Role of Cardiac Myocyte CXCR4 Expression in Development and Left Ventricular Remodeling After Acute Myocardial Infarction

Udit Agarwal, Wael Ghalayini, Feng Dong, Kristal Weber, Yong-Rui Zou, Sina Y. Rabbany, Shahin Rafii, Marc S. Penn

Rationale: Stromal cell–derived factor (SDF)-1/CXCR4 axis has an instrumental role during cardiac development and has been shown to be a potential therapeutic target for optimizing ventricular remodeling after acute myocardial infarction (AMI) and in ischemic cardiomyopathy. Although a therapeutic target, the specific role of cardiac myocyte CXCR4 (CM-CXCR4) expression following cardiogenesis and survival of cardiac myocyte and left ventricular remodeling after AMI is unknown.

Objective: We hypothesized that cardiac myocyte derived CXCR4 is critical for cardiac development, but it may have no role in adulthood secondary to the short transient expression of SDF-1 and the delayed expression of CM-CXCR4 following AMI. To address this issue, we developed congenital and conditional CM-CXCR4−/− mouse models.

Methods and Results: Two strains of CM-CXCR4flox/flox mice were generated by crossing CXCR4flox/flox mice with MCM-Cre+/- mouse and MLC2v-Cre+/- mouse on the C57BL/6J background, yielding CXCR4flox/flox MCM-Cre+/- and CXCR4flox/flox MLC2v-Cre+/- mice. Studies demonstrated recombination in both models congenitally in the MLC2v-Cre+/- mice and following tamoxifen administration in the MCM-Cre+/- mice. Surprisingly the CXCR4flox/flox MLC2v-Cre+/- are viable, had normal cardiac function, and had no evidence of ventricular septal defect. CXCR4flox/flox MCM+/- treated with tamoxifen 2 weeks before AMI demonstrated 90% decrease in cardiac CXCR4 expression 48 hours after AMI. Twenty-one days post AMI, echocardiography revealed no statistically significant difference in the wall thickness, left ventricular dimensions or ejection fraction (40.9±7.5 versus 34.4±2.6 %) in CXCR4flox/flox mice versus CM-CXCR4−/− mice regardless of strategy of Cre expression. No differences in vascular density (2369±131 versus 2471±126 vessels/mm2; CXCR4flox/flox versus CM-CXCR4−/− mouse), infarct size, collagen content, or noninfarct zone cardiac myocyte size were observed 21 days after AMI.

Conclusions: We conclude that cardiac myocyte–derived CXCR4 is not essential for cardiac development and, potentially because of the mismatch in timings of peaks of SDF-1 and CXCR4, has no major role in ventricular remodeling after AMI. (Circ Res. 2010;107:667-676.)

Key Words: stem cells ■ myocardial infarction ■ cardiogenesis

STEM cell– and gene transfer–based strategies are being pursued in an attempt to decrease infarct size, optimize ventricular remodeling, and prevent the onset of chronic heart failure in patients following acute myocardial infarction (AMI). Although benefits have been demonstrated in several clinical trials and recent metaanalyses,1–3 the mechanism pursued in an attempt to decrease infarct size, optimize ventricular remodeling, and prevent the onset of chronic heart failure in patients following acute myocardial infarction (AMI). Although benefits have been demonstrated in several clinical trials and recent metaanalyses,1–3 the mechanism potentially because of the mismatch in timings of peaks of SDF-1 and CXCR4 signaling pathway.4–8 This pathway has been implicated in stem cell survival following transplantation, homing of bone marrow–derived and cardiac progenitor stem cells to the infarct zone heart, and cardiac myocyte survival during AMI and chronic heart failure. The SDF-1/CXCR4 axis has also been shown to be critical in cardiac development.9–11 Although SDF-1/CXCR4 axis is a

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Non-standard Abbreviations and Acronyms

<table>
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<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tr>
<td>AMI</td>
<td>acute myocardial infarction</td>
</tr>
<tr>
<td>CXCR4</td>
<td>CXC chemokine receptor-4</td>
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<tr>
<td>CM-CXCR4</td>
<td>cardiac myocyte-specific CXCR4</td>
</tr>
<tr>
<td>EGFP</td>
<td>enhanced green fluorescent protein</td>
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<tr>
<td>GFP</td>
<td>green fluorescent protein</td>
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<tr>
<td>LAD</td>
<td>left anterior descending artery</td>
</tr>
<tr>
<td>LV</td>
<td>left ventricular</td>
</tr>
<tr>
<td>LVED</td>
<td>left ventricular end dimension</td>
</tr>
<tr>
<td>MCM</td>
<td>MerCreMer, tamoxifen-induced α-myosin heavy chain driven Cre</td>
</tr>
<tr>
<td>MI</td>
<td>myocardial infarction</td>
</tr>
<tr>
<td>MLC2v</td>
<td>ventricular myosin regulatory light chain-2</td>
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<tr>
<td>MSC</td>
<td>mesenchymal stem cell</td>
</tr>
<tr>
<td>pc</td>
<td>postconception</td>
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<tr>
<td>SDF</td>
<td>stromal cell–derived factor</td>
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therapeutic target of interest, its natural role in myocardial repair in adulthood is less clear because SDF-1 expression is immediate and transient and cardiac myocyte CXCR4 expression is delayed and persistent following AMI. More recently, the hypothesis has been forwarded that the basis of benefit associated with stem cell therapy following acute myocardial infarction is the restoration of the temporal alignment of SDF-1 and CXCR4. CXCR4, a G protein–coupled 7-transmembrane receptor, together with its ligand SDF-1, can play a crucial role during embryonic development and in maintaining the stem cell niche, homing of stem cells at the site of injury, and preservation of the injured tissue. During embryogenesis, CXCR4 expression starts as early as blastocyst formation and is expressed in various different stages of development and a variety of cell types. The importance of CXCR4 during development is evident in the CXCR4-null mouse. In these mice, the deficiency of CXCR4 is lethal as the developing heart is recognized. Following AMI, SDF-1 expression is elevated immediately and peaks within 24 hours. However, cardiac myocyte CXCR4 expression occurs between 48 and 72 hours after AMI. This physiological mismatch in the peak of the ligand and its receptor has led various researchers to overexpress SDF-1 in infarcted regions. The prolonged expression of SDF-1 in infarcted tissue leads to reestablishment of stem cell homing, neoangiogenesis, myocardial preservation, and increased ventricular function. Although SDF-1/CXCR4 axis has been shown to be critical in myocardial reparative process after myocardial infarction (MI), the exact role of CXCR4 derived from cardiac myocytes in ventricular remodeling is not known.

To address this question directly, we have developed a congenital (MLC-2Vcre) and conditional (MCM-cre) deletion of cardiac myocyte CXCR4 using the CXCR4flox/flox mouse. We have characterized these mice before and after AMI.

Methods
An expanded Methods section is available in the Online Data Supplement at http://circres.ahajournals.org and includes detailed information regarding immunostaining, western blotting, echocardiography, left anterior descending artery (LAD) ligation, and cardiac remodeling.

Mice Generation and In Vivo Analysis of CXCR4 Deletion
Previous studies have reported the generation of mice having CXCR4flox allele, in which exon 2 (2.2 kbp) is flanked by 2 loxP sequences. We crossed CXCR4flox/flox mice with the mice bearing a transgene of α-MyHC-MerCreMer (MCM) and MLC2v-Cre. The progeny was crossbred to yield MCM-CXCR4flox/flox and MLC2vCRE-CXCR4flox/flox mice. Tamoxifen (40 mg/kg) in corn oil was administered IP in 6-week-old MCM-CXCR4flox/flox male mouse continuously for 5 days. The genomic DNA from the hearts of 8-week-old mice from both types was isolated and subjected to PCR to estimate CXCR4 deletion. The location of the primers used to detect CXCR4 deletion is depicted in Figure 1. The primers used were as follows: forward, 5'-CCTCGGAATGGAAGAGATTATG-3'; reverse, 5'-CCTCGGAATGGAAGAGATTATG-3'. All animals were housed in an animal facility of Cleveland Clinic approved by the Association for Assessment and Accreditation of Laboratory Animal Care International, and all animal protocols were approved by Animal Research Committee. All animals had C57Bl/6J background.

SDF-1 mRNA Expression
Three days after LAD ligation, the heart were perfused with 10 mL of saline and infarcted left ventricles were cut at a level just below the ligation. The tissue was snap-frozen and stored in −80°C until used. 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.

Stem Cell Homing Studies
Mesenchymal stem cells (MSCs) were isolated and prepared as we have previously described. The cells were incubated at 37°C. Confluent cells were passaged and plated out at 1:2 to 1:3 dilutions until passage 6. Cells were assayed for their ability to differentiate into the adipogenic, chondrogenic, and osteogenic lineages. Cells were maintained in differentiation media for 2 to 3 weeks. Differentiation was validated by staining the cells with Oil Red (adipogenic lineage), Alcian blue (chondrogenic lineage), or alkaline phosphatase (osteogenic lineage). Cells were induced to express green fluorescent protein (GFP) using a lentiviral construct encoding enhanced green fluorescent protein (EGFP) under the CMV promoter. Seven days after transfection, GFP+ cells were sorted by FACS. Animals were infused via the tail vein with 250 000 GFP+ MSCs 24 hours after LAD ligation. Hearts were harvested 72 hours after MSC infusion and prepared as described above for immunostaining with a primary antibody against GFP. The number of GFP+ cells per high-power fields was quantified in the infarct border zone as described before, by blinded observers across a minimum of 4 sections and 12 fields obtained from the mid–left ventricle per animal.

Statistical Analysis
Ventricular function was analyzed with one way ANOVA. All values with P<0.05 were considered significant.
Results

Generation of Congenital Cardiac Myocyte–Specific CXCR4-Deficient Mice

CXCR4 deficiency is embryonic lethal and is known to exhibit defective hematopoiesis, neural development, and ventricular septal defects. The ventricular septum begins to form in mouse at day 11 and is complete by day 12.5 postconception (pc). MLC2v expression begins at day 9 pc in the primitive heart tube. Therefore, to assess the role of cardiac myocyte–specific CXCR4 expression in cardiac development, we generated a mouse with congenital deletion of CXCR4 specifically in cardiac myocytes using the MLC2v-Cre-mediated expression. We successfully crossed MLC2v-Cre+/H11001/MLC2v-Cre+/H11002 (kind gift from Kenneth Chien, Harvard Stem Cell Institute) to CXCR4flox/flox mice to obtain MLC2v-Cre+/MLC2v-Cre+/CXCR4flox/flox mice. To confirm the deletion of CXCR4 in cardiac myocytes, we performed PCR analysis on genomic DNA from tail and heart tissue from 8-week-old MLC2v-Cre+/MLC2v-Cre+/CXCR4flox/flox mice. A PCR analysis on genomic DNA from tail and heart was performed on 8-week-old CXCR4flox/flox, MLC2v-Cre+/MLC2v-Cre+/CXCR4flox/flox, and MLC2v-Cre+/MLC2v-Cre+/CXCR4flox/flox mice. Left and right show the presence of CXCR4 floxed allele and Cre into the genome, and the lower panel shows deletion of CXCR4 from ventricular homogenates and not from the tail of the same animal. CXCR4 staining (red) and phase contrast pictures of neonatal cardiac myocytes from CXCR4flox/flox and MLC2v-Cre+/MLC2v-Cre+/CXCR4flox/flox mice shows deletion of CXCR4 from neonatal cardiac myocytes of MLC2v-Cre+/MLC2v-Cre+/CXCR4flox/flox mice. White arrows point toward the cardiac myocytes, and the green arrow points toward noncardiac myocyte cells in culture. Representative Doppler echocardiography image showing normal outflow and inflow during systole and diastole in MLC2v-Cre+/MLC2v-Cre+/CXCR4flox/flox mouse without any evidence of a ventricular septal defect. Representative image of view through right ventricle to reveal normal IVS in MLC2v-Cre+/MLC2v-Cre+/CXCR4flox/flox mouse. Circumferential and radial strain in MLC2v-Cre+/MLC2v-Cre+/CXCR4flox/flox and CXCR4flox/flox mice showing no significant difference (n=4 in each group).
Institute, Boston, Mass) with CXCR4<sup>flox/flox</sup> (kind gift from Yong-Rui Zou, Feinstein Laboratory for Hematopoiesis, New York, NY) and CXCR4<sup>flox/flox</sup> to generate the MLC2v-Cre<sup>+/–</sup>/CXCR4<sup>flox/flox</sup> mouse and MLC2v-CRE<sup>Cre</sup>/CXCR4<sup>flox/flox</sup> mouse, respectively. All these mice were viable with litter sizes similar to that observed with MLC2v-Cre<sup>+/–</sup>. The genotype was determined by genomic PCR from the homogenate obtained from tails (Figure 1A and 1B). The loxP sites surrounds the exon 2 of CXCR4 gene (encodes >90% of protein), and to determine the deletion of exon 2 of CXCR4 gene, we used the primers that bind before and after the loxP sites. Successful recombination was observed from the ventricular homogenate and not from the tail. Immunofluorescence for CXCR4 in neonatal cardiac myocytes in the ventricular homogenate and not from the tail. Immuno-fluorescence for CXCR4 in neonatal cardiac myocytes in the ventricular homogenate and not from the tail. Immuno-fluorescence for CXCR4 in neonatal cardiac myocytes in the ventricular homogenate and not from the tail.

Table. Baseline LV Function Obtained by Echocardiographic Parameters of Mice Used in Studies

<table>
<thead>
<tr>
<th>Tamoxifen</th>
<th>Congenital Deletion</th>
<th>Conditional Deletion</th>
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<tbody>
<tr>
<td></td>
<td>CXCR4&lt;sup&gt;flox/flox&lt;/sup&gt;</td>
<td>MLC2vCre&lt;sup&gt;+/–&lt;/sup&gt; CXCR4&lt;sup&gt;flox/flox&lt;/sup&gt;</td>
</tr>
<tr>
<td>End diastole (mm)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IVS</td>
<td>1.15±0.09</td>
<td>1.14±0.06</td>
</tr>
<tr>
<td>LVPW</td>
<td>1.12±0.08</td>
<td>1.01±0.05</td>
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<tr>
<td>LVED</td>
<td>2.77±0.09</td>
<td>2.76±0.15</td>
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<tr>
<td>End systole (mm)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IVS</td>
<td>1.62±0.08</td>
<td>1.93±0.11</td>
</tr>
<tr>
<td>LVPW</td>
<td>1.67±0.05</td>
<td>1.65±0.07</td>
</tr>
<tr>
<td>LVED</td>
<td>1.37±0.14</td>
<td>1.10±0.14</td>
</tr>
<tr>
<td>Ejection fraction (%)</td>
<td>87.4±2.5</td>
<td>92.1±1.9</td>
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Values are expressed as means±SEM (n=7 in each group). *P<0.05. IVS indicates interventricular septum; LVPW, left ventricular posterior wall; LVED, left ventricular end dimension.

Effect of CXCR4 on Ventricular Remodeling and Function Before and After MI

MCM<sup>+/–</sup>/CXCR4<sup>flox/flox</sup> mice were treated with tamoxifen (40 mg/kg, IP) for 5 days at 6 weeks of age. As recently reported in studies with the MCM mouse, following tamoxifen administration, we observed a transient decrease in myocardial CXCR4 protein compared to littermates that did not receive tamoxifen (Figure 2D). The baseline ventricular function before and 14 days after tamoxifen was within normal limits, as shown in the Table. There were modest statistically significant differences in left ventricular end diastolic dimension and ejection fraction in MCM<sup>+/–</sup>/CXCR4<sup>flox/flox</sup> that received tamoxifen 2 weeks before echocardiography compared to those that did not.
Figure 2. Characterization of MCM^{1+/−}×CXCR4^{floxed/floxed} mouse. A, PCR analysis on genomic DNA from tail and heart was performed on 8-week-old CXCR4^{floxed/floxed}, MCM^{1+/−}×CXCR4^{floxed/floxed}, and MCM^{1+/−}×CXCR4^{floxed/floxed} mice. The left panel depicts a wild-type and floxed CXCR4 band; the right panel depicts CRE band in the genome; and the lower panel depicts deleted band for CXCR4, which was only detected after tamoxifen administration in the ventricular homogenates and not from the tail. B, A dose–response curve for tamoxifen-induced recombination of CXCR4^{floxed/floxed} based on quantitative PCR shows maximum deletion was achieved after 4 days of tamoxifen administration. C, Representative images of immunofluorescence for CXCR4 48 hours after AMI shows knockdown of CXCR4 in cardiac myocytes of tamoxifen-treated MCM^{1+/−}×CXCR4^{floxed/floxed} and endothelial cells positive for CXCR4 (green). Delineated area in CM-CXCR4^{1+/−} image is shown below at higher magnification to aid in identification of nucleated cardiac myocytes devoid of CXCR4 expression. D, Western blot analysis of CXCR4 48 hours after AMI on infarct region shows ~90% deletion of CXCR4. E, Representative M-mode recording of CXCR4^{floxed/floxed} and MCM^{1+/−}×CXCR4^{floxed/floxed} after tamoxifen administration.
was monitored by echocardiography. We hypothesized that because SDF-1 expression is transiently expressed for a short period of time immediately after AMI and begins to return to baseline by the time cardiac myocyte CXCR4 expression is significantly upregulated, the deletion of cardiac myocyte CXCR4 expression would not alter cardiac function or cardiac remodeling. Consistent with our hypothesis, 3 days after AMI, the ejection fraction, anterior wall thickness, and posterior wall thickness of the cardiac myocyte–specific CXCR4 mice (MCM/CXCR4flox/flox and MLC2vCRE/CXCR4flox/flox) were: ejection fraction, 57.8 ± 5.0%; anterior wall thickness, 0.71 ± 0.07 mm; and PW thickness, 0.92 ± 0.10 mm, respectively; there were not statistically different from mice that did not receive tamoxifen (ejection fraction, 50.1 ± 5.0%; anterior wall thickness, 0.73 ± 0.08 mm; and PW thickness, 1.04 ± 0.11 mm, respectively) (Figure 3A through 3F). Twenty-one days after AMI, the ventricular function and remodeling was not statistically different between the absence and presence of cardiac myocyte CXCR4 expression: ejection fraction, 34.4 ± 2.6% versus 40.9 ± 7.5%; anterior wall thickness, 0.36 ± 0.12 versus 0.32 ± 0.11 mm; and PWT, 0.81 ± 0.21 versus 0.83 ± 0.04 mm, respectively (Figure 3A through 3E).

We evaluated vascular density 21 days after AMI to determine whether cardiac myocyte CXCR4 may have had any effect on vascular density. Using isoelectin staining (Figure 4), we measured vascular density in the infarct borderline zone and found similar levels of vascular density in the presence and absence of cardiac myocyte CXCR4 (CM-CXCR4) expression (2471 ± 126 versus 2369 ± 131 vessels/mm², respectively; Figure 4A). We further quantified vascular density as vessels per cardiac myocyte in the noninfarct zone 21 days after AMI (Figure 4B). We observed no difference in vessels per myocyte between groups (vessels/cardiac myocyte: 1.15 ± 0.09 versus 1.18 ± 0.09, CXCR4flox/flox versus CM-CXCR4flox/flox, n = 5 animals per group, P = 0.61).

To further characterize the cardiac remodeling 21 days after MI (Figure 5A through 5D), we measured infarct size 21 days after LAD ligation using Masson’s trichrome stain (Figure 5B). We found no statistically significant difference in the infarct size of CXCR4flox/flox animals (47.93 ± 4.42% of the left ventricular [LV] area, n = 3) compared to CM-CXCR4flox/flox (45.89 ± 7.61% of the LV area, n = 3).

We further characterized the response to MI in the noninfarct zone to determine whether cardiac myocyte CXCR4 had a role in remodeling in the noninfarct zone. The interstitial fibrosis within the noninfarct zone of the myocardium 21 days after MI (Figure 5C) was 0.34 ± 0.11% versus 0.38 ± 0.16% of the LV area and cardiac myocyte diameter in the posterior wall remote from the infarct zone (Figure 5D) was 12.34 ± 0.80 versus 12.89 ± 0.46 μm in CXCR4flox/flox (n = 4) compared to CM-CXCR4flox/flox (n = 4).
SDF-1α mRNA levels were analyzed 3 days after LAD ligation by real-time PCR. We observed by real-time PCR that there was a 33% decrease ($P=0.08$, $n=4$ per group) in SDF-1 mRNA expression in animals that were CM-CXCR4$^{-/-}$ compared to CM-CXCR4$^{+/+}$ (Figure 6). To determine whether this decrease in SDF-1 expression significantly altered stem cell homing, we infused 250,000 GFP+ MSCs via tail vein 1 day after AMI and euthanized animals 4 days after AMI (3 days after MSC infusion).12 Immunofluorescence was used to quantify the number of MSCs per millimeter squared of tissue and revealed no significant differences in MSCs present (CXCR4$^{+/+}$: 17.4±7.33 cells/mm² versus CM-CXCR4$^{-/-}$: 17.2±3.1 cells/mm²; $P=0.99$, $n=5$ per group), suggesting that the degree of change in SDF-1 expression in the CM-CXCR4$^{-/-}$ is not sufficient to alter stem cell homing to the heart.

Discussion
Because significant body of literature has been developed that has demonstrated a critical role for the SDF-1/CXCR4 axis in cardiac development10,27 and myocardial response to cell therapy12,14,28,29 the goal of this study was to analyze the importance of cardiac myocyte derived CXCR4 expression during development and repair following AMI. To address this question we generated a congenital cardiac deletion of...
CXCR4 mouse and a conditional tamoxifen inducible cardiac myocytes specific CXCR4 deletion mouse. We characterized the baseline function of these animals and then studied the myocardial response to injury in these mice.

Cardiac Myocyte–Derived CXCR4 in Cardiac Development

SDF-1 and CXCR4 are ubiquitously expressed during embryogenesis. However, the timing and the stage specific expression of the cytokine and ligand orchestrate the migration of stem cells and development of various organs. CXCR4- and SDF-1–deficient mice present with similar phenotypes and exhibits ventricular septal defect in the membranous part of the septum. Studies have demonstrated that CXCR4 is expressed in the outflow tract and descending part of bulbus cordis by cardiac myocytes, endothelial cells and cardiac neural crest cells during septum formation.30,31 However, the contribution of specific cell type expression of CXCR4 during septum formation is not known. MLC2v is expressed in the outflow tract at day 9 pc, before formation of the septum which starts at or near day 11 pc. We chose MLC2v as a promoter to regulate cre expression to specifically remove cardiac myocyte CXCR4 expression before formation of the septum to determine whether the lack of cardiac myocyte CXCR4 would prove lethal secondary to abnormal cardiogenesis and at the very least ventricular septal defect formation. Studies have demonstrated that the absence of cardiac myocyte CXCR4 would not adversely affect left ventricular remodeling or function following AMI because SDF-1 is rapidly and transiently expressed following AMI before cardiac myocyte CXCR4 is upregulated. This hypothesis proved to be true.

Recent studies have demonstrated that MSCs with or without SDF-1 overexpression have beneficial effects on ventricular function after MI.16 Various mechanisms including stem cell homing, neoangiogenesis, and decreased myocytes death have been suggested. Studies have also demonstrated that SDF-1 overexpression initiates CXCR4 signaling in hypoxic cardiac myocytes and induce antiapoptotic pathway by Akt phosphorylation.12 CXCR4 following AMI is also involved in the recruitment of endothelial progenitor cells and MSCs to the site of injury.28,33–35 Furthermore, the role of SDF-1/CXCR4 signaling is questionable in recruitment and differentiation of cardiac stem cells into cardiac myocytes.29

It is interesting that we observed a decrease in myocardial SDF-1 expression in the CM-CXCR4−/− mice. A recent study in which adenovirus encoding CXCR4 was used to constitutively overexpress CXCR4 in the myocardium before inducing myocardial infarction demonstrated an increase in cardiac SDF-1 expression.36 Thus, it would appear, based on this study and our findings, that cardiac CXCR4 expression is correlated with SDF-1 expression in response to AMI. Importantly, with respect to our findings, we did not observe a...
significant difference in stem cell homing between hearts that had cardiac myocyte CXCR4 expression and those that did not. However, it is yet undefined and awaits future studies to determine whether the myocardial response to stem cell engraftment will be blunted in the absence of cardiac myocyte CXCR4 expression.

The MCM $^{1-2}$/CXCR4$^{flox/flox}$ mouse developed for these studies will serve as a tool to dissect out the mechanisms responsible for the beneficial effects associated with the administration of SDF-1. In particular, because baseline and post-AMI cardiac function is the same in the presence and absence of cardiac myocyte CXCR4 expression, differences in response to stem cell therapy will be able to be attributed to the absence of cardiac myocyte CXCR4. We will further be able to determine the importance and relative contribution of CXCR4 on strategies associated with enhancing cardiac myocyte survival such as ischemic preconditioning and growth factor delivery. In conclusion, deletion of cardiac myocyte CXCR4 does not alter cardiac development or function. Similarly, deletion of cardiac myocyte CXCR4 before AMI does not alter myocardial response to injury. These data, taken together, would suggest no role for cardiac myocyte CXCR4 expression in development, normal physiology, or response to injury. Future studies will need to determine whether there is a role for cardiac myocyte CXCR4 expression in the modulation of the myocardial response to injury mediated by SDF-1 and/or stem cell therapy.

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Disclosures
None.

References


Novelty and Significance

What Is Known?

- SDF-1/CXCR4 axis is important in stem cell–based myocardial repair.
- Myocardial SDF-1 expression begins to decline before cardiac myocyte CXCR4 expression is upregulated after acute myocardial infarction (MI).
- CXCR4-deficient mice die in utero with various birth defects including ventricular septal defect.

What New Information Does This Contribute?

- Cardiac myocyte CXCR4 expression is not essential for heart development.
- CXCR4 deletion from adult cardiac myocytes does not affect ventricular remodeling after MI in the absence of stem cell treatment.

The SDF-1/α/CXCR4 axis plays a major role in the recruitment of bone marrow–derived and cardiac stem cells to the infarct zone after acute myocardial infarction. Recent data suggest that cardiac myocytes upregulate CXCR4 after MI and that extended SDF-1 expression may increase cardiac myocyte survival. Assessing these effects is complicated by the fact that CXCR4-deficient mice die of various birth defects in utero. Thus, it becomes critical to dissect out the role cardiac myocyte–derived CXCR4 (CM-CXCR4) plays after MI and during heart development. To investigate these issues, we generated congenital and conditional cardiac myocyte–specific CXCR4-deficient mouse models. We found that congenital CM-CXCR4 deletion is not essential for heart development and ventricular remodeling after AMI. These are novel findings and have been reported for the first time in literature. Moreover, the mouse models we have generated in our laboratory will be useful for investigating the contribution of CM-CXCR4 to improved ventricular remodeling observed in response to stem cells delivery.
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Role of cardiac myocyte CXCR4 expression in development and left ventricular remodeling after acute myocardial infarction

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**Materials and Methods:**

**Immunostaining:** Mixed cellular population (containing cardiac myocytes, endothelial cells and fibroblast cells) of Neonatal heart was isolated from the pups (1-3 days old) of MLC2vCRE^{+/−}CXCR4^{floxflox} parents with Neomys Kit (Cellutron Life Technology Cat # nc-6031) and plated on the 35mm plate. The cells were cultured for 24 hours and then fixed in 2% paraformaldehyde, blocked with 1% BSA, 1% donkey serum solution and then stained for CXCR4 (primary antibody – 1:100 from abcam Catalog#7199 overnight at 4°C, secondary 1:1000- anti rabbit 594 from abcam for 1 hour at room temperature).

Immunostaining in tissue was performed on the hearts harvested 48 hours post MI and 21 days post MI from MCM^{/+}CXCR4^{floxflox} mice with and without tamoxifen treatment. The hearts were fixed in formalin and embedded in paraffin blocks. 6µm sections were made and stained as described. Forty eight hours post AMI tissue were stained for CXCR4 (Primary - NB100-74396 (Novus Biologicals), secondary- donkey anti rabbit IgG Alexa Fluor 488 from molecular probes) and 21 days post AMI tissue was stained to calculate vessel density with isolectin (Cat # FL-1201 from Vectorlabs) and wheat germ agglutin to quantify cardiac myocyte density (Cat #RL-1022 from Vectorlabs).

**Western Blotting** - Western blotting was done on the 48 hours post infarcted tissue homogenate of CXCR4^{floxflox} and MCM^{+/−}CXCR4^{floxflox} mice (with tamoxifen administration) as described. Briefly, the infarcted tissue was homogenized in PBS with 0.1% Triton X-100 supplemented with PMSF (100 mM), leupeptin (10 µg/ml) and aprotinin (10 µg/ml). Total protein (50 µg) from each sample was prepared in 4x laemmli buffer (200 mM Tris HCl (pH 6.8), 8% SDS, 0.1% bromophenol blue, 40% glycerol), subjected to 10% SDS PAGE and transferred on PVDF membrane. The blot was finally probed with primary antibody (1:500 in 5% milk in 1xTBST) against CXCR4 (Abcam, cat# 2074) followed by incubation with peroxidase-conjugated anti-mouse secondary antibody (1:4000 in 5% milk in 1xTBST). Chemiluminescence (Amersham Biosciences) was used to visualize the bands. Microtek scanner was used to scan the blots and the density of the bands was analysed using the NIH software, Image J.

**Cardiac remodeling** – Mice were anesthetized and their hearts were perfusion fixed with 10% phosphate-buffered formalin under normal pressure 21 days after LAD ligation. The hearts were embedded in paraffin and 5µm sections were cut from the apex to the level just below ligation and alternating sections were stained with Masson trichrome as described. SX images were taken to measure infarct size. Interstitial fibrosis was measured using the viable posterior wall of non infarcted ventricle. Cardiac myocyte diameter was measured along the shortest diameter across the nucleus. 60 such readings were taken. All parameters were measured using Image-Pro Plus (Media Cybernetics). Formula used for calculating infarct size = (epicardial infarct length/ total epicardial circumference) X 100.

**Echocardiography:** Baseline 2D-echocardiography was performed on MLC2v-Cre^{+/−}CXCR4^{floxflox}, MCM^{+/−}CXCR4^{floxflox} and CXCR4^{floxflox} mice at 6 weeks of age using 15MHz linear array transducer interfaced with a Sequoia C256 and GE vision 7 as described previously. Fourteen days post tamoxifen administration echocardiography was performed on MCM^{+/−}CXCR4^{floxflox} mice to determine the baseline function. Doppler and myocardial straining analysis was done on MLC2v-Cre^{+/−}CXCR4^{floxflox} mice at 6 weeks of age. Echocardiography was also performed 3 and 21 days post AMI. EF was calculated as the (LVEDarea-LVESarea)/LVEDarea x 100 where the LVESarea and the LVEDarea are the the end systolic and end diastolic areas of the left ventricle obtained in the parasternal long axis view.
Left Anterior Descending (LAD) artery ligation: LAD ligation was performed on 8-10 weeks old MLC2vCRE$^{+/-}$CXCR4$^{flox/flox}$, MCM$^{+/-}$CXCR4$^{flox/flox}$ (post tamoxifen injection) and CXCR4$^{flox/flox}$ mice as previously described. Briefly, the animals were anesthetized with Xylazine/ketamine, intubated and ventilated with room air at 105 breaths per minute using a rodent ventilator (Harvard Apparatus). Sternotomy was performed and LAD was identified with the help of surgical microscope (Leica M500). LAD was ligated by using 7-0 prolene. Immediate blanching and anterior wall dysfunction revealed a successful ligation. The chest and skin were closed using 6-0 prolene. The animals were removed from the ventilator and kept under oxygen until they recover from anesthesia. Only the animals which survived first 24 hours of ligation were considered for the study.

References