Myocardial CXCR4 Expression Is Required for Mesenchymal Stem Cell Mediated Repair Following Acute Myocardial Infarction

Running title: Dong et al.; Myocardial CXCR4 in cardiac repair

Feng Dong, MD, PhD1; James Harvey, MD2; Amanda Finan, BS3; Kristal Weber, BS1; Udit Agarwal, MD, PhD1; Marc Penn, MD, PhD1,4

1 Skirball Laboratory for Cardiovascular Cellular Therapeutics, Dept of Integrative Medical Sciences, Northeast Ohio Medical University, Rootstown, OH; 2 Dept of Cardiovascular Medicine, Cleveland Clinic, Cleveland, OH; 3 Dept of Molecular Biology & Microbiology, Case Western Reserve University School of Medicine, Cleveland, OH; 4 Summa Cardiovascular Institute, Summa Health System, Akron, OH

Corresponding Author:
Marc S Penn MD, PhD
Director of Research, Summa Cardiovascular Institute
Professor of Medicine and Integrated Medical Sciences
Northeast Ohio Medical University
4209 St. Rt. 44
Rootstown, Ohio 44272
Tel: 216-835-8503
Fax: 866-299-7071
E-mail: mpenn2@neomed.com

Journal Subject Codes: [110] Congestive; [27] Other Treatment; [108] Other myocardial biology
Abstract:

**Background** - Over-expression of SDF-1 in injured tissue leads to improved end-organ function. In this study we quantify the local trophic effects of mesenchymal stem cell (MSC) SDF-1 release on the effects of MSC engraftment in the myocardium after acute myocardial infarction (AMI).

**Methods and Results** - Conditional cardiac myocyte CXCR4 (CM-CXCR4) null mice were generated using tamoxifen inducible cardiac specific cre by crossing CXCR4 floxed with MCM-cre mouse. Studies were performed in littermates with (CM-CXCR4 null) or without (control) tamoxifen injection 3 weeks before AMI. One day after AMI mice received 100,000 MSC or saline via tail vein. We show αMHC-MerCreMer and the MLC-2v promoters are active in cardiac progenitor cells (CPC). MSC engraftment in WT mice decreased TUNEL+ CM (-44%, p<0.01), increased CPC recruitment (100.9%, p<0.01) and increased cardiac myosin positive area (39%, p<0.05) at 4, 7 and 21 d after AMI, respectively. MSC in WT resulted in 107.4% (p<0.05) increase in ejection fraction compared to 25.9% (p=NS) increase in CM-CXCR4 null mice. These differences occurred despite equivalent increases (16%) in vascular density in response to MSC infusion in WT and CM-CXCR4 null.

**Conclusions** - These data demonstrate that the local trophic effects of MSC require CPC- and CM-CXCR4 expression and are mediated by MSC SDF-1 secretion. Our results further demonstrate and quantify for the first time a specific paracrine mechanism of MSC engraftment. In the absence of CM-CXCR4 expression there is a significant loss of functional benefit in MSC mediated repair despite equal increases in vascular density.

**Key words:** angiogenesis; mesenchymal stem cells; myocardial infarction; stem cells; ventricular function
Introduction

Stem cell-based therapies have demonstrated that there is significant potential to improve myocardial healing after AMI.\textsuperscript{1-5} Currently adult stem cell therapy is moving forward for the prevention and treatment of cardiac dysfunction in patients with ischemic heart disease. While these studies move forward, the mechanisms of benefit remain undefined despite significant efforts. Confounding all studies to date is the fact that multiple potential mechanisms are involved and experimental manipulations in one pathway often cause confounding effects on other pathways.

SDF-1-CXCR4 axis has been shown to be critical in tissue repair in multiple organ systems including the skin, eye, kidney, brain and heart.\textsuperscript{6-10} The expression level of SDF-1 elevated at 1 h after myocardial infarction and returned to baseline by 7 days and remains down-regulated through 30 days after MI.\textsuperscript{11} The lack of myocardial SDF-1 expression beyond 7 days post-AMI is a likely explanation for the lack of efficacy of intracoronary infusion of bone marrow derived stem cells in the LATETIME trial.\textsuperscript{12} Furthermore, over-expression of SDF-1 has been shown to improve tissue repair through the recruitment of tissue specific and bone marrow derived stem cells.\textsuperscript{13} However, as yet undefined is the precise role of end-organ CXCR4 in tissue repair in response to SDF-1 expression and/or response to stem cell based repair. We have demonstrated that cardiac myocytes up-regulate CXCR4 (CM-CXCR4) expression 36-48 h after acute myocardial infarction.\textsuperscript{8} Attempts to determine the precise role of the SDF-1:CXCR4 axis in tissue repair have been confounded due to the fact that systemic inhibition of SDF-1:CXCR4 binding also results in stem cell mobilization of the bone marrow.\textsuperscript{14,15} Thus to date, inhibiting tissue SDF-1:CXCR4 binding has been studied in the setting of greater stem cell mobilization. To begin to address these mechanisms we have recently generated the conditional
CM-CXCR4 null mouse. We have further demonstrated that there is no phenotypic difference between control and CM-CXCR4 null mouse following AMI. MSC are known to express stromal cell derived factor-1 (SDF-1), thus, the goal of this study is to determine the role of MSC induced SDF-1 expression and CM-CXCR4 MSC mediated cardiac repair.

Materials and Methods

Generation of CM-CXCR4 null mouse

We have recently published the generation and characterization of the CM-CXCR4 null mouse. It was generated using a CXCR4Fr mouse (kind gift from Yong-Rui Zou, Feinstein Laboratory for Hematopoiesis, New York, NY) in a C57Bl/6 strain with the MerCreMer mouse (from The Jackson Laboratories) for inducible CM-CXCR4 null mouse. The MerCreMer mouse has cre induced by a tamoxifen inducible cardiac specific promoter and has been used extensively to induce recombination in cardiac myocytes and possibly cardiac stem cells. Mice received tamoxifen (40 mg/kg) daily for 5 days starting 4 weeks prior to LAD ligation to achieve cardiac specific cre expression. The cardiac specific cre expression leads to excision of Exon 2 of the CXCR4 gene, rendering the CM- CXCR4 null. The administration of tamoxifen led to a transient decline in cardiac function that was back to baseline 3 weeks later. Therefore, all LAD ligations in this study were performed 3 weeks after the tamoxifen injection and in animals with recovered cardiac function. Our previously data demonstrated that cardiac myocytes in the infarct border zone begin to express CM –CXCR4 36-48 hours after MI. Therefore, immunofluorescent staining and western blot were performed to quantify CXCR4 deletion two days post-MI. Our results demonstrate that the administration of tamoxifen to these mice leads to a ~90% decrease in CXCR4 protein in infarct and infarct border zone. The Animal Research
Committee approved all animal protocols. Mice were housed with free access to food and water in an American Association for Accreditation of Laboratory Animal Care–approved animal.

**MSC preparation and delivery**

Six week-old GFP mouse was sacrificed and the hind limbs were removed. Femurs were carefully cleaned of adherent soft tissue and bone marrow was flushed into a 50 ml falcon with flush medium (Alpha Medium with 2 g/L NaHCO3, 10% horse serum, 10% FBS, 1% L-Glutamine, 1% penicillin-streptomycin). The cells were filtered through a 70-μm nylon mesh filter followed by centrifugation for 5 min at 260 g and washed with PBS. The washed cells was plated in flush medium and incubated at 37°C. Nonadherent cells were removed by replacing the medium after 24 h. The cells were cultured in a monolayer at 37°C and 5% CO2 and medium was refed every 3–4 days. When cells reached 80% confluency, adherent cells was detached after incubation with 0.05% trypsin and 2 mM EDTA (Invitrogen) for 5 min. Cells were depleted of CD45+, CD34+ cells by negative selection using primary PE-conjugated antibodies; mouse anti-CD45 (BD Biosciences, San Diego, CA, USA) and mouse anti-CD34 antibodies (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). PE-positive cells were negatively selected using the EasySep PE selection kit according to the manufacturer’s instructions (Stem Cell Technologies, Vancouver, B.C., Canada). MSCs were replaced in medium and were subsequently passaged until passage 6. One day after LAD ligation mice underwent echocardiography to confirm anterior wall MI. Animals with an ejection fraction under 50% received 100,000 MSCs were suspended in 100 μl of PBS or 100μl of PBS alone was infused via tail vein injection.

**LAD ligation**

Anterior wall MI was induced in mice as described. Briefly, animals were endotracheal intubated and ventilated with room air at 100 breaths per minute using a rodent ventilator.
(Harvard Apparatus). Sternotomy was performed and the proximal LAD was identified using a surgical microscope (Leica M500) after retraction of the left atrium and ligated with 7-0 prolene. Blanching and dysfunction of the anterior wall verified LAD ligation. After LAD ligation, the animals were evaluated with echocardiography or sacrificed at different time points for organ harvest (Heart) and staining.

**Echocardiography**

Two dimension echocardiography was performed using a 15-MHz linear array transducer interfaced with a Sequoia C256 (Acuson) as previously described.20 Briefly, 3 d before LAD ligation as well as 4d, 7d, and 21d after LAD ligation, left ventricle (LV) dimensions were quantified by digitally recorded 2D clips and M-mode images in a short axis view from the mid-LV just below the papillary muscles to allow for consistent measurements from the same anatomical location in different mice. Measurements were made by two independent blinded researchers off-line using ProSolv echocardiography software. Each measurement in each animal was made from three randomly chosen m-Mode clips out of five recorded by observers blinded to the strain of the mice. Dimensions were measured between the anterior wall and posterior wall from the short axis view just below the level of the papillary muscle.

**Immunostaining**

Four days and 21 days after LAD ligation, mice were anesthetized and perfusion fixed with 10% phosphate-buffered formalin at physiological pressures. Fixed hearts were embedded in paraffin and serially cut at 4 µm from the apex to the level just below the coronary artery ligation site. Antigen retrieval was performed using 10 mM sodium citrate buffer (pH 6.0) and incubated with 1% normal blocking serum in PBS for 60 min to suppress nonspecific binding of IgG. Four days post AMI tissue were stained for GFP (1:1000, Abcam, Inc. San Francisco, CA) to calculate GFP
positive MSCs. Three weeks post AMI tissue was stained to measure vessel density and percent cardiac myosin-positive area in the infarct zone. Negative control with no fluorescent probe was used in immunostaining to control the autofluorescence.

**MSC homing following MSC infusion**

Four days post-MI tissue was prepared as described above for immunostaining. The number of GFP+ cells was quantified in the infarct zone by independent blinded researchers across a minimum of 4 sections and 12 fields obtained from the mid-LV per animal.

**TUNEL assay for assessment of apoptotic cell death**

TUNEL was performed to detect apoptotic nuclei using terminal deoxynucleotidyl transferase (TdT)-mediated in situ fluorescein-conjugated, dUTP nick end-labeling technique according to the manufacturer’s protocol (Roche, Indianapolis, IN, USA). The sections were incubated with mouse monoclonal antibody (Abcam, Inc. San Francisco, CA) to recognizing cardiac myosin heavy chain to specifically recognize apoptotic cardiomyocytes. The fluorescence staining was viewed with a confocal laser scanning microscope. The number of apoptotic cells with TUNEL-positive nuclei was counted by two independent observers blinded to treatment group and expressed as a percentage of total myocyte population. TUNEL-positive nuclei in 1000 nuclei within 4–5 cells from the infarct border zone from 5 different sections (4000- 5000 nuclei total) obtained from the mid-LV.

**Percent cardiac myosin-positive area measurement**

Three weeks post AMI tissue was prepared as described above for immunostaining. Slides were stained with the mouse anti-CM heavy chain primary antibody (Abcam, Inc. San Francisco, CA) and then with goat anti mouse IgG Alexa Fluor 594 (molecular probes, Carlsbad, CA) and mounted with aqueous mounting medium (Vectashield Mounting Medium with DAPI, H-1200;
Vector Laboratories, Burlingame, CA). Five randomly fields in the infarct zone for each sample was selected and measured by an observer blinded to treatment group with ImagePro Plus software (Media Cybernetics) as previously described.8,21

**Myocardial infarct size measurement**

Four days and 21 days after LAD ligation, mice were anesthetized and perfusion fixed with 10% phosphate-buffered formalin at physiological pressures. Fixed hearts were embedded in paraffin and serially cut at 4 μm from the apex to the level just below the coronary artery ligation site. Alternating sections were stained with Masson trichrome. The infarcted area was measured by planimetry using computer-assisted image analysis software Image-Pro Plus (Media Cybernetics). Parameters were calculated using the equations: % infarct size = epicardial infarct length/epicardial LV circumferences x100.

**Vessel density measurement**

Three weeks post AMI tissue was prepared as described above for immunostaining. sections were stained with fluorescein-conjugated isolectin (Vector Laboratories), which stains endothelium and rhodamine-conjugated wheat germ agglutinin (Vector Laboratories), which labels myocyte membranes as previously described.22 Five to ten randomly selected fields for each sample were imaged at 630x using confocal microscopy and vessel density within the infarct border zone was measured. All quantitative evaluations were performed with ImagePro Plus software (Media Cybernetics).

**Isolation of cardiac progenitor Cells (CPC) from mouse hearts**

CPCs were isolated from mouse hearts in different groups 7 days post-MI as described previously.23 Briefly, infarct zone of hearts were digested using 0.1% collagenase B and 2.4 U/mL dispase II (Roche Molecular Biochemicals) containing 2.5 mmol/L CaCl2 at 37° C for 45
minutes. Cells then were filtered through 30µm filters, washed in Hanks’s balanced salt solution (HBSS) (Invitrogen) containing 2% fetal calf serum and 10 mmol/L HEPES and were lysed with RBC lysing buffer. After washing with PBS, Cells were resuspend in 2x 10^6 nucleated cells/mL. Cells were fixed with 4% PFA and then were incubated with antibodies against the mouse CD16/32, APC rat anti mouse CD117, V450 rat-anti mouse CD45 (BD Biosciences). Flow cytometry were performed on a FACS Calibur (BD Biosciences). Data were analyzed with FlowJo software. The percentage of Ckit+/CD45- cells in the infarction zone 7d post-MI was calculated. To investigate whether αMHC-MerCreMer promoter is activated in endogenous CPC, CPCs were isolated from CM- CXCR4 null mouse hearts 3 weeks after tamoxifen injection. After fixed with 4% PFA and treated with 0.1% triton, the cells were incubated with APC rat anti mouse CD117, V450 rat-anti mouse CD45 (BD Biosciences), anti β-gal (cell signaling), and α-MHC (abcam). Immunostaining and flow cytometry were performed to investigate the recombination in CPC. Spleen cells were used as control.

**SDF-1 mRNA and protein Expression**

Four and 7 days after LAD ligation, the hearts were perfused with saline and infarcted left ventricles were cut at a level just below the ligation. The RNA was harvested using TRIzol and cDNA was synthesized using SuperScript VILO cDNA synthesis kit from Invitrogen. The real-time PCR reaction for SDF-1 and 18S was carried out using TaqMan primers on Applied Biosystems 7500 Real-Time PCR System. To identify the source of SDF-1, we performed immunofluorescence staining with paraffin embedded tissue 4 days and 3 weeks post AMI. Samples were stained for GFP (1:1000, Abcam, Inc. San Francisco, CA) and SDF-1 (1:50, Santa Cruz, Inc, Santa Cruz, CA). Negative control with no fluorescent probe was used in immunostaining to control the autofluorescene.
Statistical Analysis

Data are analyzed with SigmaPlot Statistics and presented as mean ± SEM. Comparison between 2 groups were made with a 2-tailed student t test (Figure 1). Comparisons among multiple groups were made with two-way analysis of variance (ANOVA) followed by the Tukey post-hoc analysis for Table 1, Figures 2,3,4,5, and 6. No statistics required for data in Figure 7. Three-way ANOVA was used for Figure 8. All differences were considered statistically significant at p<0.05 levels.

Results

Role of CM-CXCR4 expression on MSC homing following cell therapy

To investigate the role of CM-CXCR4 on MSC homing following cell therapy, 100,000 GFP positive MSCs (isolated from GFP mouse, passage 6) were suspeneded in 100 μl of PBS and infused via tail vein injection 24 h after AMI. Three days after infusion (four days post AMI), the hearts were harvested and embedded in paraffin. Sections were stained with an antibody to GFP and the number of GFP+ cells was quantified in the infarct zone by independent blinded observers (Figures 1 A and B). Although we have observed a 33% decrease in SDF-1 mRNA expression in CM-CXCR4 null mice compared to the control mice 3 days after LAD ligation by real-time PCR,16 no significant differences in MSC engraftment were observed between groups with or without CM-CXCR4 expression (Figure 1) suggesting that the absence of CM-CXCR4 expression and the degree of decrease in SDF-1 expression in the CM-CXCR4 null mice do not alter MSC recruitment to the heart after AMI.

Role of CM-CXCR4 expression on cardiac myocyte preservation following cell therapy

SDF-1:CXCR4 binding has been shown to induce cell survival through phosphorylation of AKT...
and the STAT3 pathways.\textsuperscript{24,25} To investigate the role of CM-CXCR4 expression on cardiac myocyte preservation following MSC delivery, the number of TUNEL+ cardiac myocytes in the infarct border zone 4 days after AMI was quantified. As seen in Figure 2, in the absence of MSC infusion there was no difference in the number of TUNEL positive cardiac myocytes in mice with or without CM-CXCR4 expression. However, consistent with our previous results in the rat,\textsuperscript{8} the infusion of MSC 1 day after AMI led to a significant 44% reduction in TUNEL+ cardiac myocytes (p<0.01 compared to the control group). Interestingly, in the absence of CM-CXCR4 expression the decrease in cardiac myocyte apoptosis was blunted by almost 50% (23%) and significantly greater than control mice treated with MSC.

**Role of CM-CXCR4 expression on infarct size and percent cardiac myosin positive area following cell therapy**

To evaluate if reduced cardiomyocyte apoptosis 4d after AMI is sufficient to decrease cardiac damage, we measured the cardiac myosin-positive area within the infarct zone by immunofluorescence 21 days after AMI. We observed that in the absence of stem cell therapy CM-CXCR4 expression does not alter the number of surviving cardiac myocytes within the infarct zone. However, in response to MSC infusion 1 d after AMI, we observed a significant increase in cardiac myosin positive area within the infarct zone (43.9%). This increase in cardiac myosin positive area in response to MSC infusion was not observed in the CM-CXCR4 null mice (Figure 3).

**Role of CM-CXCR4 expression on ventricular remodeling and cardiac function following cell therapy**

Consistent with our previous findings, in the absence of stem cell therapy CM-CXCR4 expression does not alter LV function and LV remodeling 21 d after AMI (Table 1, Figure 4).\textsuperscript{8}
The infusion of MSC 1 day after AMI led to a significant improvement in ejection fraction (EF) and decreased systolic and diastolic LV internal dimension (LVISD and LVIDD) compared to controls animals that received saline. MSC therapy also significantly increased interventricular septal thickness in systole and diastole (IVSS and IVSD) without change the systolic and diastolic thickness of the LV posterior wall (LVPWS and LVPWD) in control mice; however, in the absence of CM-CXCR4 expression there was no significant improvement in cardiac function or remodeling (EF, LVISD, LVIDD, IVSD, and IVSS) in response to MSC infusion (Table 1, Figure 1). These data demonstrate that CM-CXCR4 plays a critical role in post-MI ventricular remodeling and cardiac function following MSC engraftment.

Role of CM-CXCR4 expression on vascular density following cell therapy

To investigate if CM-CXCR4 expression may affect vascular density following MSC infusion we evaluated vascular density 21 days after AMI. Immunofluorescence using wheat germ agglutinin and isolectin was used to identify and quantify the vasculature within the infarct zone. A significant increase in the number of capillaries and small arterioles was observed in WT and CM-CXCR4 null mice that received MSC 21 d after AMI (Figure 5A and B).

Role of CM-CXCR4 expression on CPC recruitment following cell therapy

We investigated whether the αMHC-MerCreMer promoter and/or the MLC-2v-cre promoters are activated in CPC. We quantified promoter activity by immunostaining and flow cytometry by crossing the cre inducible β-gal mouse with the αMHC-MerCreMer and the MLC-2v-cre mice. Our data showed that the αMHC-MerCreMer and MLC-2v promoters are activated in endogenous CPC (Figures 7 A-C). To further investigate whether there is evidence of an extracardiac progenitor cell whose recruitment we could be affecting through the down-regulation of CXCR4 we performed flow cytometry on the cellular population isolated from the
spleen and did not observe any significant evidence of β-gal+ cells in those mice that received tamoxifen as seen in Figure 7B.

To determine if promoter activation has functional consequences in CPC we quantified CPC recruitment following AMI with and without MSC infusion. We found that MSC engraftment led to a significant increase of CPC recruitment (100.9%, p<0.01) in the heart of WT mice 7 days after AMI and that CPC recruitment in response to MSC was significantly decreased to 55.4% in CM-CXCR4 null mice (Figure 6 A-B).

Role of MSC infusion on myocardial SDF-1 expression

We wanted to confirm that MSC engraftment in the myocardium led to increased and sustained SDF-1 expression. Therefore, we quantified SDF-1 mRNA levels in the infarct zone 4 and 7 days after LAD ligation by real-time PCR. As seen in Figure 8A, SDF-1 levels decreased approximately 30-40% in all groups between days 4 and 7 with a trend towards ~50% and ~30% greater SDF-1 mRNA expression in those animals that received MSC at 4 and 7 days after AMI, respectively. To identify the source of SDF-1, we performed immunofluorescence staining with paraffin embedded tissue 4 days and 3 weeks post AMI. We observed SDF-1 expression in MSCs, cardiac myocytes and other non-MSC cells 4 days post-MI (Figure 8B). However, SDF-1 expression was only detected in engrafted MSC and not cardiac myocytes 21 days post-MI (data not shown).

Discussion

Using our novel model of CM-CXCR4 null mice, for the first time we were able to specifically dissect the effects of MSC SDF-1 expression on end organ tissue repair. In this study we demonstrated a critical role for CPC and cardiac myocyte CXCR4 expression on MSC mediated
cardiac repair. Most importantly, we observed that inhibiting CPC and cardiac myocyte response to SDF-1 by deletion of CXCR4 blocked significant improvement in cardiac function despite equivalent levels of MSC engraftment (Figure 1) and a significant increase in vascular density within the injured myocardium (Figure 5).

Our study also demonstrates that the αMHC-MerCreMer and MLC-2v promoters are activated in endogenous CPC (Figure 7A-C). Moreover, we confirmed our earlier observations that MSC engraftment leads to a significant increase in CPC recruitment in WT mice, that was significantly blunted in CM-CXCR4 null mice (100.9% vs. 55.4%) in the infarcted heart 7 days after AMI. Our data demonstrates that increases in SDF-1 expression following ischemic injury results in increased bone marrow and cardiac stem/progenitor cell homing to the myocardium that is responsible at least in part for the benefits associated with stem cell therapy. There is increasing recognition that CXCR4 expression by cardiac myocytes is responsible for ischemic preconditioning and cardiac myocyte preservation following cell therapy. Previous studies have suggested an important role for the SDF-1:CXCR4 axis in stem cell based repair; however, the strategies for dissecting the importance of CM-CXCR4 have to date been confounded because CXCR4 and SDF-1 null mice are not viable. Inhibiting CXCR4:SDF-1 binding with CXCR4 antagonists (i.e., AMD3100) is confounded because it leads to stem cell mobilization from the bone marrow leading to increased bone marrow-derived endothelial progenitor cell (EPCs) recruitment.

Our previous findings showed that myocyte CXCR4 has no role in ventricular remodeling after MI in the absence of cell therapy due to the mismatch in timings of peaks of SDF-1 and CXCR4. These results were confirmed again in our current study. Our study demonstrates prolonged SDF-1 expression in mice that received MSC infusion with trends
towards greater SDF-1 levels in WT animals that received MSC. Interestingly, we found SDF-1 expression in MSCs and non-MSC cells 4 days post-MI. The cells are responsible for SDF-1 expression includes injured cardiomyocytes, cardiac progenitor cells, endothelial cells and smooth muscle cells. However, SDF-1 was detected in MSC and not cardiac myocytes 21 days post-MI. This observation supports the concept that SDF-1 release by MSC leads to the recruitment of CXCR4+ CPC that may further release SDF-1. That we observed no difference in vascular density between WT and CM-CXCR4 null mice suggests that CPC do not have a significant role in the vascular response to MSC engraftment after AMI.

Our current and previous data demonstrate that activation of SDF-1/CXCR4 signaling leads to preservation of cardiac myocytes and improvement in cardiac function. This antiapoptotic effect of SDF-1:CXCR4 binding has previously been shown to be mediated by the PI-3 kinase/Akt signaling pathway. Our previous data showed that CXCR4 signaling does not enhance cardiomyocyte hypertrophy. Our current data indicate that CM-CXCR4 expression plays an important role on CPC recruitment, cardiac myocyte preservation left ventricular remodeling and function following cell therapy for MI. It was recently reported that the over expression of CM-CXCR4 by gene transfer showed a negative inotropic effect. Cardiac myocyte CXCR4 expression may act as a marker of an injured or dysfunctional cardiac myocyte, potentially leading to hibernation of cardiac myocytes in order to minimize energy dependent properties and further injury. Our study shows that in the absence of CM-CXCR4 expression there is a significant decrease in cardiac myocyte survival in response to MSC infusion. The decreased cardiac myocyte survival within the infarct zone leads to inhibition of significant improvements in cardiac function and remodeling 21 d after AMI in response to MSC infusion. Taken together these data indicate that an important mechanism associated with the benefits of
MSC engraftment is the delivery of SDF-1 (through MSC infusion and MSC induced endogenous CPC recruitment) to CXCR4 positive cardiac myocytes. Our current study further proves that myocardial CXCR4 is necessary for improved cardiac function in the presence of MSC after MI.

In summary, our study directly quantifies the impact of a specific stem cell paracrine factor on cardiac myocyte survival and left ventricular remodeling and function after AMI. Our findings demonstrate that myocardial CXCR4 plays a critical role in myocardial response to MSC engraftment through the improvement of the CPC recruitment and the inhibition of myocyte apoptosis post-MI. These data demonstrate that increase in vascular density is not sufficient to improve ventricular remodeling and function; increasing cardiac myocyte survival during infarct expansion is necessary. These data demonstrate that the significant mode of action of mesenchymal stem cells is the local release of SDF-1 to block end-organ cell death, and suggest that inducers of early CXCR4 could be efficacious, and that expression or prolongation of myocardial SDF-1 expression could be strategy for minimizing tissue damage in the setting of acute injury. Further studies are needed to define the effects of CXCR4 in specific cell population. Such studies could directly impact the development of clinical targets for improving cardiac function after MI and CHF.

Funding Sources: This work was supported by the Skirball Foundation (MSP) and American Heart Association SDG (FD).

Conflict of Interest Disclosures: Dr. Penn is named as an inventor on patent applications submitted by the Cleveland Clinic for the use of SDF-1 to prevent and treat tissue injury. He is the founder and CMO of Juventas Therapeutics, Inc. which has licensed the use of these patents for the commercial development of SDF-1 to prevent and treat tissue injury.
References:


**Table 1.** LV dimensions at baseline and 21d post-MI in control (wild-type) and cardiac myocyte CXCR4 null (CM-CXCR4<sup>−/−</sup>) mice

<table>
<thead>
<tr>
<th></th>
<th>IVSD (mm)</th>
<th>IVSS (mm)</th>
<th>LVIDD (mm)</th>
<th>LVISD (mm)</th>
<th>LVPWD (mm)</th>
<th>LVPWS (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Baseline</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control-Saline</td>
<td>0.92 ± 0.09</td>
<td>1.60 ± 0.05</td>
<td>2.66 ± 0.09</td>
<td>1.09 ± 0.06</td>
<td>0.81 ± 0.07</td>
<td>1.47 ± 0.07</td>
</tr>
<tr>
<td>CM-CXCR4&lt;sup&gt;−/−&lt;/sup&gt; Saline</td>
<td>0.98 ± 0.06</td>
<td>1.73 ± 0.09</td>
<td>2.95 ± 0.19</td>
<td>1.27 ± 0.12</td>
<td>0.97 ± 0.06</td>
<td>1.65 ± 0.07</td>
</tr>
<tr>
<td>Control-MSC</td>
<td>1.09 ± 0.05</td>
<td>1.79 ± 0.08</td>
<td>2.63 ± 0.18</td>
<td>1.04 ± 0.18</td>
<td>1.01 ± 0.05</td>
<td>1.53 ± 0.06</td>
</tr>
<tr>
<td>CXCR4&lt;sup&gt;−/−&lt;/sup&gt;-MSC</td>
<td>1.12 ± 0.05</td>
<td>1.89 ± 0.08</td>
<td>3.09 ± 0.22</td>
<td>1.29 ± 0.21</td>
<td>0.86 ± 0.04</td>
<td>1.59 ± 0.06</td>
</tr>
<tr>
<td><strong>21d post-MI</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control-Saline</td>
<td>0.38 ± 0.08</td>
<td>0.46 ± 0.09</td>
<td>5.03 ± 0.30</td>
<td>4.50 ± 0.36</td>
<td>0.68 ± 0.09</td>
<td>1.01 ± 0.13</td>
</tr>
<tr>
<td>CM-CXCR4&lt;sup&gt;−/−&lt;/sup&gt; Saline</td>
<td>0.38 ± 0.09</td>
<td>0.45 ± 0.12</td>
<td>5.29 ± 0.38</td>
<td>4.72 ± 0.41</td>
<td>0.74 ± 0.09</td>
<td>1.05 ± 0.12</td>
</tr>
<tr>
<td>Control-MSC</td>
<td>0.88 ± 0.08&lt;sup&gt;*&lt;/sup&gt;</td>
<td>1.06 ± 0.09&lt;sup&gt;*&lt;/sup&gt;</td>
<td>4.25 ± 0.25&lt;sup&gt;*&lt;/sup&gt;</td>
<td>2.93 ± 0.32&lt;sup&gt;**&lt;/sup&gt;</td>
<td>0.81 ± 0.08</td>
<td>1.10 ± 0.05</td>
</tr>
<tr>
<td>CXCR4&lt;sup&gt;−/−&lt;/sup&gt;-MSC</td>
<td>0.59 ± 0.12</td>
<td>0.65 ± 0.13</td>
<td>5.09 ± 0.49</td>
<td>4.35 ± 0.51</td>
<td>0.76 ± 0.10</td>
<td>1.03 ± 0.11</td>
</tr>
</tbody>
</table>

Data are expressed as means ±SEM (n = 6-11 in each group). *p<0.05 vs. corresponding saline treatment group. #p<0.05 vs. CM-CXCR4 null-MSC group. IVSD, Interventricular septal thickness in diastole; IVSS, Interventricular septal thickness in systole; LVIDD, Diastolic LV internal dimension; LVISD, Systolic LV internal dimension; LVPWD, diastolic thicknesses of the LV posterior wall; LVPWS, systolic thicknesses of the LV posterior wall.

**Figure Legends:**

**Figure 1.** Exogenous MSC homing following cell therapy: A, Representative immunofluorescent staining for GFP (FITC, green) and nuclei (DAPI, blue) within the infarct zone 4d after LAD ligation from mice that received GFP+ MSC 24 h after LAD ligation. B, Number of MSC per square millimeter within the infarct zone at 4 days after LAD ligation. Data represent mean ± SEM (n = 4 per group).

**Figure 2.** A, Confocal image of representative immunofluorescent staining for cardiac myosin heavy chain (Alexa Fluor 594, red), TUNEL (Alexa Fluor 488, green), and merged image from animals 4d after LAD ligation B, Number of TUNEL-positive cardiomyocytes in the infarct border zone 4d after LAD ligation in animals that received Saline or MSC 24 h after AMI.
Arrow heads represent positive nuclei. Data represent the mean percent TUNEL-positive cells ± SEM (n = 4 per group) *p< 0.05 vs. corresponding saline treatment group. #p<0.05 greater than treatment match control.

**Figure 3.** A, Confocal image of representative immunofluorescent staining for cardiac myosin heavy chain (Alexa Fluor 594, red) and Dapi (blue). Inset is high power image of delineated area B, Percent area positive for cardiac myosin within the infarct zone 3 wk after LAD ligation in animals that received saline or MSCs 24 h after AMI. Data represent mean ± SEM (n=5 per group) *P < 0.05 compared with Control-saline group. # p< 0.05 vs. CM-CXCR4 null -MSC group.

**Figure 4.** Role of CM-CXCR4 expression on LV function following cell therapy. Ejection fraction (EF) at baseline and 21 days after LAD ligation in CTRL (open and black bars) and CM-CXCR4 null (light and dark grey) mice. Mice were infused with saline (open and light grey bars) or MSC (black and dark grey bars) 1 day after LAD ligation. Data are means ±SEM (n= 6 to 8 per group). * p< 0.05 vs. strain matched control, # p<0.05 vs. treatment matched control.

**Figure 5.** Vascular density in the border zone 21 days post-MI. A, Confocal image of representative immunofluorescent staining. Endothelial cell staining with Isolectin (green). Corresponding merged image –Wheat Term Agglutinnin (Red) and Dapi (Blue). B, Number of vascular density. Data represent mean ± SEM (vessles/mm2, n= 4 per group). * p< 0.05 vs. corresponding saline treatment group.
Figure 6. CPC recruitment following AMI with and without MSC infusion. A, Representative flow cytometry analysis of isolated cells within the infarct zone 4d after LAD ligation from groups. B, Percent c-Kit+/CD45- cells within the infarct zone 7 days after LAD ligation. Data represent mean ± SEM (n = 4 per group). *p< 0.05 vs. WT control group.

Figure 7. A: Representative confocal images of immunofluorescent staining of cells from αMHC-MerCreMer cre inducible β-gal mice. To investigate whether the αMHC-MerCreMer promoter is activated in CPC, we crossed the αMHC-MerCreMer mouse with the cre inducible β-gal mouse. From these mice we isolated C-kit+/CD45- cells from the heart and spleen. These cells were stained for α-cardiac myosin heavy chain (Alexa Fluor 594, red), β–gal (Alexa Fluor 488, green), cell nuclei (Dapi, blue) after Triton-x-100 (0.1%) treatment. Our data show that there is recombination in the α-MHC positive cells from the heart, but no recombination in α-MHC negative splenocytes. 7-B: To further confirm that αMHC-MerCreMer promoter is activated in CPC, We quantified promoter activity by flow cytometry (FACS). We crossed the αMHC-MerCreMer mouse with the cre inducible β-gal mouse. After isolation of cells from the hearts of these mice, cells were incubated with anti-c-Kit (APC), anti-CD45 (v450), and anti-β-gal. FACS was performed on a FACSCalibur (BD Biosciences). Examples of flow cytometric analysis of β–gal and c-Kit expression in CD45 negative cells from WT (left) and MCM/β-gal mouse heart (right). Our data confirmed that the αMHC-MerCreMer is activated in endogenous CPC. 7-C: Representative confocal images of immunofluorescent staining of cells from MLC-2v-cre: cre inducible β–gal mice. In order to determine if cardiac specific cre expression leads to recombination (deletion) in CPC, we crossed the MLC-2v-cre mouse with the cre inducible β-gal mouse. From these mice we isolated C-kit+ cells from the heart and spleen. These cells were
stained for α-cardiac myosin heavy chain (MHC), β-gal and Hoechst dye. Confocal image of representative immunofluorescent staining of cells shows that there is recombination in the α-MHC positive cells from the heart, but no recombination in α-MHC negative splenocytes.

**Figure 8.** A: SDF-1 mRNA levels in the infarct zone 4 and 7 days after LAD ligation. Data represent mean ± SEM (n=3-4 per group). *p< 0.05 vs. corresponding 4 days post-MI group; # p< 0.05 vs. 7days post-MI WT control group. B: Confocal image of representative immunofluorescent staining for SDF-1 (red), GFP (green) and Dapi (blue) in samples 4d post-MI.
A.

B.

MSC / mm²

CM-CXCR4: - +
A. 

Green 575-A - CD45
CTRL
CTRL + MSC

Green 575-A - CD45
CM-CXCR4 null
CM-CXCR4 null + MSC

Red-660-A - c-Kit

B. 

% of c-Kit+/CD45- cells in the infarction zone 7d post-MI

CTRL
CM-CXCR4 null
CTRL-MSC
CM-CXCR4 null + MSC
Myocardial CXCR4 Expression Is Required for Mesenchymal Stem Cell Mediated Repair Following Acute Myocardial Infarction
Feng Dong, James Harvey, Amanda Finan, Kristal Weber, Udit Agarwal and Marc Penn

Circulation. published online June 9, 2012;
Circulation is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2012 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circ.ahajournals.org/content/early/2012/06/09/CIRCULATIONAHA.111.082453

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation is online at:
http://circ.ahajournals.org/subscriptions/