells (n=6). Colocalization of cKit and von Willebrand factor (n=4) that identified endothelial commitment occurred in 33±2% of the cKit+ cells.

An estimate of the number of cKit+ cells that were resident cardiac stem cells versus CD133+ BMPCs was obtained by dual staining for both cKit and CD133. When assessed 2 weeks after iCMSC administration, 41±5% of the cells were cKit+/CD133+, and the remainder were cKit+/CD133−. Because cKit+ resident cardiac stem cells are negative for the hematopoietic surface antigen CD133, this colocalization supports the notion that nearly half of the cKit+ cells initially came from sources other than the resident cardiac stem cell pool. In contrast, after 6 weeks, CD133+ cells returned to baseline. The sustained increase in cKit+ cells could reflect resident cardiac stem cells or cKit+ BMPCs that remained in the myocardium after mobilization by iCMSCs.

We infused MSCs into the distal aorta to determine whether increases in cKit+/CD133+ cells were related to myocardial microembolization (supplemental Figure IV). Aortic MSC infusion increased myocardial cKit+ cells from 8±3×103 to 393±82×103 cells/cm3 versus 819±209×103 cells/cm3 after iCMSC administration (both P<0.05 versus untreated). Likewise, myocardial CD133+ cells increased from 5±2×103 to 366±109×103 cells/cm3 versus 234±64×103 cells/cm3 after iCMSC administration (both P<0.05 versus untreated). Thus, increases in myocardial cKit+/CD133+ progenitor cells did not require intracoronary injection or microembolization of MSCs in the heart.

**Autologous iCMSCs Stimulate Cardiomyocytes to Reenter the Cardiac Cell Cycle**

Although iCMSCs labeled with EGFP did not differentiate into cardiomyocytes, they had a profound effect on stim-
ulating endogenous myocyte proliferation in diseased but not sham-normal hearts. Figure 5 summarizes the effects of icMSCs on myocyte nuclear Ki67 staining, a marker of proliferation. A single dose of icMSCs increased Ki67+ myocyte nuclei in LAD and in remote regions of dysfunctional hearts for up to 6 weeks (LAD 2234±892 versus 410±82 nuclei/10^6 myocyte nuclei, P<0.05). There was also a sustained increase in myocyte phospho-histone H3 staining (LAD 213±16 versus 9±5 nuclei/10^6 myocyte nuclei, P<0.05; Figure 6). In contrast, icMSCs did not increase Ki67 or phospho-histone H3–positive myocytes in sham-normals. Thus, increased cardiomyocyte proliferation after icMSC administration was restricted to the diseased heart.

Figure 2. Myocardial cKit+ cells are persistently increased after icMSC administration. The confocal microscopic images on the left show a cluster of cKit+ cells, but they were frequently seen singly throughout the heart. Autologous icMSCs increased cKit+ cells in dysfunctional LAD regions and remote normally perfused regions of hibernating hearts, as well as in sham-normal hearts. Although the levels of cKit+ cells declined 6 weeks after icMSC administration, they remained significantly elevated over untreated animals. There was dissociation in the temporal course of circulating cKit+ cells (returning to baseline after 2 weeks; Online Figure III) and myocardial cKit+ cells, which suggests stimulation of resident cardiac cKit+ cells or long-term retention of circulating BMPCs in the heart. TOPRO3 indicates nuclear stain.

Figure 3. Myocardial CD133+ cells are transiently increased after icMSC administration. Confocal images of a CD133+ cell cluster are shown on the left, but these were more often seen singly throughout the heart. Autologous icMSCs resulted in a marked increase in myocardial CD133+ cells in tissue harvested 2 weeks after administration. After icMSC administration, CD133+ cells increased in dysfunctional LAD regions and remote regions of hibernating animals, as well as in sham-normals. In hibernating tissue harvested 6 weeks after injection, CD133+ cell levels had returned to pretreatment controls. Thus, increases in myocardial CD133+ cells were transient.
Impact of Proliferative Markers on Angiogenesis, Myocyte Numbers, LV Mass, and Myocyte Diameter

Although coronary flow reserve did not change, icMSCs stimulated angiogenesis by increasing capillary density (from $1172/\mu m^2$ in untreated animals to $1441/\mu m^2$ at 2 weeks and $1383/\mu m^2$ at 6 weeks, both $P<0.05$ versus untreated animals; Figure 7). To assess the quantitative impact of myocyte proliferative markers on myocyte regeneration in vivo, we also quantified myocyte nuclear density (Figure 8). As reported previously,27 LAD myocyte nuclear density was reduced, with compensatory myocyte cellular hypertrophy in hibernating LAD regions of untreated animals. After icMSC administration, there was a progressive increase in myocyte nuclear density that became significantly higher than in untreated animals after 6 weeks (1016/\mu m^2 versus 830/\mu m^2 myocyte nuclei/\mu m^2, $P<0.05$). A similar trend was found in remote regions, although the differences did not reach statistical significance (1219/\mu m^2 versus 1027/\mu m^2 myocyte nuclei/\mu m^2). The calculated myocyte nuclear number per cubic millimeter was even larger (114 424 myocyte nuclei/mm^3 after icMSC administration versus 75 336 myocyte nuclei/mm^3 in untreated myocardium, $P<0.01$) and was accompanied by an increase in postmortem LV mass-to-body weight ratio after icMSC administration (2.8±0.1 g/kg after icMSC administration versus 2.5±0.1 g/kg in untreated animals, $P<0.05$). Importantly, the increase in LV mass was not accompanied by cellular hypertrophy, because myocyte diameter actually decreased after icMSC administration (LAD 16.0±0.4 μm to 14.5±0.4 μm after icMSC administration, $P<0.05$; Figure 8). Collectively, the increases in nuclear number and LV mass along with the reduced myocyte cell size are all consonant with increased myocyte proliferation after icMSC administration.

Autologous icMSCs Upregulate Chemokines

To ascertain potential paracrine factors responsible for the effects of icMSCs on myocyte proliferation, we quantified the myocardial expression of stromal cell–derived factor-1, monocyte chemotactic protein-1, and vascular endothelial growth factor 2 weeks after icMSC administration. In hibernating myocardium, icMSCs significantly increased stromal cell–derived factor-1 (2.1±0.2-fold, $P<0.05$ versus untreated) and monocyte chemotactic protein-1 (2.5±0.7-fold, $P<0.05$ versus untreated) but not vascular endothelial growth factor (1.1±0.1-fold, $P=NS$). Expression was not altered in sham-normal animals that received icMSCs (Online Figure V).

Discussion

There are several novel and important new findings from the present study. First, although autologous icMSCs do not
measurably differentiate into cardiac myocytes in vivo, they mobilize BMPCs, which leads to increased tissue levels of cKit+/CD133+ and cKit+/CD133− cells in normal and diseased myocardium. Second, in dysfunctional hearts with preserved perfusion, icMSCs stimulate cardiomyocytes to reenter the proliferative phase of the cell cycle, as reflected by increased Ki67+ and phospho-histone H3–positive myocytes. This is sustained for at least 6 weeks and results in substantial myocyte regeneration, as documented by significant increases in myocyte nuclear number, a reduction in cellular hypertrophy, and an increase in LV mass. Although capillary neoangiogenesis occurs, there is no improvement in coronary flow reserve. The findings support the central role of myocyte regeneration and regression of cellular hypertrophy in functional improvement, because this model is devoid of heart failure, infarction, and ongoing cell death.

Figure 5. icMSCs increase Ki67+ myocytes in hibernating myocardium. Left, Confocal photomicrograph represents Ki67+ nuclear staining (green) that is localized in a myocyte nucleus surrounded by cardiac troponin I (red). There was no alteration in Ki67+ myocytes in sham-normal myocardium after icMSC administration (left graph). In hibernating myocardium (right graph), Ki67+ myocytes increased 2 weeks after MSCs and persisted up to 6 weeks.

Figure 6. icMSCs increase phospho-histone H3–positive myocytes in hibernating myocardium. Left, Confocal images represent phospho-histone H3–positive nuclear staining (green) surrounded by cardiac troponin I (red). There was no effect of icMSCs on phospho-histone H3 in sham-normals (left graph). In contrast, icMSCs increased the number of myocytes in the mitotic phase of the cardiac cycle in hibernating myocardium, and this persisted up to 6 weeks after injection (right graph).
Effect of icMSCs on Flow, Function, Myocyte Number, and LV Mass

Previous studies have fairly consistently demonstrated the ability of a variety of MSC formulations (autologous, allogenic, and xenogenic) to improve myocardial function in animal models of infarction. Although intramyocardial MSC injection variably reduces infarct size and improves function, the mechanisms of action have remained unclear. Existing studies have largely restricted histological analysis of myocyte regeneration to the thin rim of border zone tissue, and thus, the magnitude of myocyte regeneration in the heart has not been quantified. Although some studies demonstrate that MSCs can differentiate into myocytes, other studies are consistent with our observations showing that MSC differentiation into a cardiac lineage is a rare phenomenon, with only vascular cells colabeled with MSCs transfected with AdvEGFP. Although this approach may have underestimated the retention and differentiation of MSCs into a cardiac phenotype, previous experiments using DAPI-labeled MSCs demonstrated that only 2% of icMSCs were retained in the heart after 2 weeks. A similar low retention has been demonstrated for other cell types through intracoronary or direct myocardial injection, including cardiac stem cells, embryonic stem cells, CD133+ cells, and MSCs. Thus, the rare transdifferentiation of MSCs into a cardiac phenotype reported by others cannot explain the substantial increase in cardiomyocytes and regression of cellular hypertrophy in our model.

Many laboratories have found MSCs to increase angiogenesis in infarct border zones, but their effect on maximal perfusion is unknown. Interpretation of capillary density surrounding an infarct is difficult, because MSC-mediated preservation of existing myocardium would preserve capillary density without vascular regeneration. Because our model of collateral-dependent hibernating myocardium was devoid of infarction and apoptosis when studies began, we were able to examine a large, homogeneously perfused dysfunctional region and dissociate the effects of icMSCs on vascular remodeling and myocyte proliferation from these confounding effects. We found that icMSCs stimulated capillary neoangiogenesis. Interestingly, the relative increase in capillary density in hibernating myocardium was closely matched to the increase in myocardial nuclear density after icMSC administration, which suggests that the capillary density–myocyte density ratio was maintained as myocytes

Figure 7. icMSCs increased capillary density in hibernating myocardium. Capillary density was quantified with von Willebrand factor (vWF). Upper photomicrographs show vWF staining (green) from hibernating myocardium. Animals that received icMSCs increased capillary density at 2 and 6 weeks vs untreated animals (both P<0.05 vs untreated; lower graph). Although icMSCs stimulated capillary angiogenesis, there was no functional improvement in coronary collateral resistance, because myocardial perfusion at rest and vasodilation did not change.

Figure 8. icMSCs increase LAD myocyte nuclear number and regressed cellular hypertrophy in hibernating myocardium. To determine whether the increased number of myocytes in the cell cycle led to pathological evidence of repair, we quantified myocyte nuclear density and derived myocyte number using nuclear length. icMSCs did not alter myocyte nuclear density, number, or myocyte size in sham-normal animals (left graph). In untreated hibernating myocardium, LAD myocyte nuclear density was reduced, with an increase in myocyte diameter and reduction in myocyte number. After icMSC administration, there was a progressive increase in LAD myocyte nuclear density and number (right graph). This was accompanied by a reduction in myocyte diameter and a large increase in the calculated number of myocytes, which approached normal after 6 weeks. These data indicate substantial myocyte regeneration in an experimental model that is devoid of ongoing cell death.
proliferated. Nevertheless, because capillaries contribute little resistance to perfusion, there was no significant increase in coronary flow during vasodilation, which indicates that there was no proliferation of functional coronary resistance vessels supplying the collateral-dependent region. The lack of effect on functional collateral flow is similar to findings after pharmacological mobilization of BMPCs by use of pravastatin in a similar model. This supports a primary myocyte mechanism for the improvement in regional wall thickening and indicates that capillary density changes are not a reliable surrogate measure of myocardial perfusion.

Multiple mechanisms are likely operative in producing improvements in function independent of myocardial perfusion. We have demonstrated that sarcoplasmic reticulum calcium handling proteins and a broad array of mitochondrial proteins are downregulated in chronic hibernating myocardium. Regression of cellular hypertrophy attendant with MSC-mediated myocyte proliferation would presumably restore many of the contractile and metabolic pathways altered in the hypertrophied myocytes. Other investigators have demonstrated that hypertrophied myocytes have reductions in contractile function in remodeled myocardium that reflect myofilament alterations and are independent of perfusion. It is also possible that MSCs or their paracrine mediators provide protection of the hibernating region against repetitive ischemia-induced stunning, which may contribute to a component of dysfunction in hibernating myocardium. Further studies will be required to evaluate these potential mechanisms in detail.

Sources of New Cardiomyocytes and Myocyte Turnover
Cardiomyocytes are capable of self-renewal through proliferation from an endogenous cKit+ progenitor cell population. Convincing experimental evidence suggests that self-renewal is an ongoing process, with myocyte turnover increasing with aging and with structural heart disease. Self-renewal is critical to maintain myocyte number in the face of continuous loss from even low rates of apoptosis. Other studies support the notion that BMPCs can populate the heart and adopt a cardiomyogenic phenotype as a source of myocyte renewal. The present results indicate that icMSCs are capable of increasing the cKit+/CD133+ progenitor cell pool and, in contrast to MSCs administered via intramyocardial injection, do so throughout the entire heart. The downstream development of myocytes, however, appears to be regulated by endogenous substrate factors that are unique to the diseased heart, because MSC-mediated increases in cKit+ and CD133+ cells do not promote mitosis (phospho-histone H3) or proliferation of normal cardiomyocytes. Because myocyte apoptosis was similar to that in normal hearts at the time we began our studies, increases in myocyte nuclear density after icMSC administration reflected cell regeneration. In contrast, MSC-mediated inhibition of cell death plays an important role in preserving myocytes in infarct or heart failure models.

A key question is whether the significant increase in myocyte nuclear density that occurs after icMSC administration in hibernating hearts is the result of resident cKit+ cardiac stem cells differentiating into adult myocytes, differentiation of cKit+/CD133+ BMPCs into cardiac myocytes, or stimulation of adult cardiomyocytes to reenter the cell cycle and divide. The present data support any or all of these mechanisms. For example, after 2 weeks, we demonstrated that subpopulations of cKit+ cells and CD133+ cells coexpressed GATA4, Nkx2.5, or troponin I, which supports their potential to differentiate into cardiomyocytes. After 6 weeks, however, CD133+ cells returned to baseline levels, and only cKit+ cells remained elevated. This could reflect the fact that the CD133+ cells have replenished the cardiac niche, as suggested by others using labeled bone marrow transplantation experiments in rodents, or it could indicate persistent stimulation of the cardiac stem cell pool to proliferate. Regardless of origin, the number of cKit+ cells coexpressing cardiomyocyte markers (17 cells/cm²) was lower than troponin I–positive, phospho-histone H3–positive myocytes (24 cells/cm²) or Ki67+ cardiomyocytes (350 cells/cm²). All of these were much lower than the cumulative increase in myocyte nuclear density (18 600 myocytes/cm² at 6 weeks, an average increase of 440 myocyte nuclei/cm² per day). Definitively quantifying the contribution of each of these potential sources to newly regenerated myocytes is not currently possible in the porcine model.

MSCs Increase cKit+/CD133+ and cKit+/CD133– Cells in the Myocardium
Collectively, the present results identify a novel mechanism whereby icMSCs transiently increase myocardial cKit+/CD133+ BMPCs, which is transiently followed by a persistent increase in cKit+/CD133– resident stem cells. Because CD133+ cells are not present in the normal heart, the former supports the possibility of a bone marrow origin of the progenitor cells involved in cardiac repair. On the other hand, the presence of cKit+/CD133– cells is consonant with a recent study demonstrating that intramyocardial injection of MSCs stimulated the proliferation of cKit+ cells in infarct border zones. In contrast to a localized effect at the border between normal and infarcted tissue, the intracoronary administration of MSCs produced myocyte proliferation throughout the heart and reduced cellular hypertrophy. Whether a similar benefit would occur when myocardial infarction is present is unclear, but the potential of icMSCs to stimulate myocyte proliferation and ameliorate dysfunction in remote remodeled myocardium after infarction could be as important as scar replacement.

Myocardial CD133+ and cKit+ cells increased similarly after icMSC administration in normal and hibernating hearts, as well as after the infusion of MSCs into the descending aorta. The latter finding indicates that myocardial injection or microembolization is not required and suggests a systemic action that may arise from a paracrine mediator or homing of the MSCs back to the bone marrow. Multiple paracrine factors have been implicated in explaining the beneficial actions of MSCs. In our previous studies in the Syrian hamster with heart failure, we demonstrated the potential role of paracrine factors in mobilizing BMPCs after repetitive skeletal muscle injection of porcine MSCs, MSC media, and vascular endothelial growth factor. The effects of single injections of MSC media in swine are controversial. Some of the paracrine candidates secreted by porcine MSCs include vascular endothelial growth factor, insulin-like growth factor-1, and interleukin-6, and we have recently demonstrated that these converge to activate the JAK/STAT pathway. The differential upregulation of stromal
cell–derived factor-1 and monocyte chemotactic protein-1 in hibernating myocardium after icMSC administration are but 2 of what are likely many potential candidates involved in the model of hibernating myocardium.

Given the ability of CD133+ BMPCs to coexpress cardiac lineage markers, including GATA-4, Nkx2.5, and troponin I, we propose that some of the myocyte proliferation that results from icMSCs reflects the transdifferentiation of endogenous BMPCs mobilized to the heart. Although transdifferentiation of MSCs into myocytes is not seen after icMSC administration, the present data also support the possibility that, like intramyocardial MSC injections, icMSCs promote myocyte proliferation by activation of a resident cKit+/CD133+ cardiac stem cell pool. The similarity of these changes to the functional improvement and mobilization of CD133+ and cKit+ myocardial cells after chronic administration of pravastatin in pigs with hibernating myocardium is striking.36 The present findings after icMSC administration are also consonant with recent reports that suggest that paracrine-mediated stimulation of endogenous cardiac myocyte proliferation is responsible for many of the beneficial effects seen after intracoronary administration of cardiac stem cells, as well as intramyocardial cardiosphere-derived cells, in infarct models.38,55,56 Further studies will be required to determine whether the actions of MSCs are additive to similar effects demonstrated pharmacologically with pravastatin, but a synergistic effect could result in more prominent myocyte regeneration of failing, noninfarcted myocardium.

Methodological Limitations
We did not measure circulating cKit+ and CD133+ cells 6 weeks after icMSC administration, but circulating cKit+ cells were probably low, because they were already near baseline at 2 weeks. Likewise, because tissue CD133+ cell levels returned to baseline at 6 weeks, it would seem unlikely that circulating levels would be increased. Although the present results quantify the cumulative extent of myocyte regeneration and its translational relevance in a large animal model of chronic ischemia, mechanistic insight regarding the contribution of each progenitor cell subtype will require technically challenging studies in which genetic fate mapping, bone marrow transplantation, and reporter gene imaging can be applied using rodent models of chronic ischemia.

Clinical Implications
The present study provides a rationale for the clinical evaluation of the effects of intracoronary administration of MSCs in humans with viable dysfunctional myocardium with residual perfusion. The clinical relevance of this substrate as a therapeutic target is high,57 and recent clinical trials have demonstrated no effect of revascularization on survival in ischemic heart failure.58 Viable dysfunctional myocardium is common and arises secondary to hibernating myocardium and repetitive stunning, as well as from myocyte loss and cellular hypertrophy due to LV remodeling.59,60 In contrast to avascular fibrotic scar, there is residual perfusion, which permits the distribution of intracoronary cell–based therapy by standard cardiac catheterization approaches. Importantly, although much preclinical investigation has focused on heart failure due to postinfarction remodel-


20. Gneechi M, He H, Liang OD, Melo LG, Morello F, Mu H, Noisieux N, Zhang L, Pratt RE, Inglaw JS, Drav IJ. Paracrine action accounts for marked protection of ischemic heart by Akt-modified mesenchymal stem cells. *Nat Med*. 2005;11:367–368.


49. Kuhn B, del Monte F, Hajjar RJ, Chang YS, Lebeche D, Arab S, Keating

48. Orlic D, Kajstura J, Chimenti S, Jakoniuk I, Anderson SM, Li B, Pickel J,

46. Kajstura J, Gurusamy N, Ogorek B, Goichberg P, Clavo-Rondon C,


390:834–838.

(VEGF) as a key therapeutic trophic factor in bone marrow mesenchymal

infarction by self-proliferation and selective homing of bone marrow-


51. Nguyen BK, Mahan S, Perrault LP, Tanguay JF, Tardif JC, Stevens LM,

Borie M, Harel F, Mansour S, Neveux N. Improved function and myocardial


55. Tang XL, Rokosz G, Sanganalumath SK, Yuan F, Sato H, Mu J, Dai S, Li


56. Chimenti I, Smith RR, Li TS, Gerstenblith G, Messina E, Giacomello A,


Suzuki et al. MSCs and Cardiac Repair in Hibernating Myocardium

**Novelty and Significance**

**What Is Known?**

- Intramyocardial injection of mesenchymal stem cells (MSCs) improves myocardial function in animal models of infarction.
- MSCs variably effect myocyte proliferation, inhibit myocyte death, and reduce scar volume, but the relative importance of each of these mechanisms to repair is unknown.
- Although controversial, transdifferentiation of MSCs into cardiomyocytes and stimulation of resident cardiac stem cells has been demonstrated in infarct border zones, but the importance of these localized changes to improved global function is unknown.

**What New Information Does This Article Contribute?**

- Intracoronary MSCs improve regional function independent of flow in swine with hibernating myocardium.
- Functional improvement arises from de novo myocyte proliferation, and the resultant increase in cardiomyocytes leads to regression of cellular hypertrophy throughout the dysfunctional region.
- Although intracoronary MSCs do not transdifferentiate into cardiomyocytes, they increase myocardial bone marrow progenitor cells and resident cardiac stem cells; both subtypes can transdifferentiate into cardiomyocytes.

Many stem cell therapies are effective at improving function and increasing capillary density after infarction in animal models. Nevertheless, viable dysfunctional states due to hibernating myocardium, stunning, and LV remodeling rather than scar underlie the contractile dysfunction seen in many patients with ischemic cardiomyopathy. Our studies, in a model devoid of scar and ongoing apoptosis, demonstrate the quantitative impact of MSC-mediated cardiomyocyte proliferation when residual perfusion is present. We show that readily implemented infusion of MSCs to all of the major coronary arteries improves contractile function as a result of a marked increase in myocyte numbers and secondary regression of cellular hypertrophy in hibernating myocardium. There is no evidence that the myocytes arise from transdifferentiation of MSCs when administered by this route. Rather, de novo myocyte regeneration arises from transdifferentiation of myocardial bone marrow (CD133+ ) and resident (cKit+ ) progenitor cells that are increased after MSCs in a fashion similar to that demonstrated pharmacologically with pravastatin. Although the relative contributions of each source of new myocytes remains to be defined, the results suggests that intracoronary MSC administration to patients with viable dysfunctional myocardium with residual perfusion may afford a more favorable substrate for repair than fibrotic scar.
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Autologous Mesenchymal Stem Cells Mobilize cKit\(^+\) and CD133\(^+\) Bone Marrow Progenitor Cells and Improve Regional Function in Hibernating Myocardium

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Autologous Mesenchymal Stem Cells Mobilize cKit+ and CD133+ Bone Marrow Progenitor Cells and Improve Regional Function in Hibernating Myocardium


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Abbreviated Title – MSCs and Cardiac Repair in Hibernating Myocardium

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Materials and Methods

Procedures and protocols conformed to institutional guidelines for the care and use of animals in research.

Effects of Intracoronary Mesenchymal Stem Cells (icMSCs) on Flow and Function in Hibernating Myocardium

Hibernating myocardium was produced as previously described\(^1\). 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 limited pericardiotomy, the proximal LAD was instrumented with a Delrin occluder (1.5 mm). 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 fentanyl (1-2 mg/kg q.d.). Animals were subsequently observed and brought back for studies beginning at three months, a time when we have consistently demonstrated the development of hibernating myocardium with stable reductions in flow and regional function without evidence of infarction or necrosis\(^1\).

Collection in vitro Expansion and Intracoronary Infusion of Autologous Porcine MSCs

Autologous bone marrow was sampled for isolation and expansion of MSCs 2-months after instrumentation as previously described\(^2\). Approximately 30 ml of bone marrow was aspirated from the sternum under propofol sedation. Extracted samples were heparinized using a 1:10 (v:v) ratio of preservative-free heparin to bone marrow aspirate and placed in a Ficoll gradient (BD Vacutainer CPT with Sodium Heparin) for isolation of the buffy coat. Samples were centrifuged at 2250 RPM (1160 RCF) for 20 minutes at 25°C and 2 ml of plasma was cryopreserved until MSC implantation. For each sample, 1.5-2 ml of monocyte/lymphocyte layer was removed from the buffy coat layer, washed twice in Hanks Balanced Salt Solution (HBSS), resuspended in 10 ml ADMEM (Invotrogen/Gibco Inc.) with 10% FBS and plated on separate plastic 100 mm tissue culture dishes (BD Biosciences). Samples were incubated in a humidified atmosphere at 37°C and 5% CO₂ and adherent cells were permitted to attach to the bottom of the plastic dishes. Non-adherent cells were easily detached by successive washings with HBSS at 2 and 4 days after initial plating followed by replacement of fresh media. Upon reaching confluency (5-6 days after initial plating), all cultures were virtually devoid of non-adherent cells and trypsinedized (0.25% trypsin in HBSS solution) and expanded in plastic 150 mm tissue culture dishes (BD Biosciences). Culture media was changed every 4-5 days and after trypsinizations. After 3-weeks cultivation we collected mesenchymal stem cells for implantation (nominal 44 x 10⁶ cells). In four animals, half of the cells were labeled with AdvEGFP (green fluorescent protein, Biomedical Research Scientific Co).

Prior to administration, we filtered cell suspensions through a 30 µm pore filter to circumvent cell aggregation (MACS pre-separation filters, Miltenyi Biotec) and suspended them in HBSS solution (30ml in total) for intracoronary infusion as described below\(^3\). We infused approximately 10-15 x 10⁶ cells into each of the three proximal coronary arteries including the
stump of the occluded LAD. The cell suspensions were each slowly infused over 15 minutes with no untoward hemodynamic changes or electrocardiographic evidence of myocardial ischemia.

Overall Design and Experimental Groups

A total of 34 pigs were used for the study (hibernating animals n=17, sham animals n=17). Pigs with hibernating myocardium (n=17) underwent initial studies 3-months after instrumentation. We have previously demonstrated that reductions in flow, function and flow reserve remain unchanged between 3-months and 5-months after instrumentation in this model1. To confirm the physiological stability of animals with hibernating myocardium prior to icMSC injection, we performed baseline physiological studies before icMSC injection at 3-months and 4-months after instrumentation. After the baseline study was repeated at 4-months, we administered intracoronary MSCs to ten animals while seven served as untreated controls. Hearts from icMSC treated animals were removed 2-weeks (n=6) or 6-weeks (n=4) after injection. The serial studies in hibernating myocardium are summarized in Online Figure I.

We also assessed the effects of icMSCs on hemodynamics and myocardial pathology in normal animals that received MSCs with hearts harvested 2-weeks after administration (n=6). In a second group (n=6), we infused MSCs into the distal aorta (n=6) to circumvent microembolization and determine whether systemic administration of MSCs increased myocardial progenitor cells in normal swine. Finally, five normal animals provided myocardial tissue to assess immunohistochemistry in untreated sham controls.

Serial Physiological Studies – Animals with hibernating myocardium (n=17) underwent physiological studies at 3-months, 4-months and 5-months after initial instrumentation. For each of the studies, sedation was initiated with Telazol (100 mg/ml)/xylazine (100 mg/ml) mixture (0.017 ml/lb I.M.) and maintained with propofol (5-10 mg/kg/hr i.v.). Under sterile conditions, we inserted a 6-Fr introducer into the left brachial artery. A Millar catheter was inserted into the left ventricular (LV) apex for microsphere injection. The introducer side port was used to monitor aortic pressure and provide a reference blood withdrawal for microspheres. Animals were heparinized (100 U/kg), and hemodynamics allowed to equilibrate for at least 30-minutes. Regional wall-thickening was assessed with transthoracic echocardiography from a right parasternal approach4. All pigs employed for the study showed anterior dysfunction but dyskinesis was not present under any condition. Systolic wall-thickening (ΔWT=ESWT-EDWT; %WT=ΔWT/EDWTx100) was measured in dysfunctional LAD regions as well as remote normally perfused regions of the same heart. Ventricular dimensions and LV mass were calculated from echocardiographic dimensions using ASE criteria. After echocardiographic and hemodynamic measurements, we assessed myocardial perfusion with microspheres at rest and following pharmacological vasodilation using adenosine (0.9mg/kg/min iv) while phenylephrine was infused and titrated to maintain mean blood pressure at ~100 mmHg. At the end of each study, catheters were removed and pigs were brought back to the animal facility.

At the end of the studies, animals were euthanized under general anesthesia. The LV was rapidly excised, weighed and sectioned into 1-cm rings parallel to the AV groove from apex to
Thin rings above each major ring were incubated in TTC to assess infarction. Additional samples were taken for quantifying flow and histology.

**Microsphere Flow** - Regional perfusion was assessed using 15μm microspheres labeled with fluorescing dyes. We injected ~3x10⁶ microspheres into the left ventricle while a reference sample was withdrawn at 6 ml/min for 90-seconds. At the end of the study, samples were taken from a midventricular ring, divided into twelve circumferential wedges with each cut into 3 transmural layers as described before. Dyes were extracted using standard techniques and fluorescence quantified at selected excitation wavelengths.

Serial changes in flow were evaluated several ways. In each animal the circumferential flow distribution during adenosine was analyzed to identify the hibernating risk region as compared to normal regions where flow increased 4-6 folds. From this, we evaluated weighted average flow from samples in the central hypoperfused region (hibernating LAD) or normally-perfused remote region. Samples with intermediate vasodilated flows were considered border regions and excluded from this analysis. We compared flow values in animals receiving icMSCs to untreated swine with hibernating myocardium. We also evaluated relative coronary flow reserve. For these measurements relative perfusion was determined by dividing the flow in LAD regions by the corresponding average full-thickness value from normal myocardium. Finally, absolute coronary flow reserve was assessed by comparing flow in each region during vasodilation to the corresponding values at rest.

**Effects of icMSCs on Circulating cKit⁺ and CD133⁺ Bone Marrow Progenitor Cells**

We assessed the effect of icMSCs on circulating bone marrow progenitor cells (cKit⁺ or CD133⁺) as well as myocardial function and histopathology in normal myocardium in uninstrumented swine (n=6). Mononuclear cells were isolated from peripheral blood (30 ml) using the Becton Dickinson CPT cell separation system before, 3 days and 2 weeks after icMSC treatment. WBC counts were performed using an automated hemocytometer while monocyte counts were done using a manual hemocytometer. Approximately 10-20 x 10⁶ mononuclear cells were analyzed by FACS after staining for cKit (CD117, eBioscience), CD133 (PE conjugated, Miltenyi Biotech) and CD 45 (PE-Cy5 conjugated, BD Pharmingen). Isotype controls were used as negative controls. Single stains were also performed to determine quality control and for multi-channel compensation. Data were expressed as progenitor cells (cKit⁺ and CD45⁻, CD133⁺ and CD45⁻) per million mononuclear cells. All counts were corrected for the absolute mononuclear cell count. Immunohistochemical analysis and morphometric analysis of excised tissue is summarized below.

**RNA extraction and RT-PCR**

Total RNA was isolated with RNeasy fibrous tissue kit (Qiagen) and reverse transcription performed using M-MLV reverse transcriptase from Promega and oligo(dT)1.5 primer. QPCR was performed using iQ SYBR green supermix(Bio-Rad) and relative quantification of gene expression was calculated using the comparative method (ΔΔCT) with β-2-microglobulin(B2M) used as the endogenous control. Primer sequences are as follow.
Beta 2 macroglobulin:
AAACGGAAAGCCAAATTACC(5’) and ATCCACACGTAGGAGTGA(3’),

VEGF-R2:
CTTTGTGCGAGGTATCCAGA(5’) and GCTGATCATGCTGGGAAT(3’),
c-Kit:
GAGAATAGGCTCATACTAG(5’) and GCCAAGTCTCTGTGAATACA(3’).

MCP1:
CACCAGCAGCAAGTGTCCA (5’) and TCCAGGTGCTATGGAGTC (3’).

SDF1:
CCTTGCCGATTCTTTGAGAG (5’) and CAATTTTGGGTCAATGCACA(3’).

VEGF:
CTACCTCCACCATGCCAAAGT (5’) and ACACAGGACGGCTTGAAGAT (3’).

Myocardial Histopathology and Immunohistochemistry

Myocyte Nuclear Density and Morphometry - Samples approximately midway between the base and apex that were immediately adjacent to the LAD (hibernating) and posterior descending arteries (normal) were fixed (10% formalin) and paraffin-embedded. Point-counting of trichrome-stained sections was used to quantify connective tissue. PAS stained sections were used to quantify myocyte diameter and nuclear length (100 longitudinal myocytes per region) in subendocardial and subepicardial thirds of the left ventricle. Myocyte diameter was assessed 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. We also assessed regional myocyte nuclear density as previously described. By incorporating myocyte nuclear length and the other measurements, we also calculated the number myocyte nuclei per mm³ of myocardium.

Immunohistochemistry Assessment of Myocyte Proliferation - All of the antibodies have been successfully used in the pig by our group and other laboratories. Paraffin-fixed tissue sections with 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 myocyte filaments as previously described by our laboratory in swine. Myocardial levels of CD45 negative hematopoietic progenitor cells (CD45 antibody, 1:200, AbD serotec) were quantified using the cell surface marker CD133 (Miltenyi biotec, 1:200) and cKit
(AbD serotec, 1:200). To characterize mast cells we additionally used mast cell marker (CD68 antibody, 1:200, Santa Cruz). To preserve CD133 and cKit antigens, we conducted the quantitative analysis using frozen tissue sections. GATA-4 (Santa Cruz, 1:100), Nkx2.5 (R&D, 1:100) and cTnI antibodies were used to identify whether the tissue BMPCs were differentiating into a myocyte lineage. Samples were post-treated with FITC conjugated anti-mouse and TexasRed conjugated anti-rabbit antibody (Dako). Nuclei were stained with TO-PRO-3 (Molecular Probes) or DAPI (Vectashield). Image acquisition was performed with a confocal microscope (Bio-Rad MRC 1024) and AxioImager equipped with ApoTome (Zeiss). Both Ki-67 myocytes and phospho-histone-H3 were counted and evaluated as positive nuclei per myocyte nuclear density as previously section described. The number of CD133\(^+\) and cKit\(^+\) cells in myocardium were also expressed in relation to myocyte nuclear density or cells per cm\(^3\). Data represent the averages from 260±28 fields (45±5mm\(^2\)) examined per slide.

Capillary density quantification - Paraffin-fixed tissue sections were incubated with Factor VIII-related antigen (Biocare Medical). Samples were post-treated with FITC conjugated anti-mouse antibody (Dako). Nuclei were stained with DAPI. Image acquisition was performed with a Zeiss's Axioimager fluorescence microscope at x200 magnification. The number of capillaries were quantified by ImageJ software using the analyze particle feature. 10 random fields (1.478mm\(^2\)) were selected and data were expressed as capillary number per tissue area (mm\(^2\)).

Statistics

Data are expressed as mean ± standard error. Differences after treatment with icMSCs and comparisons between the hibernating and normally perfused remote regions of the same heart were assessed using paired t-tests. Differences among icMSCs treated animals and age-matched untreated animals were assessed using a two-way ANOVA and the post-hoc Holm-Sidak test (Sigma Stat 3.0). Differences of p<0.05 were considered significant.

Results

Stability of Hibernating Myocardium Prior to Injection of MSCs

To demonstrate stability of dysfunctional myocardium as well as coronary flow reserve, we evaluated animals with hibernating myocardium over a 4-week period prior to injection of MSCs. Online Figure II demonstrates stable reductions in regional LAD%WT and flow reserve in the 1 month interval preceding icMSC infusion (n=10). Thus, there was no time-dependent improvement or deterioration in the primary physiological parameters once hibernating myocardium was present. Likewise, untreated animals with hibernating myocardium (Figure 1 of main manuscript) demonstrated stable reductions in LAD%WT and coronary flow reserve until the final study. The serial effects of icMSCs on animals with hibernating myocardium (n=10, 2-week and 6-week pooled), sham normals receiving icMSCs (n=6) and untreated controls with hibernating myocardium (n=7) were compared (Online Tables I-II). In animals with a chronic LAD occlusion, LAD wall-thickening at the initial study (4-months) was severely
reduced compared to normal remote regions and no different between animals that were untreated vs. those subsequently receiving icMSCs.

**icMSCs increase circulating cKit\(^+\) and CD133\(^+\) BMPCs**

Three days after icMSCs, circulating cKit\(^+\) and CD133\(^+\) cells increased four-fold and remained significantly elevated (Online Figure III). After 2-weeks, cKit\(^+\) and CD133\(^+\) cells declined but remained elevated.

**Intracoronary and distal aortic infusion of MSCs increase myocardial cKit\(^+\) and CD133\(^+\) BMPCs**

We infused MSCs into the distal aorta to determine whether the increase in cKit\(^+\)/CD133\(^+\) cells was related to myocardial microembolization (Online Figure IV). Aortic infusion increased myocardial cKit\(^+\) cells from \(8\pm3\times10^3\) to \(819\pm209\times10^3\) cells/cm\(^3\) after icMSCs and \(393\pm82\times10^3\) cells/cm\(^3\) after aortic MSC infusion (both \(p<0.05\) vs. untreated). Likewise, myocardial CD133\(^+\) cells increased from \(5\pm2\times10^3\) to \(234\pm64\times10^3\) cells/cm\(^3\) after icMSCs and \(366\pm109\times10^3\) cells /cm\(^3\) after aortic MSC infusion (both \(p<0.05\) vs. untreated). Thus, increases in cKit\(^+\)/CD133\(^+\) myocardial progenitor cells did not require icMSC injection or microembolization of MSCs in the heart.

**Autologous icMSCs Upregulate Chemokines**

To ascertain potential paracrine factors responsible for the effects of icMSCs on myocyte proliferation we quantified the myocardial expression of SDF-1, MCP-1 and VEGF 2-weeks after icMSCs. In hibernating myocardium icMSCs significantly increased SDF-1 (2.1\pm0.2 fold, \(p<0.05\) vs. untreated) and MCP-1 (2.5\pm0.7 fold, \(p<0.05\) vs. untreated) but not VEGF (1.1\pm0.1 fold, p-ns). Expression was not altered in sham-normal animals receiving icMSCs (Online Figure V).

**Specificity of the cKit and CD133 Antibodies for Porcine Tissue and their Absence on Porcine MSCs**

To confirm specificity of the cKit and CD133 antibodies we used porcine spleen and intestine as positive controls. Splenic hematopoietic stem cells were positive for CD133 (green, Online Figure VIa.). Intestinal mast cells were positive for cKit (green, Online Figure VIb). In contrast, porcine MSCs expanded in culture were completely negative for CD133 as well as cKit (Online Figure VIc and Vid). We previously reported a similar result in porcine MSCs\(^3\).

**Immunohistochemical Characterization of Porcine MSCs**

We assessed cKit expression in cultured porcine MSCs using RT-PCR. RNA was isolated from cultured porcine MSCs, and qPCR was used to obtain the threshold cycles for the house-keeping gene beta-2-microglobulin (b2M), VEGF receptor-1 (VEGF-R1), VEGF-R2, and
c-kit. Porcine MSCs have previously been shown to be positive for VEGF-R1 and negative for VEGF-R2. Amplification of VEGF-R1 and the housekeeping gene beta-2-microglobulin occurred at a threshold of ~25 cycles. In contrast, VEGF-R2 and cKit had much higher threshold cycles of ~35. This supports the conclusion from immunohistochemistry that the porcine MSCs we used for intracoronary administration were cKit negative (Online Figure VII).

Exclusion of cKit⁺ Cells as Mast Cells

Intestinal mast cells were positive for cKit (green, Online Figure VIIIa) as well as CD68 (a mast cell marker, white, Online Figure VIIIb) and CD45 (a hematopoietic cell marker, red, Online Figure VIIIc). The quantified data represent CD45⁻ cells and thus, did not include mast cells (Online Figure IX).

Frequency of Ki67 in myocardial cKit⁺ cells in Hibernating Myocardium

We performed Ki67 staining in cKit⁺ cells and found Ki67⁺/cKit⁺ cells in 9.2±3.2 % (Online Figure X). There was no effect on the frequency of myocardial cKit⁺/Ki67⁺ cells after icMSCs as compared to untreated animals. The percentage of Ki67⁺ cells is much higher than the TnI positive myocytes (0.2 – 0.4% for Ki67).

Cardiogenic Committed Porgenitor Cells Increase After icMSCs in Hibernating Myocardium

Co-staining with cardiac Troponin I was used to confirm cardiogenic commitment of progenitor cells (n=6). cTnI positive cells were not detected in untreated animals. After icMSCs, cKit⁺/cTnI⁺ cells were seen in 5.6±1.9% of the cKit⁺ cells (Online Figure XI). Thus, icMSCs directed cardiac commitment in cKit⁺ resident cells.

Cardiac Porgenitor Cells express endothelial markers in Hibernating Myocardium

Co-staining with von Willebrand factor was used to determine endothelial commitment of progenitor cells in icMSCs and untreated animals (n=4 respectively, Online Figure XII). Co-localization of cKit and VonWillebrand factor identifying endothelial commitment occurred in 33±2% of the cKit⁺ cells.
Supplemental References


Experimental Protocols

Sterile Surgery

3-month Study (Pre-MSCs)

MSCs 2weeks (n=6)

4-month Initial Study (Pre-MSCs)

Final Study

MSCs 6weeks (n=4)

icMSCs 44x10^6 cells (n=10)

Sterile Surgery

4-month Initial Study (n=7)

Final Study

Untreated

Hibernating Myocardium

Online Figure I.
Online Figure II. Stability of hibernating myocardium prior to icMSCs.
The results summarize serial coronary flow and function in closed chest swine with hibernating myocardium (n=10). Measurements of LAD wall thickening were depressed as compared to remote myocardium and coronary flow reserve was critically impaired and unable to increase in response to adenosine. In swine with hibernating myocardium, regional wall thickening and coronary flow reserve remained stable before treatment (3-month and 4-month). These results demonstrate stability of the physiological parameters before treatment.
Online Figure III. icMSCs increase circulating cKit\(^+\) and CD133\(^+\) BMPCs. There was no effect of icMSCs on the total mononuclear cell count in peripheral blood. Within three days after icMSCs, the number of circulating cKit\(^+\) and CD133\(^+\) cells were increased in comparison to initial values. Circulating cKit\(^+\) BMPCs and CD133\(^+\) BMPCs declined but remained elevated 2-weeks after a single injection of autologous MSCs.
Online Figure IV. Both intracoronary and distal aortic infusion of MSCs increase myocardial cKit\(^+\) and CD133\(^+\) cells. MSCs were injected into the distal aorta to determine the role of cardiac microembolization in increasing myocardial progenitor cells. Data 2-weeks after injection were compared to untreated and icMSCs in sham animals. Myocardial cKit\(^+\) cells (left graph) increased significantly after icMSCs as well as distal aortic infusion of MSCs. Likewise, myocardial CD133\(^+\) cells (right graph) increased to a similar extent after icMSCs and distal aortic infusion of MSCs. Thus, increases in myocardial progenitor cell levels did not require direct intracoronary injection and were not related to microembolization.
Online Figure V. Increased myocardial expression of paracrine factors after icMSCs in hibernating myocardium.

Heart tissue was harvested two weeks after icMSC administration. Myocardial RNA was isolated for quantitative PCR analysis (n=24) of gene expression. In hibernating myocardium icMSCs significantly increased SDF1 (2.1±0.2 fold) and MCP1 (2.5±0.7 fold) but not VEGF (1.1±0.1 fold) expression. In contrast, expression was not altered after administering icMSCs in sham myocardium.
Online Figure VI. Porcine positive tissue controls and porcine MSC staining for CD133 and cKit. Pig spleen was used as a standard tissue control to identify CD133$^+$ cells (VIa.). Pig intestine was used as a positive control for cKit$^+$ cells (VIb.). Porcine MSCs were all negative for CD133 (VIc.) as well as cKit (VID.). Nuclei were stained blue with DAPI.
Online Figure VII. Porcine MSCs do not express c-kit.
RNA was isolated from cultured porcine MSCs, and quantitative PCR was used to obtain the threshold cycles in comparison to the house-keeping gene beta-2-microglobulin (b2M), VEGF receptor-1 (VEGF-R1), VEGF-R2, and c-kit. We have previously shown that porcine MSCs are positive for VEGF-R1 and negative for VEGF-R2. Amplification of VEGF-R1 and the housekeeping gene beta-2-microglobulin had thresholds for amplification of ~25 cycles. In contrast, VEGF-R2 and c-Kit had threshold cycles of ~35. This, in conjunction with the negative immunohistochemical staining provided in Supplemental Figure 6, supports the conclusion that there is little expression of cKit in porcine MSCs.
Online Figure VIII. Porcine mast cells express CD68, cKit and CD45 in intestine. Intestinal tissue was used to assess co-localization of mast cells, cKit+ cells and hematopoietic cells. Most of the cKit+ cells (green, VIIIa.) were CD68+ (mast cell marker, white, VIIIb) and CD45+ (hematopoietic marker, red, VIIIc). This indicates that porcine mast cells are both cKit+ and CD45+. Therefore, myocardial cKit+/CD45- cells are not compatible with a mast cell origin.
Online Figure IX. cKit+/CD45− cell and cKit−/CD45+ cells in the heart. cKit is stained as green (one arrow) and CD45 is stained as white (double arrows). There is one cKit+/CD45− cell and two cKit−/CD45+ cells in the image. cKit+/CD45− cells are considered as cardiac progenitor cells and counted separately.
Online Figure X. Frequency of Ki67 staining in myocardial cKit+ cells in hibernating myocardium.
As shown on the upper image, there were two myocardial cKit+ cells (green) co-expressing Ki67 (red) in the nuclei. The lower graph show the frequency that Ki-67 was expressed in myocardial cKit+ BMPCs. There was no effect of icMSCs on the frequency of myocardial cKit+/Ki67+.
Online Figure XI. Co-localization of troponin I (TnI) in cardiac progenitor cells.
After icMSCs, co-staining with TnI was seen in 5.6±1.9% of the cKit⁺ cells. In contrast, cKit⁺/cTnI⁺ cells were not detected in untreated animals.
Online Figure XII. Cardiac progenitor cells express endothelial markers in hibernating myocardium. Co-staining with von Willebrand Factor (vWF) was used to demonstrate endothelial commitment of a subpopulation of cKit+ cells after icMSCs.