Bone Marrow Mesenchymal Stem Cells Stimulate Cardiac Stem Cell Proliferation and Differentiation

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Rationale: The regenerative potential of the heart is insufficient to fully restore functioning myocardium after injury, motivating the quest for a cell-based replacement strategy. Bone marrow-derived mesenchymal stem cells (MSCs) have the capacity for cardiac repair that appears to exceed their capacity for differentiation into cardiac myocytes.

Objective: Here, we test the hypothesis that bone marrow derived MSCs stimulate the proliferation and differentiation of endogenous cardiac stem cells (CSCs) as part of their regenerative repertoire.

Methods And Results: Female Yorkshire pigs (n=31) underwent experimental myocardial infarction (MI), and 3 days later, received transendocardial injections of allogeneic male bone marrow–derived MSCs, MSC concentrated conditioned medium (CCM), or placebo (Plasmalyte). A no-injection control group was also studied. MSCs engrafted and differentiated into cardiomyocytes and vascular structures. In addition, endogenous c-kit+ CSCs increased 20-fold in MSC-treated animals versus controls (P<0.001), there was a 6-fold increase in GATA-4+ CSCs in MSC versus control (P<0.001), and mitotic myocytes increased 4-fold (P=0.005).

Conclusions: MSCs stimulate host CSCs, a new mechanism of action underlying successful cell-based therapeutics. (Circ Res. 2010;107:913-922.)

Key Words: myocardial infarction • mesenchymal stem cells • cardiac stem cells • myocardial regeneration

The quest to restore damaged organs is one of the major challenges in medicine.1,2 Recently, it has been demonstrated both experimentally3 and in patients4,5 that the heart has the capacity to replace cardiac myocytes throughout life, but that this response is inadequate to compensate for major injuries such as myocardial infarction. These recent observations coupled with the description of endogenous cardiac stem cells6–9 have raised enthusiasm for tapping this intrinsic property therapeutically. Another avenue of cardiac cell-based therapy has used diverse adult cell sources such as fat, bone marrow, umbilical cord blood, and adipose tissue.10,11 Some of these cell types, particularly mesenchymal stem cells, have properties that could allow them to stimulate endogenous cardiac repair in a regulated manner.12,13

Bone marrow (BM)-derived MSCs regulate hematopoietic and other stem cell niches14–16 while maintaining substantial multilineage differentiation capacity.17 Notably, recent studies document that interactions with MSCs are essential to the stimulation of in vitro proliferation and differentiation of other progenitor cell populations, in a process requiring direct cell-cell contacts.16,18–20 Currently, in experimental models, MSCs exert major functional recovery in the injured heart,12,21–24 through incompletely understood mechanisms.25–27 Here, we hypothesized that MSCs stimulate cardiac repair through cell-autonomous effects that stimulate host myocardial precursor cells to amplify and differentiate into cardiomyocytes.6,22,28 To address this prediction, we injected green fluorescent protein (GFP)-labeled, male porcine MSCs into the infarct and border zone in female pigs 3 days following myocardial infarction (MI); another group received injection of concentrated conditioned medium (CCM), so as to test whether secreted factors alone would be sufficient to stimulate host cardiac repair.
Methods

An expanded Methods section describing all procedures and protocols is available in the Online Data Supplement at http://circres.ahajournals.org.

This study was reviewed and approved by the University of Miami Institutional Animal Care and Use Committee and complies with all Federal and State guidelines concerning the use of animals in research and teaching as defined by The Guide For the Care and Use of Laboratory Animals (NIH Publication No. 80-23, revised 1985).

For this study, 31 healthy female Yorkshire swine weighing 25 to 35 kg underwent experimental myocardial infarction (MI) followed by reperfusion (Online Figure I). The study was conducted in 2 phases. In the first phase, 3 groups were studied: Yorkshire pigs received transendocardial injections (TEI) (Stiletto, Boston Scientific, Natick, Mass) of 75x10⁶ GFP labeled MSCs (n=8), Placebo (n=8) or no injection (n=3) 3 days following the MI. Animals were euthanized at 24 hours (n=2 placebo and n=2 MSCs treated), 72 hours (n=3 placebo and n=3 MSCs treated) and 2 weeks (n=3 MSCs treated n=3 placebo and n=3 control) after transplantation to study the fate of the allogeneic cells.

In the second phase, 2 groups were studied: The Yorkshire pigs were randomized to receive TEI of 100x10⁶ cells of male GFP-labeled MSCs or their 10⁻⁵ concentrated condition medium 3 days after MI and followed by MRI analyses (Siemens Symphony, Erlangen, Germany) at multiple time points (baseline; 2 days after MI; 4 days, 2 weeks, and 8 weeks after injection) to assess the amount of functional recovery. The animals were euthanized at 2 weeks (n=3 CCM and n=3 MSC-treated) and 8 weeks (n=3 CCM and n=3 MSC-treated) after injections.

**GFP Transduction of MSCs**

Passage 1 (P1) MSCs were plated in a T25-cm² flask and transduced with lentiviral green fluorescence protein (GFP). At approximately 50% confluence the media was removed and replaced with 5 mL of transduction media consisting of Alpha MEM plus 20% FCS plus 8 µg/mL polybrene and 10 µL of lentiviral vector LV-173GFP (Lentigen, Gaithersburg, Md). The culture flask was incubated for 72 hours total, with fresh transduction media being added every 24 hour. The next day the media was removed and Alpha MEM plus 20%FCS added to the culture. The flask was further incubated until confluent.

### Table. Myocardial Infarct Phenotype Before and After Treatment

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<th>Baseline</th>
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<th>Week 2 After Injection</th>
<th>Week 8 After Injection</th>
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Values determined by cMRI. *Probability values significant between groups; † and ‡, probability values significant within group.
Cultures were expanded with each passage of the GFP+ MSC until sufficient numbers of MSC were obtained. The cells were then frozen in liquid nitrogen until needed. Before injection, the cells were thawed rapidly and washed to remove DMSO, then resuspended in PBS plus 1% human serum albumin to the required cell dose.

Transendocardial Injections ofMSCs

Delivery of the cells at the sites of myocardial injury was performed as previously described. Briefly, left ventriculograms from 2 different angiographic projections (left [−30°] and right [+30°] anterior oblique) were used to manually map the endocardial contours of the left ventricles in both projections. The infarcted myocardial zone (IZ) and border zone (BZ) were then delineated on the contours, and a total of 15 injections were performed in each animal, with each injection containing 0.5 mL of the injectate. Each injection was fluoroscopically guided to distribute cells evenly throughout the entire infarct and border zones.

Histology

For microscopic evaluation, the regions of interest were selected from each of the transverse ventricular sections based on CMRI and gross pathology findings: (1). One sample from the middle of the scarred, infarcted tissue. (2) One sample containing the left border of the infarct along with nonscarred tissue; one sample containing the right border of the infarct along with nonscarred tissue (these 2 samples were defined as the border zones macroscopically). Microscopically, within these samples, border zones were defined as the areas that were 1 to 1 one-half high-power fields distant from scarred zones. (3) One sample from the posterior noninfarcted left ventricular (LV) wall. Confocal analysis was performed as previously described.

Statistical Analysis

All the values are presented as means±SEM. All analyses were performed by using the SPSS for Windows version 15.0 (SPSS Inc, Chicago, Ill). Differences between groups following immunohisto- logical evaluation were compared by using 1-way ANOVA. Differences between groups in ejection fraction and infarct size based on cMRI were calculated by using 2-way repeated-measures ANOVA. The Tukey’s test was used for the post hoc analysis. A level of P≤0.05 was considered statistically significant.

Results

MSC Engraftment Reduces Infarct Size

As shown by serial cardiac MRI (cMRI), MI led to a reduced ejection fraction (27.9±1.1% and 25.8±3.1% for MSC and CCM groups respectively, P=NS, P<0.001 versus baseline) and scar tissue that comprised ~25% of the left ventricle (24.9±2.4% and 24.4±3.2% of the left ventricles of the MSC and CCM groups, respectively, P=NS; Table and Figure 1).

Consistent with previous observations, as early as 4 days following TEI of MSCs, the absolute size of the myocardial infarct was reduced by 8.3±1.8% (% LV mass) in the animals treated with MSCs, while remaining unchanged in the CCM group (P=0.018; Figure 1). Eight weeks following MI, myocardial scar was reduced to 10.9±5.1% of the LV in the MSC-treated animals, whereas CCM caused a mild reduction in the absolute IS by 2.3±1.3% (P=0.002 between groups) (Figure 1A). In addition, by 2 weeks the MSC group had a significant improvement in EF compared to post-MI (P<0.05), which persisted through the 8-week follow-up period (Figure 1B).

Localization of Injected MSCs in Infarcted Hearts

Engraftment and lineage tracing of transplanted MSCs were assessed with confocal immunofluorescence. As determined by both GFP and Y-chromosome tags, MSCs occupied IZ and BZ but not the healthy, remote areas of the infarcted myocardium.

Interestingly, whereas MSCs lacked markers of cardiovascular lineage in vitro (Figure 2A through 2C), evidence for their cardiac commitment could be documented within 24 hours (Figure 3A; Online Figures IV, VI, VIII, and XI), and by 2 weeks MSCs had differentiated into new, mature cardiomyocytes and vascular structures (Figure 3C through 3E; Online Video I; Online Figures II, V, and X). The number of MSCs committed to cardiomyocytic lineage was quantified based on the expression of GFP+ and/or Y-chromosome+ tags colocalized with the cardiomyocyte specific-markers GATA-4 and α-sarcomeric actinin. Commitment of MSCs peaked at 3-days postimplantation (640±240 cells/cm3 GFP+ /GATA-4+ cells/cm3 at 24 hour versus 1980±360 GFP+/GATA-4+ cells/cm3 at 72 hours) (Online Figure IV) and the extent of myocardial chimerism remained constant in the regenerated hearts (Figure 4A). The number of MSCs committed to vascular lineage was quantified based on the expression of GFP+ and/or
MSCs Engraftment Induces C-kit+ CSC Recruitment

Next, we tested the hypothesis that MSCs, in addition to direct tissue replacement could also contribute to myocardial repair by supporting endogenous CSCs to regenerate myocardium. Interestingly, 2 weeks after MSC injection, the MSC-treated hearts exhibited chimeric clusters containing both immature MSCs of exogenous origin and endogenous CSCs (Figures 4, 5; Online Figure III, A through C; Online Figure XII). These clusters were mainly localized within the IZ and BZ but not the noninfarcted, remote myocardium or the non-MSC treated hearts; important cell-cell interactions between the MSCs and endogenous CSCs could be documented (Figures 4D, 4G, and 4H and Figure 5; Online Figure IV and VII). Two weeks later, coronary vascular chimerism was still present within the myocardium (Figure 3E).

MSCs Stimulate Cardiomyocyte Cell Cycling

In addition to activating c-kit+ cardiac precursors to enter cardiomyocytic lineages, MSCs also stimulated cardiomyocyte replication (Figure 6 and Online Figure IX). Host cardiomyocyte turnover peaked at 2 weeks after therapy as indicated by the expression of the mitotic marker of serine 10-phosphorylated Histone-H3 (phospho-H3), and was 4-fold greater in the IZ and BZ of the MSCs treated hearts compared to the CCM group (Figure 6A). The number of mitotic cardiomyocytes in the MSC-group was significantly higher in both the IZ (14,220±4,750 versus 3,480±590 cardiomyocytes/cm³, P=0.03) and BZ (12,810±3,720 versus 3,190±470 cardiomyocytes/cm³, P=0.005) compared to the CCM-treated animals (Figure 6A). Colocalization of GFP and/or Y-chromosome tags with phospho-H3+ cardiomyocytes could be rarely documented, indicating that the majority of amplifying cardiomyocytes detected were of host origin (data not shown). The levels of cycling cardiomyocytes in the MSC-treated group decreased by 2 months, and no differences could be observed between groups (Figure 6B). However, the total number of newly formed cardiomyocytes of both donor and host origin was still greater in the BZ of the MSCs treated animals compared to the CCM-treated animals (2,310±640 versus 450±120 total new cardiomyocytes/cm³ in the BZ of MSCs and CCM-treated respectively, P=0.02), illustrating that direct tissue replacement by differentiated MSCs contributed significantly to the regeneration of the BZ areas (Figure 6B).
MSCs Stimulate Proliferation and Myocardial Commitment of c-kit+ CSCs In Vitro

We next performed in vitro experiments to study the function and origin of endogenous CSCs in relation to MSCs (Figure 7 and Online Figure XIII). Fresh (n=12) or cryopreserved (n=26) endomyocardial biopsies from porcine hearts were cultured for one week with or without MSCs as feeder layers (Figure 7B–7D and Online Figure XIII, A–C). In additional control experiments, MSCs were cultured under the same conditions without a myocardial biopsy. After 3 days, myocardial biopsies were harvested from biopsies cultured with and without MSCs respectively (P=0.003). Similar to our in situ findings, immunocytochemical analysis documented the development of connexin-43 mediated cell-cell interactions between GFP+ and c-kit+ cells (Figure 7B). In contrast, c-kit+ cells purified from biopsies cultured without MSCs had a large, antigen-presenting cell-morphology that did not proliferate (Figure 7D).

In comparison to previous studies, purification of c-kit+ CSCs from single biopsy samples was accelerated by coculture with MSCs, and facilitated an outgrowth of highly cardiomyocytic CSCs; greater than 90% of the cells expressed the cardiac transcription factors Nkx2.5 and GATA-4 and the ATP-binding cassette transporter MDR1 while lacking expression of the VEGF-receptor, KDR (Figure 7; Online Figure III, D through G; Online Figure XIII).

Porcine CSCs Differentiate Into Myocytes by Coculturing With Neonatal Cardiomyocytes

To test the capacity of the CSCs to differentiate into myocytes we performed cocultures with neonatal rat cardiomyocytes (NRCMs). Porcine CSCs were seeded on transwell membranes, and placed on top of NRCM monolayers. After 3 to 4 days in coculture, CSCs illustrated phenotypic characteristics of mature cardiomyocytes, evidenced by a striated cytoskeleton expressing cardiac troponin-I (Online Video II). Importantly, differentiation of CSCs into cardiomyocytes in a transwell culture system which prevents the development of direct cell contact with NRCMs, excludes cell-fusion and demonstrates a cardiomyocytic phenotype of this adult cardiac precursor cell type.

Discussion

Here, we demonstrate that bone marrow derived MSCs, when injected into the post–myocardial infarct porcine heart, facilitate substantial cardiac recovery involving host cell–based repair, as well as MSC engraftment and differentiation. Differentiation of MSCs occurs acutely after transplantation, and MSCs stimulate endogenous cardiomyocyte turnover in 2 likely related ways: first, by stimulating endogenous c-kit+ CSCs; and second, by enhancing cardiomyocyte cell cycling. Together these phenomena represent the spectrum of changes necessary for the amplification and differentiation of new adult cardiomyocytes. The interaction between cardiac derived c-kit cells and bone marrow derived MSCs could be replicated in vitro, where the coculturing amplified c-kit cell expansion and differentiation.

Efficacy of MSCs

Several previous studies from our group and others demonstrate in large animal models major degrees of infarct size reduction and functional recovery with MSC cell therapy. We have previously documented reappearance
of myocardial tissue in the border zones of infarction including a rim of tissue on the endocardial surface that is associated with improved tissue perfusion and recovery of regional function. Despite the reproducible demonstration of major cardiac recovery with MSC therapy and as shown in both animal models and humans, the underlying mechanism of action has been a challenge to demonstrate given the rarity of differentiated MSC-derived myocytes in the post infarction heart.

In the present study, we demonstrate a multifold mechanism of action for this effect that includes MSC engraftment and trilineage differentiation. Importantly, MSCs interact with host cardiac progenitor cells, promoting their recruitment and/or expansion and differentiation. In addition, there is evidence of extensive myocyte mitosis which very likely represents the terminal stage of cycling of cardiac progenitor cell–derived myocytes. It is also possible that this observation could represent cell cycling of mature myocytes as has been suggested to occur in the zebrafish. The engraftment of MSCs appears to be necessary as tissue recovery is not achieved with the injection of MSC conditioned media.

**Direct Versus Indirect Tissue Replacement Following MSC-Based Cardiac Repair**

The present findings argue against a solely paracrine mechanism for MSC stimulated cardiac repair and strongly support that transplanted MSCs engraft and respond directly to cues of injury in infarcted myocardium. By using dual labeling with GFP and sex mismatch strategies to trace allograft fate, we document that transplanted MSCs exhibit features of differentiated cardiomyocytes suggesting direct replacement of myocardial tissue by chimeric CMs. Without excluding the possibility of cell fusion between exogenous and endogenous cells, our data indicate that MSCs generate new CMs and that this process occurs during the first 24 hours following transplantation. However, even though the newly produced chimeric CMs last for at least 2 months and contribute significantly to the total number of new CMs regenerating the injured heart, their number remains unlikely to

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**Figure 4.** MSCs stimulate endogenous CSCs. **A**, Graph depicting the contribution of cardiomyocyte precursors following exogenous administration of MSCs (green line) and endogenous CSCs (orange line) during cardiac repair after MI. MSC differentiation occurs rapidly after delivery. At 2 weeks, MSCs activate endogenous expansion of c-kit CSCs (orange line). **B**, Two weeks following TEG, the number of C-kit− cells coexpressing GATA-4 is greater in MSCs versus non-MSCs treated hearts. The cardiomyocyte precursors are preferentially located in the IZ and BZ of the MI, indicating an active process of endogenous regeneration (P<0.019 and P<0.0001). **C** and **D**, The 2-week-old chimeric myocardium contains mature cardiomyocytes (open arrow), immature MSCs (inset), and cardiac precursors of MSCs origin (arrow), coupled to host myocardium by connexin-43 gap junctions. Interestingly, endogenous c-kit− CSCs are found in close proximity to MSCs (D). **E**, Cluster of c-kit− CSCs in an MSC-treated heart; numerous CSCs are committed to cardiac lineage documented by GATA-4 and MDR-1 coexpression (arrows). **F**, Few isolated c-kit− cells were found in non-MSC–treated animals.

**Figure 5.** MSCs stimulate amplification of endogenous c-kit+ CSCs 2 weeks after injection. **A**, Recruitment of c-kit+ CSCs in the MSC-treated vs nontreated hearts and distribution of the c-kit cells within the different zones (P<0.001). **B**, Endogenous c-kit+ CSCs develop putative mechanical connections with the infarcted myocardium as indicated by colocalization with N-cadherin (inset). **C**, Large cluster of c-kit+ CSCs connected to each other and to adjacent GFP+ MSCs by connexin-43 (inset, yellow arrowheads). **D** through **F**, Representative figures illustrating the noninflammatory/mast cell phenotype of the c-kit+ CSCs. Whereas clusters of CD68pos/c-kitneg cells could be detected in the non-MSC–treated hearts (D), the MSC-treated hearts were rich in c-kitpos/CD68neg (E) and CD3pos clusters of CSCs (F). Mean values ± SEM (n=6 MSCs; n=3 placebo; n=3 CCM; and n=3 control).
account in total for the extensive degree of cardiac recovery documented by cMRI. Accordingly we demonstrate alternative mechanisms for cell autonomous, MSC mediated cardiac repair. MSCs stimulated a series of secondary endogenous responses that caused substantial amounts of adult CMs and, more importantly, immature CSCs to proliferate and replenish the damaged regions with new CMs of host origin.

Our findings permit an estimate of the degree of myocardial tissue regenerated by varying mechanisms. Using rates of appearance of various cell types as a percentage of total new myocytes within the infarct border zones, we estimate that MSCs could contribute up to ~8% of the newly formed cardiomyocytes.39,40 Chimeric cardiomyocytes require less than 2 weeks to reach a mature appearance and their presence at 2 months argues that they are not rejected. However, a greater extent of cardiac repair originates endogenously. We document the concerted appearance of c-kit+ CSCs and phospho-H3+ mitotic CMs5,37 (likely representing transient amplifying myocytes originating from CSCs) that peaked 2 weeks after therapy. Together, CSCs and mitotic CMs are estimated to contribute at least 45% of the new myocardial mass. Interestingly, the abundance of both CSCs and transient amplifying myocytes decline in parallel by 8 weeks further suggesting a linkage between the appearance of CSCs and transient amplifying cells.5,41 This phenomenon strongly supports the notion that repeated application of cell therapy may lead to further declines in scar tissue within injured myocardium.

None of the above effects occurred when CCM was used as treatment following MI, highlighting that even though paracrine signaling can be a major contributor to cardiac regeneration, the degrees of repair that occur with cell injections cannot be achieved with single applications of factors secreted from MSCs. Indeed, although this may simply be an issue of pharmacokinetics as specific paracrine and autocrine pathways are shown to stimulate endogenous tissue repair, MSC delivery clearly provides an enormous advantage in that a single delivery provides a long-standing and sustained biological effect.

These findings offer a broader perspective on the biology of regeneration and should be viewed in the context of several other major advancements. First, substantial efforts are underway to genetically manipulate MSCs to enhance their survival and/or to use them as delivery vectors42; indeed our findings showing cell-cell coupling of MSCs support this approach. Second, successful cardiac repair in our model was based on interaction of administered MSCs with host cells, and in this regard there are other attractive candidates for host cells such as recently described endogenous multipotent circulating progenitors.43 As mentioned above, a major hypothesis underlying regenerative effects of MSCs has been termed the “paracrine hypothesis” in which a number of paracrine44 and autocrine factors such as nucleostemin45 drive regeneration of injured hearts. Our findings are not at odds with this hypothesis, and likely MSCs orchestrate a broad array of reparative effects. As a corollary to the paracrine hypothesis, MSCs release immunomodulatory cytokines as part of their secretome46,47 that could enhance long term graft tolerance following heart transplantation. Finally, it is highly likely that MSCs will have therapeutic potential in a broad array of cardiac disorders in addition to ischemic heart disease.

**MSCs Support Expansion of the Local CSCs Pool and Enhance Their Cardiomyocytic Potentials**

Two key questions that are addressed here warrant mention. First, is whether the clusters of c-kit+ CSCs present in the MSC-treated hearts represent cells mobilized from extracardiac tissues such as the bone marrow and circulating blood, or whether they originated from the heart. Secondly, we ad-
many more c-kit were 10-fold more cells with coculture than without, yielding 2.2% of the cells without coculture. However, there versus 5.0 /H11006 /H11021 /H11005 /H11006 19 each). *
P while lacking the mast-cell surface epitope CD68 are large, quiescent cells with a macrophages morphology (D). The fraction of the c-kit cells that were produced from the cocultures was 3.4 ± 0.9% of the total cell number in coculture versus 5.0 ± 2.2% of the cells without coculture. However, there were 10-fold more cells with coculture than without, yielding many more c-kit cells (1.01 ± 10^6 cells/coculture versus 0.9 ± 10^5 cells/biopsy alone panned 1 week after plating the organotypic cultures in each group). In comparison, coculture with MSCs egress small, semiaherent CSCs that renew their population constantly (C).  

**Conclusion**

To address the potential cardiac origin of the c-kit cells, we cocultured heart biopsies on MSC lawns demonstrating that the MSC-feeder layers stimulated the expansion of c-kit CSCs. The CSCs that resulted from MSC coculture were more than 90% positive for Nkx2–5, a phenotype previously found only in the developing fetal hearts. In addition, the adult CSCs exhibit significantly enhanced cardiomyocytic potentials compared to CSCs that exist in the adult myocardium, and suggest that bone marrow MSCs may provide significant information to link cardiac development to repair. Thus, endogenous CSCs clusters detected in situ following MSCs implantation seem unlikely to originate from distant tissues such as the bone marrow, because their isolation from single heart biopsies suggests they stem from the cardiac pool. Although our results do not exclude the possibility of mobilization from distant sources (indeed the circulating multipotent cells could represent a master reservoir of tissue specific precursors), these results confirm the presence of precursor reservoirs within the heart itself, and provide evidence that therapeutic CSCs can be harnessed from endomyocardial biopsies. With regard to differentiation, both in vivo and ex vivo studies document lineage commitment and potential differentiation of c-kit cells.

**Timing and Mode of Delivery**

It is not firmly established whether selecting different routes, timing, and doses when delivering the MSCs would have a greater or less impact on prevention of remodeling following MI. Comparing our previous and present laboratory findings, we believe that hastening or delaying cell therapy may not have as a robust an effect as to the targeted delivery of MSCs 3 days after MI. Importantly, we have found that late treatment following full infarct healing, with the goal of reversing infarct remodeling can produce substantial recovery. In this setting, the degree of infarct size reduction, although substantial, is not as great as that observed with treatment in the early postinfarction period.

Certain technical features of our study warrant mention. We used a transendocardial injection in a reperfused MI model. Most investigators have used intravenous or intracoronary routes to deliver cells in infarcted heart tissue assuming that, in this way, important paracrine actions of the infused cells could still be harnessed without risking unnecessary myocardial damage. However, our study provides novel insights to delivering cell-based therapies and, besides providing further evidence that TEIs can be a safe and efficient method to deliver MSCs suggests that TEIs delivery to the infarct border zone may provide an optimal substrate for cell engraftment and interaction with host cells. Indeed, it is attractive to speculate that delivery of boluses of MSCs may provide an ideal mechanism to foster reconstitution of cardiac stem cell niches.

**Study Limitations**

With regard to the adequacy of the conditioned media generated from porcine MSCs cultures, we used the technique described by Maitra et al with minor modifications to concentrate the media 10-fold. We acknowledge that we cannot fully rule out the importance of secreted factors contained within the conditioned media. Moreover, it is well described that several factors secreted by MSCs are shown to recruit CSCs. The fact that our CCM produced little to no reparative effects could be attributable to a pharmacological effect and that single dosing could not produce the sustained effects observed with MSCs. Work is ongoing to define the factors produced by MSCs that stimulate endogenous repair in cell nonautonomous manners.

**Figure 7. Development of cardiac stem cell niches ex vivo.** A, MSCs stimulate outgrowth of c-kit CSCs from endomyocardial biopsies. B, Immunostaining of the primary cell cultures documents interactions between GFP MSCs (green) and c-kit cells (red) as indicated by colocalization with connexin-43 (white); these clusters resemble cardiac stem cell niches. C and D, c-kit cells outgrowing after a week from the biopsy alone are large, quiescent cells with a macrophages morphology. The fraction of the c-kit cells that were produced from the cocultures was 3.4 ± 0.9% of the total cell number in coculture versus 5.0 ± 2.2% of the cells without coculture. However, there were 10-fold more cells with coculture than without, yielding many more c-kit cells (1.01 ± 10^6 cells/coculture versus 0.9 ± 10^5 cells/biopsy alone panned 1 week after plating the organotypic cultures in each group). In comparison, coculture with MSCs egress small, semiaherent CSCs that renew their population constantly (C). E and F, Immunocytological stainings of c-kit CSCs purified and expanded from the organotypic cocultures with MSCs illustrate the high percentage of c-kit cells coexpressing MDR1 (E, yellow) and Nkx2–5 (F, white), while lacking the mast-cell surface epitope CD68 (E, white).

Mean values ± SEM (n = 19 each). *P < 0.005.
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Disclosures
The University of Miami has applied for a patent on intellectual property generated from this study that lists Joshua M. Hare and Konstantinos E. Hatzistergos as inventors.

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The bulk of regenerative process is mediated by interactions between Mesenchymal stem cells (MSCs) can directly regenerate new myo-

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Despite these endogenous regenerative mechanisms, cardiac injury●

The adult mammalian heart can no longer be viewed as a postmitotic●

What New Information Does This Article Contribute?●

What Is Known?●

- The adult mammalian heart can no longer be viewed as a postmitotic organ, because it has reservoirs of stem cells and is capable of generating substantial numbers of myocytes that enter mitosis.
- Despite these endogenous regenerative mechanisms, cardiac injury attributable to ischemic and other mechanisms remains the leading cause of morbidity and mortality, which has prompted the quest to harness cell-based therapies.
- One of the most promising sources for adult cell therapy is the mesenchymal stem cell found in bone marrow and other sources.

Mesenchymal stem cells (MSCs) can directly regenerate new myocardium when injected into infarcted hearts, by differentiating into cardiomyocytes and coronary vessels.
- The bulk of regenerative process is mediated by interactions between MSCs and endogenous cardiac progenitor cells (CPCs), which cause the latter to amplify and differentiate into cardiomyocytes of host origin.
- MSCs can be used ex vivo to enhance the production of CPCs from single endomyocardial biopsies into therapeutic quantities.

Novelty and Significance

Emerging clinical and preclinical studies demonstrate the therapeutic capacity of MSCs following acute and chronic ischemic cardiac injury. Despite these promising results, whether MSCs engraft and differentiate into myocytes remains controversial, prompting a paracrine hypothesis that MSCs exert therapeutic effects through secreted factors. In this study, we illustrate that MSCs reduce infarct size by regenerating only a maximum of ~8% of newly formed myocardium. The majority of new tissue formed originates from host mechanisms that require interactions with the MSCs. We show for the first time that MSCs prompt a paracrine hypothesis that MSCs may be therapeutic following acute and chronic ischemic cardiac injury.
Bone Marrow Mesenchymal Stem Cells Stimulate Cardiac Stem Cell Proliferation and Differentiation

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SUPPLEMENTAL MATERIAL

Mesenchymal Stem Cell Isolation, Harvest and Labeling

Swine MSCs were isolated and expanded from a single, healthy male Yorkshire donor as previously described\(^1\):\(^2\). Briefly, bone marrow was obtained from the iliac crest, and aspirates were passed through a density gradient to eliminate undesired cell types and were plated with 25ml MEM Alpha media (Mediatech, Manassas, VA) containing 20% fetal calf Serum (Hyclone, Logan, UT) in 162 cm\(^2\) culture flasks (Fisher Scientific, Pittsburgh, PA). At 5-7 days after plating, non-adherent cells were washed away during medium changes and the remaining, plastic adherent, purified MSC population was expanded in culture. The MSC population was then harvested and transduced with green fluorescent protein (Lenti-GFP vector, Lentigen) according to manufacturers’ instructions. All used cells were harvested when they reached 80–90% confluence at passage 5. Labelled MSCs were placed in a cryopreservation solution consisting of 10% DMSO, 5% porcine serum albumin, and 85% Plasmalyte. Cells were placed in cryo bags at a concentration of 5–10 million MSCs/ml and frozen in a control-rate freezer to -180°C until the day of implantation. By using trypan blue staining, the viability of all thawed MSC lots was verified to be >85% before use in the study.

Generation of Concentrated Conditioned Medium (CCM) from MSCs.

Concentrated conditioned medium was generated according to previously described protocols\(^3\), with minor modifications. Confluent cultures of pig MSCs were charged with fresh alpha MEM plus 20% FCS and cultured at 37°C in 5% CO\(_2\) for 7 days. The media was collected and concentrated 10X using an Amicon Ultrafiltration unit with a
10,000 molecular weight retention membrane. The 10x concentrated CM was sterilized using a 0.22 um filter and frozen at -20°C in 10 ml aliquots

**Organotypic Cultures**

Cardiac biopsies were collected from the right ventricular septal wall of 7 Yorkshire swine with or without myocardial infarction. The biopsies were harvested and kept in cold Hank’s Balanced Salt Solution (Lonza) containing 1% penicillin/streptomycin until processing. After washing thoroughly with DMEM (GIBCO), samples were minced in ~1mm³ cubes and digested at 37°C in a solution containing DMEM/F12 (GIBCO), 20% FBS, 1% penicillin/streptomycin and 200 units/ml Collagenase-Type II (Worthington). Following that, whole lysates were collected, washed twice with DMEM, resuspended in DMEM/F12, 20% FBS, 1% penicillin/streptomycin and plated in T-25 tissue culture flasks (Corning) that contained 2-3 x10⁵ GFP⁺ porcine MSCs or not. After 1 week, samples were collected by trypsinization and c-kit⁺ cells were purified by repeated immune panning in a Petri dish as previously described. After 2-3 days in culture, the isolated c-kit⁺ cells were trypsinized and re-plated with F12K (GIBCO), 5% FBS, 10ng/ml bFGF (peprotech), 20ng/ml LIF (Sigma) and 1% penicillin/streptomycin where they grew for 7-10 days. Next, only the non-adherent fraction was collected and expanded as semi-adherent cells in tissue culture dishes containing DMEM/F12, 2% FBS, Insulin-Transferrin-Selenite (Sigma), 10ng/ml bFGF, 10ng/ml LIF, 20ng/ml EGF (peprotech), 100ng/ml SCF (Peprotech) and 1% penicillin/streptomycin. Subsequent immunocytochemical evaluation was performed on cytospin preparations according to manufacturers’ instructions (Sakura Finetek).

**In vitro differentiation assays**
To test the differentiation capacity of CSCs into cardiac myocytes, we performed co-cultures with neonatal rat cardiac myocytes (NRCMs). Briefly, NRCMs were isolated as previously described and plated at a density of 1x10^5 NRCMs/cm² in 12-well plates (Corning) containing collagen-coated glass coverslips. CSCs were then co-cultured with NRCMs in a 1/3 ratio, directly or indirectly using transwell inserts with a 0.4 μm pore size (BD). Co-cultures were maintained with NRCM medium consisting of DMEM (GIBCO), insulin-transferrin-selenite (Sigma), 2mg/ml bovine serum albumin, 20μg/ml ascorbic acid, 1% penicillin-streptomycin and incubated for up to 2 weeks in humidified incubator at 37 °C and 5% CO2. For immunocytochemical evaluation, cells were fixed in 4% paraformaldehyde for 20min at RT, 24h, 72h and 2 weeks after plating.

**Induction of Myocardial Infarction and transendocardial Injections**

Thirty one healthy female Yorkshire swine weighed 25-35 kg, were included in this study. Experimental myocardial infarction was generated according to our previously described protocols. Briefly, the right common carotid artery was canulated under anesthesia induced with ketamine (33 mg/kg, IM) and maintained with isoflurane (1.5-2.0%). MI was induced by accessing the Left Anterior Descending (LAD) coronary artery and occluding it after the first diagonal branch by inflating a coronary angioplasty balloon (2.75x15mm) for 60min followed by reperfusion. All animals were adequately heparinized during the procedure. The study was conducted in 2-phases. In the first phase, we sought to explore the mechanisms underlying MSCs-based cardiac repair, therefore animals received intramyocardial injections of allogeneic GFP labelled porcine MSCs (75x 10^6 cells) or Placebo (Plasmalyte alone, Baxter Edwards Critical Care, Deerfield, IL), three days after MI. The second phase was
designed to address whether MSCs-implantation is necessary for successful cardiac repair or their secreted factors alone could exert similar effects; therefore animals were randomized to receive intramyocardial injections of allogeneic GFP labelled porcine MSCs (100x $10^6$ cells) or the rich in secreted factors conditioned medium were the MSCs had been expanded into, concentrated 10x. All investigators involved in this study were blinded. All injections were performed under fluoroscopy, with a pistol-needle tip injection catheter advanced to the LV through a steerable guide catheter (Stiletto, Boston Scientific, Natick, Massachusetts). Hypokinetic, akinetic, and dyskinetic areas were identified during contrast ventriculography, and injections were performed within and at the borders of the dysfunctional area, as defined by bi-plane ventriculography. A total of 15 injections were performed in each animal, with each injection containing 0.5 ml of the injectate. Each injection was fluoroscopically guided to distribute cells evenly throughout the entire infarct and border zones.

**Cardiac MRI**

For the second phase, therapeutic effect on cardiac function was assessed by Cardiac MR imaging (cMRI), as previously described. Cardiac structure and function were monitored at baseline, 1 day prior to injections, 4 days, 2 weeks and 8 weeks post-injections. Serial cMRI images were acquired with a four channel phase array, 1.5T MR Scanner (Siemens Symphony, Erlangen, Germany) in anesthetized animals with electrocardiography gating and short breath-hold acquisition. The protocol for cine-cMRI has been described before. Briefly, LV Global function was assessed in steady state free precession with a number of slices to cover the entire LV from apex to base. Imaging parameters were as follow: Echo delay time(TE)=1.9 ms, repetition time(TR)= 4.2 ms; flip angle 45°; 256 x 160 matrix; 8 mm slice
thickness/ no gap; 28 cm field of view (FOV) and 1 number of signal average (NSA). Cine images were analyzed with research comprehensive software validated by the Cardiology MR group at Lund University, Sweden (http://segment.heiberg.se).

The protocol included an intravenous bolus of Gadolinium-DTPA (0.1 mmol/kg, 5 m/s.; Magnevist™, Berlex, Wayne) through a peripheral intravenous line. Images were acquired 15 minutes later at the same location as the short axis cine-images. Imaging parameters were TR=7.3, TE=3.3, TI=200 ms; flip angle=25°, 256x196 matrix; 8 mm slice thickness gap 31.2 kHz, 28 cm FOV and 2 NSA. The infarction zone was delineated using an automatic edge detection algorithm, as described.

**Histology**

For the first phase of the study, microscopic evaluation between the treated (n=3), placebo (n=3) and control (n=3) Yorkshire pigs was performed at 2 weeks after the intramyocardial injections. Moreover, in order to assess the time course of MSCs engraftment and differentiation, 8 more animals were sacrificed at 24h (n=2 placebo and n=2 MSCs treated) and 72h (n=3 placebo and n=3 MSCs treated) post-injections. For the second phase of the study, microscopic evaluation between the MSCs and CCM-treated pigs was performed at 2 weeks (n=3 each) and 8 weeks (n=3 each) after TEIs. All animals were humanely euthanized through intravenous infusion with KCL to arrest the hearts in diastole. The explanted hearts were then washed in ice-cold phosphate buffer saline (PBS) to remove any residual blood, followed by perfusions through the left and right coronary arteries with 10% buffered formalin. Heart chambers were then filled with dental impression material (Imprint, 3M ESPE) to preserve heart's shape during fixation. The hearts were then fixed for 24h in 10% buffered formalin and sliced transversely into seven to eight -4mm thick
slices using a commercial meat cutter, weighted and digitally photographed. Representative samples were selected from the infarcted (IZ), border (BZ) and remote areas (RZ) of each slice, and embedded in paraffin (FFPE) for immunohistochemical evaluation. These regions of interest were selected based on CMRI and gross pathology findings; samples were harvested for each of the transverse ventricular sections as follows: (i). One sample from the middle of the scarred, infarcted tissue. (ii) One sample containing the left border of the infarct along with non-scarred tissue; one sample containing the right border of the infarct along with non-scarred tissue. The last two were defined as the border zones macroscopically. Microscopically, within these samples, border zones were defined as the areas that were 1-1 ½ high power fields distant from scarred zones. (iii) One sample from the posterior non-infarcted LV wall. Hematoxylin and Eosin (H&E), as well as Masson’s Trichrome staining were used for the primary histological examination. For confocal immunofluorescence quantification, 4-5μm thick FFPE slides from each region (IZ, BZ, RZ) were evaluated. The total numbers of positively-stained cells were quantified per slide to calculate the number of cells per unit volume (cm³) on each sample. Morphometric analysis was performed by using a custom research package (Image J, NIH, Bethesda, Maryland).

**Immunofluorescence Confocal Microscopy**

Immunofluorescence studies were carried out on 4μm-thick paraffin sections, according to previously described protocols. Briefly, after deparaffinizing and rehydrating the tissue sections, antigen unmasking was performed by microwaving the slides for 20min in citrate buffer Solution, pH=6 (Dako, Carpenteria, CA). The sections were blocked for 1h at RT with 10% normal donkey serum (Chemicon
International Inc, Temecula, CA), followed by 1h incubation at 37°C with the primary antibody. The following antibodies were used: C-kit (kindly provided by Dr. Revilla⁹), α-sarcomeric actinin, α-smooth muscle actin, α-smooth muscle myosin heavy chain, Connexin-43 (Sigma, Saint Louis, MO), N-cadherin, anti-GFP, Laminin, Phospho-Histone H3, cardiac troponin-I (Abcam, Cambridge, MA), GATA-4, MDR1, VE-cadherin, CD3, CD14, CD68 (Santa Cruz Biotechnologies, Santa Cruz, CA), activated Caspase-3 (BD Biosciences, San Jose, CA), Nkx2.5 (R&D systems Inc, Minneapolis, MN), Factor VIII-related antigen (Biocare Medical, Concord, CA), cardiac myosin light chain-2 (Novus Biologicals, Littleton, CO) and KDR (Cell Signaling, Boston, MA). Consequently, the antibodies were visualized by incubating the sections for 1h at 37°C with FITC, Cy3 and Cy5-conjugated F(ab')₂ fragments of affinity-purified secondary antibodies (Jackson Immunoresearch, West Grove, PA). Slides were counterstained with DAPI, mounted with ProLong Antifade Gold reagent (Invitrogen, Carlsbad, California) and stored at 4°C until further examination. Microscopic evaluations and image acquisitions were performed with a Zeiss LSM-510 Confocal Microscope (Carl Zeiss MicroImaging, Inc. Thornwood, NY). The Zeiss Axiovision software (release 4.7.1.0, Carl Zeiss Imaging Solutions, GmbH) was used for 3D rendering of the confocal Z-stack images.

**Fluorescence in Situ Hybridization**

Fluorescence in Situ Hybridization (FISH) was employed to detect the Y-chromosome of the sex-mismatched transplanted allogeneic MSCs in the female porcine hearts. The Y-chromosome containing cells were detected by hybridizing the tissue samples with Cy3-conjugated porcine Y chromosome paints (StarFISH, Cambio Ltd, Cambridge, UK) according to manufacturers’ instructions. Briefly,
following deparaffinization and rehydration, the samples were microwaved for 20 min in citrate Buffer, pH=6 (Dako). After cooling for 30 min at RT, tissues were digested for 3 min at 37°C with pepsin, washed with 2X SSC buffer (Invitrogen) and dehydrated through serial ethanol washing steps. The samples were air-dried and the probe was applied. After covering the samples with a coverslip and sealing them with rubber cement, the samples were placed in the hybridizer (Dako) for denaturation (10 min at 80°C) followed by overnight hybridization at 37°C. The next day, samples were washed with 2X SSC, mounted with DAPI and covered as previously described.


Legends for supplementary video files

**Online Video I.** A 3D reconstructed z-stack confocal series, documenting Y-chromosome (yellow) containing cardiomyocytes (cardiac Troponin-I in red) in the border zone of an MSCs-treated heart 2 months after transplantation.

**Online Video II.** A 3D reconstructed confocal Z-stack series of a differentiated c-kit⁺ CSCs expressing cardiac Troponin I (red). The CSCs were co-cultured with NRCMs in transwell inserts; the striated pattern of cTnI illustrates their full differentiation capacity into mature cardiomyocytes.
Supplementary Figures

Online Figure I

Online figure I. A schematic of the study sequence and timing of procedures.
Online Figure II. Confocal evaluation of FISH controls. Representative negative (A) and positive (B) controls of FISH for the porcine Y-chromosome probes. Negative control is from a female and positive control from a male porcine myocardium. Y-chromosome probes are Cy-3 conjugated (red). Green fluorescence corresponds to the level of autofluorescence. False positive signals in negative controls, show up as yellow fluorescence after overlapping the emission signals following by laser light excitation at 543nm and 488nm (LP560 and BP505-530 filter sets respectively). No Y-chromosome positive cells could be detected in the control female hearts. The sensitivity of FISH in male controls is 45.5±2.1%.
Online Figure III. Phenotype of adult porcine c-kit⁺ CSCs. (A-C) C-kit⁺ CSCs detected in the infarcted myocardium of MSCs-treated swine co-express MDR-1 (green, A), GATA-4 (white, A), N-cadherin (white, B) and Connexin-43 (C, yellow). (D-G) Isolation and expansion of c-kit⁺ CSCs from organotypic cultures of porcine myocardial biopsies supported by MSCs. The c-kit⁺ CSCs are more than 90% MDR-1⁺ (green, D), GATA-4⁺ (white, F) and Nkx2-5⁺ (white, G), while also expressing connexin-43 (green, E).
Online Figure IV

Online figure IV. Dual labelling confocal analysis of the allografts fate. (A), Bargraphs illustrating the cardiovascular fate of MSCs during the first 3 days post-transplantation. Red bars correspond to myocytic (GATA-4+) and yellow bars to vascular (Factor-VIII related antigen+) commitment of the detected GFP+ cells. (B), Dual labelling confocal analysis demonstrating the successful co-localization of GFP (green) with Y-chromosome (red) cells. (C), A male porcine heart that has not received GFP+ cells, documenting the successful detection of Y-chromosome (red), and no GFP signal. (D) A panoramic stitched confocal image illustrating a GFP+ (green) cell stream in the infarcted porcine heart. Yellow arrows indicate co-expression of GATA-4 (white). The insets highlight one of these cells [left inset depicts DAPI (blue) and GATA-4(white) stains; GFP (green) is added in the right inset].
Online Figure V. Cardiomyocyte differentiation of MSCs. Chimeric cardiomyocytes were detected by Confocal Immunofluorescence analysis. Co-localization of α-sarcomeric actinin (red, A) with Y-chromosome (green, B) were used to identify mature cardiomyocytes of exogenous origin. Laminin (white, C) was also used to illustrate morphology of adult myocytes. DAPI (blue, D) was used to stain nuclei. (E) Merged image of panels A-D. Yellow arrows indicate Y-chromosome+ chimeric cardiomyocytes.
Online Figure VI. Cardiomyocytic commitment of MSCs 72h after transplantation. (A) A GFP\(^+\) MSC (green) co-expressing the cardiomyocyte-specific homeobox transcription factor Nkx2-5 (white, arrow). (B), A GFP\(^+\) MSC (green) co-expressing the cardiac muscle specific isoform of troponin-I (red).
Online Figure VII. Vascular commitment of MSCs 72h after transplantation. (A) GFP+ MSCs (green) co-expressing the endothelial cells-specific VE-cadherin (red). (B), A cluster of GFP+ MSCs (green) co-expressing the vascular smooth muscle cell specific protein, smooth muscle myosin heavy chain (red). (C), A cluster of GFP+ cells (green) co-localized with a vascular smooth muscle cell specific protein, α-smooth muscle actin (red).
Online Figure VIII. Putative electrical and mechanical coupling of MSCs. (A,B), Within the first 3 days after implantation, GFP+ MSCs (green) could be detected to co-express the gap-junctional protein Connexin-43 (red, A) indicating putative electrical coupling with the host myocardium, and the adherence glycoprotein N-cadherin (red, B), indicating putative mechanical coupling with the host myocardium.
Online Figure IX. Karyo and cytokinesis of mitotic cardiomyocytes.
A mitotic cardiomyocyte in the MSCs-treated hearts expressing ser-10 phosphorylated Histone-H3 (white, inset). The laminin staining (green) highlights the separation of the cardiomyocyte into two daughter cells, implying that cell division – rather than cell ploidy - occurs.
Online Figure X. Chimeric myocardium exhibits potential electrical coupling. Co-localization of a sarcomeric-actinin (red, A) with Y-chromosome containing cardiomyocytes (white, B) and connexin-43 (green, C) illustrates the potential of the newly formed, chimeric tissue to couple electrically to the host myocardium. DAPI (blue, D) was used to stain nuclei. (E) Merged image of panels A-D. Yellow arrows indicate Y-chromosome⁺ chimeric cardiomyocytes.
Online Figure XI

Online Figure XI. Potential Mechanical coupling of MSCs to the host myocardium. (A), expression of α-sarcomeric actinin (red) illustrates a surviving rim of myocardial tissue within the infarcted myocardium. (B) Expression of the adherence protein N-cadherin (white) between surviving cardiomyocytes and non-cardiomyocytes. (C) A cluster of GFP+ MSCs cells is present within the infarcted tissue. (D) DAPI staining illustrates the nuclear content in the area. (E,E1) The transplanted GFP+ MSCs express N-cadherin indicating their potential to develop adherence connections with the host tissue.
Online Figure XII. Cell-Cell interactions between endogenous and exogenous stem cells. (A,B) Immature MSCs (green) are found within the host myocardium to interact with resident c-kit^+ CSCs (red) by connexin-43 (A, white) and N-cadherin (B, white, arrow) connections, closely resembling cardiac stem cell niches.
Online figure XIII. Organotypic Cultures of porcine heart biopsies with porcine MSCs. (A,B) A small number of cells outgrow from the biopsies cultured alone (A); Organotypic cultures with MSCs have become confluent while, some GFP+ MSCs have infiltrated the heart samples (B). Panels A, B were obtained at 5 days in organotypic cultures. (C) Immunostaining on cytospins from the primary co-cultures illustrating large cell clusters containing c-kit+ and GFP+ cells in proximity. These clusters resemble stem cell niches. (D-D3), Cytospins of purified c-kit+ CSCs, illustrating co-localization with MDR1, while lack of the surface marker CD68 from the vast majority of them excludes an inflammatory or mast cell phenotype (E-E2) Lack of KDR from c-kit+ porcine CSCs. (F-F1) Nkx2-5, is expressed in more than 90% of the CSCs.