Stimulatory Effects of Mesenchymal Stem Cells on cKit+ Cardiac Stem Cells Are Mediated by SDF1/CXCR4 and SCF/cKit Signaling Pathways

Konstantinos E. Hatzistergos, Dieter Saur, Barbara Seidler, Wayne Balkan, Matthew Breton, Krystalenia Valasaki, Lauro M. Takeuchi, Ana Marie Landin, Aisha Khan, Joshua M. Hare

Rationale: Culture-expanded cells originating from cardiac tissue that express the cell surface receptor cKit are undergoing clinical testing as a cell source for heart failure and congenital heart disease. Although accumulating data support that mesenchymal stem cells (MSCs) enhance the efficacy of cardiac cKit+ cells (CSCs), the underlying mechanism for this synergistic effect remains incompletely understood.

Objective: To test the hypothesis that MSCs stimulate endogenous CSCs to proliferate, migrate, and differentiate via the SDF1/CXCR4 and stem cell factor/cKit pathways.

Methods and Results: Using genetic lineage-tracing approaches, we show that in the postnatal murine heart, cKit+ cells proliferate, migrate, and form cardiomyocytes, but not endothelial cells. CSCs exhibit marked chemotactic and proliferative responses when cocultured with MSCs but not with cardiac stromal cells. Antagonism of the CXCR4 pathway with AMD3100 (an SDF1/CXCR4 antagonist) inhibited MSC-induced CSC chemotaxis but stimulated CSC cardiomyogenesis ($P<0.0001$). Furthermore, MSCs enhanced CSC proliferation via the stem cell factor/cKit and SDF1/CXCR4 pathways ($P<0.0001$).

Conclusions: Together these findings show that MSCs exhibit profound, yet differential, effects on CSC migration, proliferation, and differentiation and suggest a mechanism underlying the improved cardiac regeneration associated with combination therapy using CSCs and MSCs. These findings have important therapeutic implications for cell-based therapy strategies that use mixtures of CSCs and MSCs. (Circ Res. 2016;119:921-930. DOI: 10.1161/CIRCRESAHA.116.309281.)

Key Words: coculture techniques ■ mesenchymal stromal cells ■ myocytes, cardiac ■ receptors, CXCR4 ■ stem cell factor

The optimal source of cells for tissue regeneration of the human heart remains controversial. The capacity of the cardiac cKit+ cell to form cardiomyocytes in several murine models has recently intensified this controversy by suggesting a relatively low contribution of this cell type to endogenous cardiomyogenesis. Several findings suggest that the cardiomyogenic capacity of this cell may be endogenously suppressed, on the one hand, and may be augmented in a therapeutic situation, on the other. In particular, an increasing number of experimental and clinical studies (NCT02501811) support the idea that therapeutic responses may be substantially augmented by the interaction of 2 cell types: mesenchymal stem cells (MSCs) and cardiac-derived cKit+ cardiac stem cells (CSCs).

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Here, we hypothesized that MSCs use the SDF1/CXCR4 and stem cell factor (SCF)/cKit signaling pathways to regulate the proliferation, migration, and differentiation of endogenous CSCs. Using a previously described cKitflk^ER(T2)^/ knockin allele, we show that cKit marks postnatal CSCs in the mammalian heart from which a relatively small number of cardiomyocytes are generated after birth. The degree to which the postnatal heart activates endogenous CSCs may be significantly enhanced via cell–cell interactions with MSCs. These interactions are cooperatively regulated via the SDF1/CXCR4 and SCF/cKit signaling pathways (Online Figure I). Thus, MSC–CSC interactions offer...
promoted the outgrowth of both EGFP+ and the red fluorescent (control), and cell migration was assessed using EGFP epifluorescence imaging every other day for 5 days (Figure 6A). Although EGFP-negative cells grew out from myocardial explants, the presence or absence of AMD3100, an inhibitor of the SDF1/CXCR4 signaling pathway, plays an important role in cardiomyocyte functionality and proliferation. Tamoxifen-pulsed PN3 MSCs promoted expansion of CSCs via the SCF/cKit signaling pathway because the spindle-like, Nkx2.5+ in vitro phenotype of postnatal CSCs closely matches the phenotype of the primordial neural crest-derived cKit+ cardiac stem cells that we recently described. Collectively, these findings support that the EGFP+ cells in the postnatal heart are CSCs. Their migratory capacity is limited in chemotactic responsiveness to the postnatal cardiac stroma but is substantially augmented by MSCs.

**Methods**

An expanded Methods section describing all procedures and protocols is available in the Online Data Supplement.

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 (National Institutes of Health, revised 2011). The cKIt+/cKitCreERT2/+;IRG (iPSC) mice have been described elsewhere. Induced pluripotent stem cells (iPSCs) were generated from adult cKIt+/cKitCreERT2+/IRG tail-tip fibroblasts as previously described. Genotyping, tamoxifen injections, gene-expression analysis, lineage-tracing, and histological analysis were performed as previously described. Manufacturing of human and porcine MSCs and CSCs was performed as previously described.

**Results**

**MSCs Stimulate Chemotaxis in Postnatal CSCs**

To characterize the properties of postnatal CSCs, we pulsed postnatal day 2 (PN2) cKIt+/cKitCreERT2+/IRG neonates with a single subcutaneous injection of tamoxifen (n=9) and collected their hearts 24 hours later to analyze enhanced green fluorescent protein (EGFP) expression in culture (Figure 1A). Live tissue imaging and confocal immunofluorescence showed that EGFP marked a noncontractile, cardiac troponin T-negative, proliferative cell population (Online Movie I; Figure 1B through 1I). Importantly, EGFP+ CSCs were consistently present within the myocardial explants (Figure 2B and 2C). Unlike splenic EGFP+ cells, which homogenously expressed CD45+ or CD68+ and exhibited a blast-like phenotype (Figure 2E and 2I), the cardiac EGFP+ cardiac explant-derived cells did not express CD45 or CD68 and had a spindle-like morphology (Figure 2G and 2J), indicating that the EGFP+ cardiac explant-derived cells have a distinct nonepithelioid lineage. Coexpression of Nkx2.5 corroborated the CSC lineage of EGFP+ cardiac explant-derived cells (Figure 3). Notably, the spindle-like, Nkx2.5+ in vitro phenotype of postnatal CSCs closely matches the phenotype of the primordial neural crest-derived cKit+ cardiac stem cells that we recently described. Collectively, these findings support that the EGFP+ cells in the postnatal heart are CSCs. Their migratory capacity is limited in chemotactic responsiveness to the postnatal cardiac stroma but is substantially augmented by MSCs.

**Dichotomous Effects of the SDF1/CXCR4 Pathway on CSC Migration and Differentiation**

SDF1/CXCR4 signaling participates in the mobilization of CSCs in the heart and plays an important role in cardiac neural crest cell migration. It has also been suggested that the therapeutic effect of MSCs is partly attributed to their rich SDF1α secretion. Therefore, we tested the hypothesis that the SDF1/CXCR4 pathway underlies the migratory and differentiation effects of MSCs on CSCs.

We first explored whether the SDF1/CXCR4 signaling pathway is normally active in CSCs, during cardiomyogenesis. Accordingly, we utilized a previously described in vitro model of mouse cardiogenesis, whereby embryoid bodies from induced pluripotent stem cells carrying the cKIt+/cKitCreERT2+/IRG alleles (iPSCs) are directed to undergo stage-specific differentiation into CSCs via transient bone morphogenetic protein (BMP) antagonism (Figure 4A). Similar to postnatal CSCs, iPSC-derived CSCs were migratory EGFP+/Nkx2.5+ cells and were exclusively detected as cells outgrowing from—but not within—the embryoid bodies (Figure 4B through 4D and Movie II). Differentiation of iPSCs to CSCs resulted in stage-specific induction of SDF1α and CXCR4 gene expression (Figure 4E and 4F), suggesting that activation of the SDF1/CXCR4 signaling pathway plays an important role in the development of cardiomyogenic CSCs.

Next, we tested the role of SDF1/CXCR4 in postnatal CSCs. Myocardial explant culture assays (n=2) were generated as described above, to analyze the emergence of EGFP+ cells in the presence or absence of AMD3100, an inhibitor of the SDF1/CXCR4 axis. Consistent with the previous finding, exposure to AMD3100 did not block outgrowth of the red fluorescent protein variant DsRed-express+ cells, but prevented EGFP+ CSCs from migrating from the cultured explants in the presence of porcine MSCs, strongly suggesting that the chemotactic effect of MSCs on CSCs is regulated via the SDF1/CXCR4 signaling pathway (Figure 5B through 5D). Moreover, exposure to AMD3100 resulted in a ~29-fold increase in the rate of CSC differentiation into spontaneously contracting cardiomyocytes (from 0.57±0.57% to 16.4±4.03% EGFP+ beating cells; p=0.0001; Figure 5B; Online Movie III).

**MSCs Promote Expansion of CSCs via the SCF/cKit Signaling Pathway**

We investigated whether the increased abundance of EGFP+ cells reflects increased mobilization because of the chemotactic responsiveness of CSCs to MSCs, or to an increase in CSC proliferation. Tamoxifen-pulsed PN3 cKIt+/cKitCreERT2+/IRG myocardial explant culture assays (n=6) were performed as described above with or without porcine MSCs, and the growth of EGFP+ CSCs was continuously quantified using live tissue epifluorescence imaging every other day for 5 days (Figure 6A). Although EGFP-negative cells grew out from myocardial explants,
reaching >50% confluency within 5 days of culture, growth of EGFP+ CSCs was less robust although their number increased significantly over time (Figure 6B). Intriguingly, in the absence of MSCs, EGFP+ cell expansion occurred primarily within the explanted tissue, whereas in the presence of MSCs, CSCs not only proliferated within the explant but also migrated out and expanded freely from the myocardial explants within the MSC feeder layers (Figures 2 and 5). However, the abundance of CSCs was not significantly affected by the presence of MSCs compared with controls (Figure 6B).

We previously showed that the combination of MSC feeders and exogenous SCF, the ligand of cKit, significantly improves the ex vivo propagation of CSCs.9 Consequently, we investigated whether the SCF/cKit signaling pathway is important for CSC migration and proliferation. Accordingly, tamoxifen-pulsed PN3 cKitCreERT2;IRG myocardial explant culture assays were performed as described above with or without porcine MSCs (n=6), and the growth of EGFP+ cells was quantified using live tissue epifluorescence imaging in the presence or absence of recombinant murine SCF (Figure 6A). Surprisingly, the presence of both MSCs and exogenous SCF produced a 3.5-fold increase in the abundance of EGFP+ cells compared with MSCs alone (Figure 6B through 6D; P=0.001). The effects of SCF and MSCs on the abundance of EGFP+ cells were abrogated when we neutralized SCF/cKit signaling with an anti–murine cKit antibody (Figure 6E). Similarly, EGFP+ cell abundance was significantly reduced (P≤0.0001) in the presence of the SDF1/CXCR4 pathway inhibitor, AMD3100 (Figure 6E through 6G).

Interestingly, modulation of the SCF/cKit signaling pathway, either through its exogenous activation with recombinant murine SCF or its neutralization with an anti–murine cKit antibody, did not significantly alter EGFP+ cell migration and differentiation (Figure 6E and 6H).

A Proportion of Myocardium Is Generated After Birth by Neonatal cKit+ Cells

We next tested whether postnatal CSCs generate new cardiomyocytes in vivo.27–29 cKitCreERT2;IRG neonates (n=6) were pulsed with tamoxifen on PN3 and PN4, and genetic fate-mapping analysis was performed at PN7 (Figure 7A). Expression of EGFP was conditional on the expression of both the cKitCreERT2/+ and IRG alleles and did not occur without tamoxifen induction (Figure 7B).

At PN7, EGFP epifluorescence was detected in all expected cKit-expressing tissues, including blood, skin, testes, intestine, lungs,3,30–32 kidney (Figure 7C), and teeth (Figure 7D). Using live tissue imaging, EGFP+ cells were visualized throughout the heart, including spontaneously beating cardiomyocytes within the left and right ventricular walls and the ventricular apex, suggesting that a proportion of neonatal cardiac cells expressed cKit at the time of tamoxifen treatment (Figure 7E; Online Movies III through VI).

A mean of 76.9.3±4.5 EGFP+ cells were detected per heart section analyzed, 20.9±2.4% of which were tropomyosin+ ventricular cardiomyocytes (Figure 7F and 7G; Online Movie VII). In addition, 6.5±1.7% of atrial EGFP+ cells...
coexpressed tropomyosin, indicative of fully differentiated atrial cardiomyocytes (Figure 7G). Intriguingly, we detected only 1 EGFP+/Tropomyosin+ mononucleated ventricular cardiomyocyte undergoing mitosis in the 2 neonatal mouse hearts in which we quantified cKit-mediated cardiomyogenesis, suggesting that cKit-dependent cardiomyogenesis and cardiomyocyte proliferation represent 2 distinct mechanisms by which the neonatal mouse heart generates cardiomyocytes (Figure 7F). In addition, and consistent with our previous finding, the expression of EGFP was observed in neuronal-like cardiac cells, ventricular epicardium, and cardiac valves, but not in coronary vascular cells (Figure 7G through 7K).

Figure 2. Mesenchymal stem cells (MSCs) stimulate outgrowth of cKit+ cardiac stem cells (CSCs) from cardiac explants. A, Schematic of the ex vivo lineage-tracing experiments to assess the effect of MSCs on cKit+ cardiac cells. B and C, Ex vivo culture of a cKitCreERT2;IRG myocardial explant on days (d) 3 (B) and d5 (C), after coculture with MSCs. D, Representative flow cytometric analysis of EGFP and cKit-allophycocyanin (APC) colocalization in the spleen. E, Live epifluorescence imaging of EGFP and the red fluorescent protein variant DsRed-express (DSRED) in spleen cells of A, before fluorescence-activated cell sorter (FACS) analysis. F, Representative flow cytometric analysis of EGFP and cKit-APC colocalization in the heart. G, Live epifluorescence imaging of EGFP and DSRED in heart cells of C, before FACS analysis. H, Summary of Cre-mediated recombination efficiency in cKit+ cardiac and spleen cells (n=3 per group). I, Immunostaining of neonatal spleen explant-derived cells with a cyanine-5 CD45/CD68 antibody cocktail (pseudocolored red). All spleen EGFP+ cells have a blast-like morphology and exhibit strong CD45/CD68 immunoreactivity. J, Immunostaining of neonatal cardiac explant-derived cells from the same mouse, with a cyanine-5 CD45/CD68 antibody cocktail. All cardiac EGFP+ cells have a spindle cell-like morphology and are negative for CD45/CD68. Scale bars, 200 μm. EGFP indicates enhanced green fluorescent protein.

Figure 3. Colocalization of EGFP and Nkx2.5 in cardiac explant-derived cells from cKitCreERT2;IRG mice. A–C, Fluorescent immunocolocalization of EGFP (A) and Nkx2.5 (B) in cardiac explant-derived CSCs. D, E, and F, Blown-up images of the areas delineated with insets in A, B, and C, respectively. Scale bars, 200 μm. EGFP indicates enhanced green fluorescent protein.
Stimulation of Dilated Cardiomyopathy Human CSCs by Human MSCs

Finally, we sought to investigate the clinical relevance of our neonatal mice and iPSCs findings and tested whether isolated CSCs from chronically diseased human (h) hearts (hCSCs) are chemotactically responsive to hMSCs, under both normal and hypoxic conditions. We prepared hCSCs from 3 dilated cardiomyopathy patients admitted to our center, after percutaneous endomyocardial biopsy as previously described.8 We used 3 hMSCs lines previously established in our laboratory from healthy donors (Online Figure IV A).13 Flow cytometric analysis indicated that 98.85±0.5% of human CSCs expressed cKit (Online Figure IVB through IVD). In addition, similar to mouse CSCs, hCSCs expressed Nkx2.5 and the neural crest-specific marker Pax3 (Online Figure IVE and IVF). A fluo-8 intracellular Ca2+ mobilization-based assay of CXCR4 activity, with a Chem-1 cell line transduced to overexpress human CXCR4 and the G protein Gα15, illustrated activation of the human SDF1/CXCR4 pathway by hMSCs; and that this effect is blocked in the presence of 1 μmol/L AMD3100 (data not shown).

The migratory capacity of hCSCs was tested in a transwell migration assay. Accordingly, 1×10^5 hCSCs were suspended in serum-free medium and loaded into the upper wells of Boyden chambers. To test hCSC chemotactic responsiveness to hMSCs, the lower wells of the Boyden chambers were coated 24 hours earlier with 1×10^5 mitotically inactivated hMSCs in serum-free medium. hCSCs were allowed to migrate for 24 hours, in either 20% O2 or 0.5% O2 (Online Figure VA). Quantification of the migrated cells corroborated a marked increase in hCSCs mobilization by hMSCs. The chemotactic effect of hMSCs was abrogated in the presence of AMD3100 (Online Figure VB through VE). Interestingly, exposure of the cell cultures to 0.5% O2 resulted in acute loss of the migratory activity of hCSCs, which could be partially rescued in the presence of hMSC (Online Figure VE). Gene-expression analysis illustrated that exposure of hCSCs and hMSCs to hypoxia did not affect the expression of CXCR4, which was highly expressed in hCSCs but not in hMSCs (Online Figure VF), but resulted in a significant downregulation of SDF1α expression, both in hCSCs and hMSCs (Online Figure VG).

**Figure 4.** Expression of SDF1α and CXCR4 during derivation of cardiac cKit+ cells (CSCs) from iPSC. A, Schematic of the experimental design. B, Confocal immunofluorescence illustrates that the generation of CSCs from iPSC involves migration of CSCs from the embryoid bodies (EBs, solid line). C and D, High magnification of the area delineated by the inset in B illustrates colocalization of EGFP with Nkx2.5 in the migrated CSC derivatives. E and F, Gene-expression analysis during differentiation of iPSC to CSCs shows a dramatic upregulation in SDF1α and CXCR4. n=3; Scale bars, 100 μm. EGFP indicates enhanced green fluorescent protein.

**Discussion**

A major hypothesis in the field of cell-based therapy is that different cell types from different tissue origins can have additive effects on tissue repair. One particular promising example is that of cKit+ CSCs and MSCs, a concept that has formed the basis for several clinical trials, including the CONCERT-HF (Combination of Mesenchymal and C-kit+ Cardiac Stem Cells as Regenerative Therapy for Heart Failure trial).7–10,16,17,33–35 Here, we have uncovered the signaling mechanisms responsible for these interactions. We have shown that SDF1α and SCF
secreted by bone marrow–derived MSCs have important effects on CSCs. SDF1α promotes migration of cardiac-derived CSCs and enhances their lineage commitment toward contractile cardiac myocytes, whereas the cKit receptor activation by SCF plays an important role in CSC proliferation (Online Figure I). These data provide the mechanistic basis by which MSCs activate endogenous CSCs to migrate, proliferate, differentiate, and stimulate repair in the diseased heart.

Our data are in agreement with earlier studies documenting that cell–cell interactions between MSCs and CSCs are associated with enhanced regeneration and therapeutic benefit.9,10,14,18,25,36 We previously showed in a large animal model of myocardial infarction that transplantation of MSCs produces significant improvements in heart function and scar size reduction and that this effect is partly attributed to a transient mobilization of CSCs at the sites of MSCs engraftment.9 Furthermore, we9,8 and others10 recently showed that when MSCs and CSCs are cotransplanted as a cell mixture, the therapeutic benefit may be further enhanced.

Here, we have used several in vivo and ex vivo sophisticated experimental models, to test these cellular interactions. Our ex vivo genetic lineage-tracing studies, and our experiments with human CSCs and MSCs, document a chemotactic effect of MSCs on CSCs that relies on the SDF1/CXCR4 signaling pathway. Although our transwell experiments with human CSCs and MSCs indicate that direct cell–cell interactions between MSCs and CSCs are not necessary for in vitro chemotaxis, experiments in large animal models of ischemic heart disease show that administering the MSC secretome is not a therapeutically sufficient substitute for MSC transplantation.9 Accordingly, these data do not exclude cell–cell effects, in addition to paracrine effects, as the underlying mechanisms of cell-based therapies.

We observed that MSCs significantly enhance CSC growth rates in the presence of SCF. Not only based on previous reports23,37 but also based on the white-spotting phenotype that is often produced from mutations in the cKit and SCF loci, we expected that the SCF/cKit signaling pathway would...
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affect CSCs mobilization by MSCs. However, we did not find evidence that modulation of SCF/cKit is sufficient to control CSC mobilization. Importantly, cKit^{CreERT2/+}−labeled cells in other postnatal tissues exhibited full ex vivo migratory capacity (Online Figure III), suggesting that the observed effect is unlikely because of an underlying cKit haploinsufficiency. Notably, consistent with our findings, a recent study reported that the activation of SCF/cKit signaling alone is not sufficient to regulate CSC migration and requires transactivation of SDF1/CXCR4.23

A recent report showed that Ephrin signaling also regulates CSC-trafficking CSC.38,39 MSCs express Ephrins40; therefore, it is possible that part of the chemotactic effects of MSCs on CSCs that were documented in this study are Ephrin mediated. Interestingly, developmental studies indicate that the 2 signaling pathways may control different aspects of cardiac neural crest cell migration.41 Signaling through Ephrins has been suggested to provide directional guidance, whereas SDF1/CXCR4 is thought to regulate motility of cardiac neural crest cells. Our findings suggest that SDF1/CXCR4-mediated inhibition of CSC migration may be required for triggering differentiation into cardiomyocytes. It would be interesting to explore whether MSCs could also enhance directional guidance of CSCs into the damaged myocardium via ephrin-mediated signaling.

Using the cKit^{CreERT2/+};IRG reporter mouse line, we were able to track the fate of cKit+ cells in the heart (≈60% EGFP+). Although this finding...
seems to contrast with other recent cKit lineage-tracing studies,3,5,6 we think that this discrepancy reflects an apparent lower fate-mapping sensitivity of the cKitCreERT2/+ mouse compared with the other 6 cKit knockin models.12 Apparently, although all 7 cKit knockin alleles3–6 exhibit similar sensitivity in tracing cKit-derived cardiomyocytes,12,43 the cKit CreERT2/+ allele specifically marks the cardiomyogenic over the vasculogenic postnatal cKit+ lineages because if they were of same ancestry, we should have detected at least a few vascular derivatives with the cKitCreERT2/+ allele.

We also examined whether the expression of reporter genes in cardiomyocytes indicates CSC differentiation4,44,45 or in situ expression of cKit in cardiomyocytes.6 Our in vitro experiments with cells derived from mice with the cKitCreERT2/+ allele show that expression of reporter genes marks noncontractile, proliferative, and migratory cKit+ cells, and not differentiated cardiomyocytes. These findings are consistent with previous reports.1,4,44,46 Nonetheless, because of the apparently lower cardiac sensitivity of the cKitCreERT2/+ allele compared with other cKit knockins,3,6 we do not exclude the possibility that a population of cardiomyocytes, undetectable by our reagents, express cKit.

To this end, we would like to acknowledge many limitations of the knockin models compared with transgenic approaches, which may have influenced the cKit lineage-tracing findings reported by us and others.3,5,6 For example, coat color differences between the wild-type and cKit knockin mice likely suggest improper function of neural crest cells because of cKit haploinsufficiency. It is possible that this deficit also affected cardiac cKit+ cells to some extent; therefore, our findings may have somewhat underestimated the role of cKit+ cells in the heart.

In summary, our findings indicate that postnatal CSCs are bona fide cardiomyogenic stem cells from which new cardiomyocytes are generated after birth. We further show that a biologically important relationship exists between MSCs and CSCs, which involves the SCF/cKit and the SDF1/CXCR4 pathways, and may be harnessed for therapeutically enhancing the degree of cardiomyogenesis from endogenous CSCs. Together these findings support the development of novel cell combination-based therapies for the prevention and treatment of heart disease.

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Dr Hare discloses a relationship with Veston Inc that includes equity, board membership, and consulting. Dr Hatzistergos and K. Valasaci disclose a relationship with Veston Inc that includes equity. Veston Inc did not participate in funding this work. The other authors report no conflicts.

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**What Is Known?**
- Because bone marrow MSCs stimulate endogenous CSCs, the idea of mixing MSCs and CSCs to create a novel and potent cell-based therapeutic has arisen.
- Although mixtures of MSCs and CSCs have enhanced therapeutic effects in porcine models, the precise molecular and cellular mechanisms for this interactive effect are not fully known.

**What New Information Does This Article Contribute?**
- Using organotypic coculturing and genetic labeling of CSCs, this study shows that MSCs stimulate profoundly the abundance and migration of CSCs.
- MSCs promote CSC proliferation via the SCF/cKit signaling axis.
- MSCs induce CSC migration and differentiation into de novo cardiomyocytes via regulation of the SDF1/CXCR4 signaling axis.

**Summary**
Combining MSC and CSCs is a potential means to augment therapeutic cell-based repair of the injured heart. Although animal models support the idea that these mixtures have enhanced efficacy to reduce infarct scar in acute myocardial infarction and enhanced left ventricular performance in chronic heart failure, the mechanistic basis for MSC–CSC interactions action are incompletely understood. Here, we show that MSCs stimulate CSCs to proliferate, migrate, and differentiate in de novo cardiomyocytes, by regulating the SCF/cKit and SDF1/CXCR4 signaling pathways. Using iPSC modeling, we demonstrate that developmental activation of SDF1/CXCR4 signaling is involved in CSC specification, migration, and differentiation into Nkx2.5+ cardiomyocytes. Paradoxically, genetic lineage tracing in neonatal mice reveals loss of migratory and proliferative activity of postnatal CSCs, which may be recovered via MSC-dependent activation of SDF1/CXCR4. Antagonism of MSC-mediated SDF1/CXCR4 signaling by AMD3100 inhibits postnatal CSC migration and proliferation but promotes differentiation into beating cardiomyocytes. On the other hand, SCF/cKit signaling enhances CSC proliferation but not migration. Human MSCs elicit similar effects in culture-expanded adult human CSCs. Together, these findings offer novel insights into the mechanisms of cell-based cardiac repair and support the concept that MSC–CSC interactions may be deployed therapeutically to enhance cardiac regenerative repair.
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DETAILED METHODS.

Mice. The \( \text{cKit}^{\text{CreERT2/}} \) and \( \text{IRG} \), mice that were employed in this study, have been previously described \(^1\).

Tamoxifen-induced recombination. To induce \( \text{CreER}^{\text{T2}} \)-mediated recombination in \( \text{cKit}^{\text{CreERT2}};\text{IRG} \) neonates, tamoxifen (Sigma) was dissolved in peanut oil (Sigma) at a final concentration of 20mg/ml by shaking overnight at 37°C, and was administered via single subcutaneous injections (50µl/injection) at selected time points. For inducing \( \text{CreER}^{\text{T2}} \)-mediated recombination \textit{in-vitro}, the active metabolite of tamoxifen, (Z)-4-hydroxytamoxifen (4-OH tamoxifen; Sigma), was dissolved in 100% ethanol (Sigma) and used at a final concentration of 1µM, supplemented in the culture medium.

Porcine MSC isolation, expansion and inactivation. Swine MSCs were isolated and expanded from a single, healthy Yorkshire donor as previously described \((7, 44)\). 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 Bovine Serum (Hyclone) in 162cm\(^2\) 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. All used cells were harvested when they reached 80–90% confluence at passage 3-4, and inactivated for 3h at 37°C with 10µg/ml of mitomycin-C (Sigma). Inactivated porcine MSCs were collected by trypsinization and cryopreserved in liquid nitrogen until use. For all experiments, MSCs were seeded at a concentration of 2x10\(^5\) cells/ml a day before use.

\textit{Ex-vivo} analysis of mouse CSCs. Three-day old neonatal mouse hearts (PN3) were harvested, washed in ice-cold HBSS and cleaned from unwanted tissues under a stereomicroscope (VistaVision). They were then transferred in a tissue-cultured hood and, after
additional washing steps with DMEM (GIBCO) under sterile conditions, were minced into ~2-3 mm³ fragments and digested in a solution of DMEM/F12 (GIBCO), 20% FBS, 1% penicillin/streptomycin and 200 units/ml Collagenase-Type II solution (Worthington) at 37°C. Digested tissue explants were then collected and washed twice with DMEM to remove residual enzyme. Single tissue fragments were then hand-picked under sterile conditions with a stereomicroscope and a micropipette, and cultured individually in each well of gelatin-coated 24-well plates, with or without 1x10⁵ MSCs. Samples were fed every other day. The basic myocardial explant feeding medium consisted of DMEM/F12, 15% FBS (Atlas), 1% penicillin/streptomycin (GIBCO), 1% β-mercaptoethanol (GIBCO), 1000 units/ml recombinant mouse LIF (Millipore), 1 ng/ml recombinant mouse bFGF (Peprotech), and 0.1 mM nonessential amino acids (GIBCO). The impact of SCF was tested by supplementing the medium with 100 ng/ml recombinant murine SCF (Peprotech). For cKit neutralization, 100 ng/ml of rat anti-murine cKit antibody (eBioscience, clone 2B8) were supplemented to the medium. For assessing the role of SDF1/CXCR4, AMD3100 (Sigma) was supplemented to the medium, at a final concentration of 1 µM. Samples were then monitored daily, and growth of EGFP⁺ cells was quantified every other day for 5-8 consecutive days based on EGFP epifluorescence, under a fluorescent microscope (Olympus IX81). Analysis of Cre-recombination efficiency was performed using flow cytometric analysis of EGFP epifluorescence and cKit immunofluorescence co-localization (anti-cKit APC, eBiosciences) in a BD LSR-II analyzer. To explore whether DSRED⁺ cardiac explant derived cells correspond to CSCs or could activate expression of cKit in culture, DSRED⁺ cells were manually picked from 1-week old, 4-OH treated cardiac explant cultures under a fluorescence microscope (EVOS), and propagated in Geltrex-coated dishes (Invitrogen) for 50 days, in the presence of 4-OH tamoxifen.

**Generation of CSCs from iPSCkit.** The generation of mouse iPSCkit has been previously described. Differentiation into mouse CSCs was performed as previously described. Briefly, iPSCkit were differentiated into embryoid bodies (EBs), via transient antagonism of the bone
morphogenetic protein pathway (BMP) with Noggin (R&D systems) or the small molecule dorsomorphin (Tocris). 4-OH tamoxifen was supplemented in the medium from EB-Day 10 onwards, in order to monitor the induction of EGFP+ CSCs. Successful differentiation of mouse iPSC into CSCs was monitored with gene-expression analysis as well as the development of spontaneously contracting EGFP+ embryoid bodies after EB-Day 10.

**Human CSCs and MSCs.** All human (h) cells were manufactured by the Foundation for Accreditation of Cellular Therapy (FACT)-accredited Good Manufacturing Practice (GMP) Cell Production Facility at the Interdisciplinary Stem Cell Institute, University of Miami Miller School of Medicine, as previously described. hMSCs were derived from the bone marrow (iliac crest aspiration) of three healthy donors. hCSCs were isolated from single,1-2 mm endomyocardial biopsies, obtained from the septal wall of the right ventricle, as previously described. Briefly, for hCSC extraction, biopsies were grown in Ham’s F12 medium (Life Technologies, Grand Island, NY) containing 10% fetal bovine serum (FBS), 0.2mM L-glutathione, 5 mU/mL erythropoietin, 10 ng/mL human fibroblast growth factor (Recombinant Human FGF-basic PeproTech), and 1% glutamine/penicillin/streptomycin (GPS; Life Technologies). hCSCs that migrated out of biopsies were isolated using the Human CD117 Microbeads kit (Miltenyi cat#130-091-332) and processed through the MACS separation system (Miltenyi). hMSCs were released for further studies after meeting the following criteria: negative for mycoplasma via polymerase chain reaction, ≥70% cell viability, growth assay via colony forming unit-fibroblasts assay, positive for CD105 (>80%) and negative for CD45 by flow cytometry, and no growth of bacteria. hCSCs were released for further studies after meeting the following criteria: negative for mycoplasma via polymerase chain reaction, ≥70% cell viability, growth assay via colony forming unit-fibroblasts assay, positive for CD117 (>80%) and negative for CD45 by flow cytometry, and no growth of bacteria. The following antibodies were used for flow cytometry:

IgG2bk-APC Isotype control (e-Biosciences 174031-82); cKit APC (e-Biosciences 17-1179-42);
IgG1 FITC Isotype control (Beckman C, IM0639U); CD45 FITC (Beckman, IM0782U); CD105 PE (Fitzgerald, 61R-610cHUPE).

**Migration Assays.** For the trans-well migration assays, 1x10^5 cryopreserved hCSCs were suspended in cell migration medium (DMEM (GIBCO), 1% glutamine/penicillin/streptomycin (GPS; Life Technologies), and 0.1% bovine serum albumin fraction V (Thermo Fisher Scientific) and plated in triplicates on the upper well of a modified Boyden chamber with 8µm pores (Corning), in 24 well plates (Corning). For testing the MSCs chemotaxis, 1x10^5 cryopreserved hMSCs were re-suspended in cell migration medium and plated in triplicates in the 24-well plates, one day before thawing the hCSCs. Treatment with AMD 3100 was performed as described above. All samples were cultured under 5% CO\textsubscript{2} atmosphere at 37°C, either in 20% O\textsubscript{2} or in a hypoxia chamber at 0.5% O\textsubscript{2} (Coy Labs). For quantification of cell migration, cells at the upper chamber of the Boyden chamber were wiped with cotton swab 24h after plating. The lower chamber of the transwell insert was fixed for 10min with 70% ethanol and the migrated cells were visualized with crystal violet (Sigma) and manually quantified in an inverted light microscope (Zeiss) using a hemocytometer.

**Immunohistochemistry and confocal microscopy.** Samples were fixed for 1-1.5 h in 4% PFA (EMS) at room temperature followed by overnight incubation in 30% sucrose (Calbiochem) at 4°C. Next day, samples were embedded in OCT (EMS) and flash-frozen in liquid nitrogen. Cryosectioning was performed as previously described (46). For immunohistochemistry, 10µm-thick samples were post-fixed for 10min with 4% PFA and blocked for 1h at RT with 10% normal donkey serum (Chemicon), followed by overnight incubation at 4°C with the primary antibody. Subsequently, samples were visualized by incubating the sections for 1h at 37°C with FITC, Cy3 and Cy5- conjugated F(ab')\textsubscript{2} fragments of affinity-purified secondary antibodies (Jackson Immunoresearch) or Alexa 488 and Alexa 546 dyes (Molecular probes). The primary antibodies used were EGFP (1:500 dilution, Aves GFP-1020), Nkx2.5 (1:50 dilution, Santa Cruz Biotechnologies, SC-14033 and SC-8697), CD68 (1:100 dilution, Santa Cruz Biotechnologies,
SC-9139), CD45 (1:100 dilution, Abcam, ab10558), Cardiac Troponin I (1:100, Abcam, ab10231) and Tropomyosin (1:100 dilution, Abcam ab7786). Samples were counterstained with DAPI, mounted with ProLong Antifade Gold reagent (Molecular Probes,) and stored at 4°C until further examination. Microscopic evaluations and image acquisitions were performed with a Zeiss LSM-710 Confocal Microscope (Carl Zeiss MicroImaging, Inc. Thornwood, NY), using the Zeiss ZEN software (version 2009, Carl Zeiss Imaging Solutions, GmbH); or with an Olympus IX81 fluorescent microscope using the Image Pro Premier software (Version 9.04; 64-bit; Media Cybernetics).

**Gene-expression analysis.** Total RNA was extracted from iPSC at selected time points before and during the course of their differentiation into cardiomyocytes, using the RNeasy mini plus kit, according to manufacturers’ instructions (Qiagen). Complementary DNA synthesis was performed using the high capacity cDNA reverse transcription kit, according to manufacturers’ instructions (Applied Biosystems). Quantitative PCR was performed using Taqman Universal Master mix in a iQ5 real time PCR detection system (BioRad). All samples were run in triplicates and normalized to a GAPDH endogenous control. Relative fold change was calculated using the $2^{-\Delta\Delta Ct}$ method. The IDs for the Taqman Gene-expression assays are the following: SDF1α: Hs03676656_mH; CXCR4: Hs00607978_s1; GAPDH: Hs02758991_g1.

**CXCR4 activity assays.** We employed a Chem-1 cell line, transduced to overexpress human CXCR4 and the G protein Gα15 (Millipore, HTS004RTA), in which the effects of CXCR4 modulators can be genetically tracked via fluorescence-based assays of intracellular Ca²⁺ mobilization. The CXCR4-Chem1 cell line was loaded with a Fluo-8 Ca²⁺ dye (Abcam, ab112129), and dose-dependent responses of intracellular Ca²⁺ mobilization to recombinant human SDF1α (Thermo Scientific), AMD3100 (Abcam), or hMSC were assessed in a 96-well plate format, with a microplate reader (Molecular Devices, Spectramax, M5), according to manufacturer’s instructions. For the condition medium, 1x10⁵ hMSCs per well were plated in 6-well plates with regular hMSC
medium. The next day, the medium was replaced with 2ml of serum-free CXCR4-Chem1 cell medium (Millipore), and returned to the incubator for 24h. For the 96-well assay, 20µl of CXCR4-Chem1 hMSC-conditioned medium were used per well.

Statistics.
Statistical analyses were performed using GraphPad Prism version 5.00 for Windows. A two-way repeated measures ANOVA, followed by Bonferroni post-hoc tests, was employed for comparing in-vitro quantification data of EGFP+ cells. A one-way ANOVA, followed by Tukey’s post-hoc tests, was performed to compare migration of human cells and gene-expression analysis. All data met the assumptions of the tests. To analyze the effect of AMD3100 in cardiomyocyte differentiation of CSCs, a Mann-Whitney test was employed to compare differences between groups. This test was chosen due to a non-normal distribution of the values and significant differences in the variation within groups (Bartlett’s test). A p<0.05 was considered statistically significant. All values are reported as mean±SEM.

Study Approval.
The heart failure patients and healthy MSC subjects recruited for this study are enrolled in POSEIDON-DCM (NCT01392625), “A Phase I/II, Randomized Pilot Study of the Comparative Safety and Efficacy of Transendocardial Injection of Autologous Mesenchymal Stem Cells Versus Allogeneic Mesenchymal Stem Cells in Patients with Nonischemic Dilated Cardiomyopathy”. This study had institutional review board approval from the University of Miami Miller School of Medicine, and all patients gave written informed consent prior to inclusion in the study 2. The animal studies were 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 (National Institutes of Health, revised 2011).
Online Figure I. Summary Figure. Lineage fate-mapping analysis illustrates that postnatal CSCs (green, oval), are a proliferative and migratory cell population that generate de-novo cardiomyocytes (green, rounded rectangle) in the neonatal mouse heart (red). Cell-cell interactions with MSCs (grey) enhance proliferation and migration of CSCs, via the SCF/cKit (green gradient) and SDF1/CXCR4 (blue gradient) signaling pathways. Blocking the SDF1/CXCR4 pathway by AMD3100, inhibits proliferation and migration of CSCs, while inducing their differentiation into cardiomyocytes.
Online Figure II. Explant-derived cells are not CSCs. A-B, Live-cell epifluorescence imaging of DSRED and EGFP expression in DSRED+ explant-derived cells, manually-picked from $cKit^{CreERT2/+};IRG$ heart explant cultures, and propagated for 50 days in the presence of 4-OH tamoxifen. DSRED+ cells do not acquire expression of EGFP with prolonged culture indicating that they are not CSCs. C-D, Live-cell epifluorescence imaging of DSRED and EGFP expression in EGFP$^+$ explant-derived cells, manually-picked from the same $cKit^{CreERT2/+};IRG$ heart explant culture that cells in panels A-F were derived, and propagated for 50 days in the presence of 4-OH tamoxifen. EGFP, enhanced green fluorescent protein. Scale bars, 200µm.
Online Figure III. Migratory capacity of cardiac- and dental-explant-derived cells. A, Live-tissue epifluorescence of an explanted myocardial sample, 4 days after plating on a gelatin-coated vessel. Cell outgrowth from myocardial explants is robust. However, expression of EGFP is confined in cells within the explant tissue borders. B, Live-tissue epifluorescence of an explanted developing incisor. In contrast to the myocardial explant, dental explant-derived EGFP⁺ cells hold full capacity to migrate along with DSRED⁺ cells, suggesting that the lack of migratory activity of cardiac EGFP⁺ cells is unlikely to be due to the hypomorphic Kit-CreERT2/+ knocked-in allele. Inset depicts a low-power, magnification, live-tissue epifluorescent image of the incisor. EGFP, enhanced green fluorescent protein. Scale bars, 200µm.
Online Figure IV. Characterization of hMSCs and hCSCs. A, Flow cytometric analysis of CD105 and CD45 expression in the 3 hMSCs lines. B-C, Representative examples of cKit expression in culture-expanded CSCs. D, Flow cytometric analysis of cKit and CD45 expression in the 3 hCSCs lines E, Gene-expression analysis of Nkx2.5 in hCSCs and hMSCs. F, Gene-expression analysis of Pax3 in hCSCs and hMSCs.
Online Figure V. Human MSCs stimulate hCSCs from patients with dilated cardiomyopathy. A-C, Fluorescent immunocolocalization of EGFP (A) and Nkx2.5 (B) in cardiac explant derived cells. Panels D-F are blown-up images of the areas delineated with insets in panels A-C, respectively. AMD, AMD3100. Scale bars, 200µm.
Supplemental References.


Supplemental Legends for Video Files.

Online Video I. Explant culture of $cKit^{\text{CreERT2}};IRG$ neonatal mouse heart. EGFP epifluorescence in a myocardial explant from a tamoxifen-pulsed PN3 $cKit^{\text{CreERT2}};IRG$ mouse heart. EGFP becomes detectable within 48h after culture. EGFP$^+$ CSCs are primarily located within the spontaneously beating myocardial explant, whereas cells outgrowing from the tissue are exclusively non-contractile DSRED$^+$ cells.

Online Video II. Modeling CSCs migration and differentiation in iPSCs. Live epifluorescent imaging illustrating that the development of spontaneously contracting EGFP$^+$ clusters of iPSC$^{\text{kit}}$-derived CSCs involves their outgrowth from the embryoid bodies. Note that the EGFP$^+$ cells retained within the embryoid body (bottom right corner) are not contractile.

Online Video III. AMD3100 induces differentiation of CSCs. Spontaneously contracting EGFP$^+$ cells in a PN3 $cKit^{\text{CreERT2}};IRG$ myocardial explant, four days after culturing in the presence of AMD3100. The EGFP$^+$ cell in green inset depicted at higher magnification in the right panel.

Online Video IV. De-novo postnatal cardiomyogenesis from CSCs in the right ventricular myocardium. Live-tissue epifluorescent imaging in the right ventricle of a PN7 spontaneously beating $cKit^{\text{CreERT2}};IRG$ mouse heart illustrates contribution of postnatal CSCs in spontaneously beating, right-ventricular EGFP$^+$ cardiomyocytes.

Online Video V. De-novo postnatal cardiomyogenesis from CSCs in the left ventricular myocardium. Live-tissue epifluorescent imaging in the left ventricle of a PN7 spontaneously beating $cKit^{\text{CreERT2}};IRG$ mouse heart illustrates contribution of postnatal CSCs in spontaneously beating, left-ventricular EGFP$^+$ cardiomyocytes.

Online Video VI. De-novo postnatal cardiomyogenesis from CSCs in the cardiac apex. Live-tissue epifluorescent imaging in the apex of a PN7 spontaneously beating $cKit^{\text{CreERT2}};IRG$
mouse heart illustrates contribution of postnatal CSCs in spontaneously beating, EGFP+ cardiomyocytes.

**Online Video VII. In-vivo cardiomyocyte differentiation of neonatal CSCs.** Confocal z-stack imaging illustrates cardiomyocyte differentiation of postnatal CSCs within the neonatal mouse heart, as indicated by the co-localization of EGFP (green) with Tropomyosin (red). DAPI is depicted in white.