Deletion of CXCR4 in cardiomyocytes exacerbates cardiac dysfunction following isoproterenol administration

ER Wang, AA Jarrah, L Benard, J Chen, M Schwarzkopf, L Hadri and ST Tarzami

ORIGINAL ARTICLE

Heart disease is not only the leading cause of death, disability and healthcare expense in the US, but also the leading cause of death worldwide, and treatments to reduce cardiac damage could have significant health and fiscal impacts.1–3 Cardiac hypertrophy is a prevalent complication of chronic hypertension and an independent risk factor for heart failure and death.9 Current therapies assume that hypertrophy is mediated by neurohormonal activation of G-protein signaling pathways.5 Both α- and β-stimulation, operating through different mechanisms, appear to have growth-promoting effects on cardiac myocytes.6 The cardiac β-adrenergic signaling system mediates most effects of circulating catecholamines and represents the most powerful regulatory input in the heart.7,8 Sustained β-adrenergic receptor (β-AR) activation was shown to enhance the synthesis of myocardial proteins. This effect was mediated via stimulation of myocardial growth factors, upregulation of nuclear proto-oncogenes, induction of cardiac oxidative stress, as well as activation of mitogen-activated protein kinases and phosphatidylinositol 3-kinase.9,10 Catecholamine-induced cardiac hypertrophy is associated with reduced contractile responses to adrenergic agonists, an effect attributed to the downregulation of myocardial β-ARs, uncoupling of the β-ARs and adenylate cyclase, in addition to modifications of downstream cAMP-mediated signaling.7,8 In compensated cardiac hypertrophy, these changes are associated with preserved or even enhanced basal ventricular systolic function due to increased sarcoplasmic reticulum calcium (Ca2+).

Recently, CXCR4 and its ligand, stromal cell-derived factor-1α (SDF-1α), have been considered therapeutic targets in cardiovascular disease.11,12 SDF-1/CXCR4 axis was identified as a key factor in the recruitment of stem cells to areas of tissue injury in multiple organ systems. CXCR4 is a member of the G-protein-coupled receptor family, and agonists or antagonists of G-protein-coupled receptors have been used to treat diseases of every major organ system including the cardiovascular system, becoming the most successful molecular targets in clinical medicine.13–15 Given the keen interest in using SDF-1α as a therapeutic target, it is essential to determine the direct effects of SDF-1α on myocardial function. CXCR4-activation pathways are well studied in leukocytes, however, we cannot assume that CXCR4 functions in an identical capacity in cardiomyocytes. CXCR4 is a widely expressed chemokine receptor that is essential for development, hematopoiesis, organogenesis and vascularization.16

We have previously reported that CXCR4 physically interacts with the β2-ARs and diminishes downstream signaling.17 Activation of CXCR4 by SDF-1α leads to a decrease in β-AR-induced PKA activity as assessed by cAMP accumulation and PKA-dependent phosphorylation of phospholamban, an inhibitor of SERCA2a that regulates calcium handling. Calcium handling is known to be disrupted in heart failure, and interventions aimed at improving cell calcium cycling may represent a promising approach to heart failure therapy.18 Utilizing the transverse aortic constriction model in mice to induce hypertrophy and heart failure, we recently reported that overexpression of CXCR4 by gene transfer successfully prevented cardiac remodeling during chronic pressure overload.19 Cardiac overexpression of CXCR4 in mice with pressure overload prevented ventricular remodeling, preserved capillary density and maintained function, by a mechanism that has not yet been fully understood.

Over the years, many myocardial signaling pathways have been implicated in regulating the cardiac response to increased pressure or volume load, for example, Ga(13)-mediated activation
of the small GTPase RhoA, leading to hematopoietic and cardiac defects. The phenotypic similarity of the SDF-1α and CXCR4 genetically deficient mice suggests that this ligand-receptor pair comprises a monogamous signaling unit in vivo. Interestingly, cardiomyocyte-specific deletion of CXCR4 produces viable mice, which we confirmed independently in this study. Briefly, cardiomyocytes were enzymatically isolated and cells were tested for the presence of CXCR4. Quantitative real-time–PCR (qRT–PCR) using RNA isolated from these CXCR4-KO cardiomyocytes demonstrates significantly less CXCR4 RNA levels compared with wild type (Figure 1a). Western blot analysis using protein lysates from these isolated cardiomyocytes confirms our expectations of significantly lower CXCR4 protein expression (Figure 1b). These observations were also validated by immunohistochemistry analysis revealing significant reduction in CXCR4 protein expression (Figure 1c).

CXCR4 overexpression improves cardiac function in CXCR4-KO mice following isoproterenol treatment

Our study assessed whether the absence of CXCR4 worsens cardiac hypertrophy during sustained exposure to isoproterenol; an agonist of β-ARs. Knockout and wild-type mice were surgically

**RESULTS**

Generation of CXCR4 cardiac-specific knockout (CXCR4-KO) mice To better understand the underlying mechanisms and to uncover SDF-1α/CXCR4’s potential role in regulating β-AR mediated hypertrophic signaling, we generated CXCR4-KO mice. Cardiac specific CXCR4-KO were required because whole body deletion of either CXCR4 or SDF-1α results in an embryonic lethal mutation, leading to hematopoietic and cardiac defects. The phenotypic similarity of the SDF-1α and CXCR4 genetically deficient mice suggests that this ligand-receptor pair comprises a monogamous signaling unit in vivo. Interestingly, cardiomyocyte-specific deletion of CXCR4 produces viable mice, which we confirmed independently in this study. Briefly, cardiomyocytes were enzymatically isolated and cells were tested for the presence of CXCR4. Quantitative real-time–PCR (qRT–PCR) using RNA isolated from these CXCR4-KO cardiomyocytes demonstrates significantly less CXCR4 RNA levels compared with wild type (Figure 1a). Western blot analysis using protein lysates from these isolated cardiomyocytes confirms our expectations of significantly lower CXCR4 protein expression (Figure 1b). These observations were also validated by immunohistochemistry analysis revealing significant reduction in CXCR4 protein expression (Figure 1c).

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implanted with a subcutaneous osmotic pump delivering 30 mg kg$^{-1}$ per day isoproterenol. To measure cardiac performance, echocardiography was performed at baseline, 7 days and 14 days post implantation. Echocardiography is an ideal method to track heart function in treated mice over time because of its noninvasive nature. Mice were left undisturbed for an additional week to maximize the remodeling process. Hemodynamic measurements were obtained at the end of the study (three weeks post implantation) using a pressure-volume conductance catheter. To confirm that the observed phenotypes were the result of CXCR4 ablation, we overexpressed CXCR4 using AAV9 gene transfer to see whether it would rescue the phenotype. AAV9 constructs encoding CXCR4 along with a LacZ control were generated; titration, purity and the most efficient dose for CXCR4 overexpression in the murine heart had been previously determined.\textsuperscript{19} AAV9.CXCR4 was injected via the tail vein one month before pump insertion.

Ejection fraction (EF), fractional shortening (FS), end-systolic and end-diastolic left ventricular internal dimensions, interventricular septum thickness, and left ventricular posterior wall thickness were assessed (Table 1 and Supplementary Table S1). Representative images of short axis m-mode are shown (Figure 2a). At three weeks post pump insertion, in vivo hemodynamics were collected and heart weight–body weight (HW:BW) ratio was calculated (Table 2 and Figures 2b–d). Representative sets of pressure-volume loops from all treated groups were selected (Figure 2c), as well as increased end-systolic and end-diastolic volumes (Tables 1 and 2) and greater HW:BW ratio following (Figure 2c), as well as increased end-systolic and end-diastolic volumes (Tables 1 and 2) and greater HW:BW ratio following isoproterenol treatment (Figure 2d). CXCR4-KO mice that had been injected with AAV9.CXCR4 were rescued from cardiac dysfunction and performance was restored to control group treated control group, suggesting some initial concentric hypertrophy. Eccentric hypertrophy was not seen using m-mode images and the formula $V = \frac{5}{6} \times \text{area} \times \text{length}$ showed an increase in end-diastolic volume among all groups following isoproterenol treatment (Table 1).

The CXCR4-KO mice treated with AAV9-LacZ clearly show significant LV remodeling and decreased cardiac contractility as evidenced by the echocardiography and PV loop data. The echocardiography data shows a 50% increase in the LV end-diastolic volume and over 100% increase in the LV end-systolic volume and significantly lower EF two weeks after isoproterenol infusion (Table 1). Similar results were obtained by PV loop (Table 2). Therefore, the decrease in EF could be explained by decreases in systolic function and depressed cardiac contractility. However, this is not reflected in the hemodynamic assessment of cardiac contractility such as Pes or ESPVR, dp/dt max and PRSW. Out of those parameters, the ESPVR is the most reliable parameter because it is not affected by loading conditions or afterload. Although the ESPVR in the CXCR4-KO mice was lower than the other groups, it did not reach statistical significance; however, the

| Baseline | +AAV9.LacZ | +AAV9.CXCR4 | +AAV9.LacZ | +AAV9.CXCR4 |
|---------|------------|------------|------------|------------|
| n       | 6          | 6          | 5          | 5          |
| ISVd (mm) | 0.73 ± 0.05 | 0.80 ± 0.05 | 0.79 ± 0.03 | 0.74 ± 0.01 |
| LVIDd (mm) | 3.69 ± 0.09 | 3.57 ± 0.10 | 3.52 ± 0.18 | 3.78 ± 0.22 |
| LVPWd (mm) | 0.78 ± 0.06 | 0.76 ± 0.04 | 0.80 ± 0.04 | 0.72 ± 0.06 |
| IVSs (mm) | 1.63 ± 0.10 | 1.65 ± 0.06 | 1.67 ± 0.05 | 1.65 ± 0.02 |
| LVIDs (mm) | 1.48 ± 0.09 | 1.52 ± 0.11 | 1.54 ± 0.13 | 1.65 ± 0.15 |
| LVPWs (mm) | 1.70 ± 0.05 | 1.63 ± 0.04 | 1.64 ± 0.06 | 1.60 ± 0.11 |
| %FS | 60.2 ± 1.6 | 57.8 ± 1.8 | 56.6 ± 1.8 | 56.7 ± 1.6 |
| EDV (ml) | 79.1 ± 7.0 | 74.3 ± 5.6 | 710.5 ± 54.4 | 805.3 ± 117.1 |
| ESV (ml) | 18.9 ± 1.5 | 16.5 ± 1.7 | 17.9 ± 2.2 | 194 ± 2.6 |
| %EF | 76.0 ± 1.6 | 78.1 ± 0.8 | 751 ± 1.9 | 75.6 ± 1.7 |
| HR (b.p.m.) | 559 ± 13 | 592 ± 30 | 523 ± 38 | 557 ± 24 |

<sup>Abbreviations:</sup> EDV, end-diastolic volume; EF, ejection fraction; ESV, end-systolic volume; FS, fractional shortening; HR, heart rate; IVSd, end-diastolic interventricular septum thickness; IVSs, end-systolic interventricular septum thickness; LVIDd, end-diastolic left ventricular internal dimension; LVIDs, end-systolic left ventricular internal dimension; LVPWd, end-diastolic left ventricular posterior wall thickness; LVPWs, end-systolic left ventricular posterior wall thickness. One-way analysis of variance (ANOVA) with Tukey's post hoc test was performed between all groups at each time point. Asterisks indicate significance in comparison with the CXCR4-KO + AAV9.LacZ group.

$V_{0}$ (theoretical volume when no pressure is generated) was shifted to the right compared with the other three groups (Figure 2b and Supplementary Figures S2a and b). Together, a lower ESPVR and a rightward shift in V0 account for depressed contractility. Unfortunately, this is a limitation of the PV loop system and it is not feasible to adjust the ESPVR value for the rightward shift in V0.
Collectively, echocardiography and in vivo hemodynamics revealed maintenance of ventricular volumes and ESPVR with AAV9.CXCR4 (Figure 2 and Tables 1 and 2). Taken together, these changes indicate a role for the SDF-1/CXCR4 axis in regulating cardiac function in the stressed condition. These findings suggest a significant role for the SDF-1/CXCR4 axis in regulating cardiac function in the stressed condition. It has been traditionally assumed that SDF-1α would affect its target cells by selectively interacting with the CXCR4; however, recent work revealed that SDF-1α also binds to the chemokine receptor CXCR7 (SDF-1α alternative receptor). Subsequent studies indicated that CXCR7 might have diverse functions in different cell types and biological conditions. To uncover whether the absence of CXCR4 alters CXCR7 expression in the heart, CXCR7 mRNA levels were assessed. Although a trend of upregulation in CXCR7 expression appears in both AAV9.LacZ- and AAV9.CXCR4-treated groups, there were no statistically significant changes observed among these groups (Figure 3c), suggesting that its expression is not regulated by the presence or absence of hypertrophy. It has also been reported that re-expression of β-MHC in distinct regions of the hypertrophic heart would affect its target cells by selectively interacting with the CXCR4-mediated contractions and improves cardiac function.

Changes in the expression of hypertrophy-related genes
The expression patterns of cardiac hypertrophy-related genes have been well documented and widely used as markers for hypertrophy. We first looked at the expression of B-type natriuretic peptide (BNP). BNP is a hormone produced by the heart that is released in response to changes in pressure that occur during the development of heart failure, and as such, BNP expression level is considered to be an indicator for heart failure. qRT-PCR revealed a marked upregulation in BNP mRNA expression in CXCR4-KO LacZ-treated mice (Figure 3a). Overexpressing AAV9.CXCR4 inhibited BNP upregulation and levels matched those of the control groups (CXCR4-f/f). Next, we looked at the expression of β-myosin heavy chain (β-MHC) as a marker of pathological hypertrophy. It has also been reported that re-expression of β-MHC occurs in distinct regions of the hypertrophic heart. It has been traditionally assumed that SDF-1α would affect its target cells by selectively interacting with the CXCR4; however, recent work revealed that SDF-1α also binds to the chemokine receptor CXCR7 (SDF-1α alternative receptor). Subsequent studies indicated that CXCR7 might have diverse functions in different cell types and biological conditions. To uncover whether the absence of CXCR4 alters CXCR7 expression in the heart, CXCR7 mRNA levels were assessed. Although a trend of upregulation in CXCR7 expression appears in both AAV9.LacZ- and AAV9.CXCR4-treated groups, there were no statistically significant changes observed among these groups (Figure 3c), suggesting that its expression is not regulated by the presence or absence of hypertrophy.
model only abolished CXCR4 in cardiomyocytes, this may be due with isoproterenol caused a marked increase in CXCR4 expression saline vehicle pump (Figure 3d, right panel). However, treatment CXCR4 fl reduction in CXCR4 protein expression between CXCR4-KO and of data from three different experiments showed signi
ing in the hearts of the experimental animals by western blot before myocardial CXCR4. Last, CXCR4 protein expression was quantied via western blot. Representative western blots of CXCR4 from three animals per group are shown in the left panel. Asterisks indicate signi
cance in comparison with the CXCR4-KO + AAV9.LacZ group. Table 2. In vivo hemodynamics: pressure-volume data were analyzed using IOX2 software

| 14 Days infusion     | CXCR4-KO +AAV9.LacZ +AAV9.CXCR4 | CXCR4 fl/fliplex +AAV9.LacZ +AAV9.CXCR4 |
|---------------------|--------------------------------|----------------------------------------|
| n                   | 6                              | 5                                      | 5                             | 4                             |
| Pes (mm Hg)         | 96.5 ± 7.9                     | 90.6 ± 5.1                             | 99.6 ± 10.1                   | 105.3 ± 12.9                  |
| Ped (mm Hg)         | 6.2 ± 1.0                      | 8.6 ± 1.7                              | 8.0 ± 1.4                     | 8.5 ± 0.5                     |
| dP/dt max (mm Hg s⁻¹) | 5780 ± 393                   | 4677 ± 339*                            | 5173 ± 313                   | 5308 ± 1008                   |
| dP/dtmin (mm Hg s⁻¹) | −4765 ± 175                   | −3778 ± 340                            | −4155 ± 475                  | −4029 ± 840                   |
| Tau (ms)            | 10.3 ± 0.9                     | 12.6 ± 1.0                             | 11.6 ± 0.7                   | 11.2 ± 0.4                    |
| EDV (μl)            | 86.0 ± 7.9                     | 49.8 ± 3.8**                           | 50.6 ± 3.7**                 | 54.7 ± 2.5**                  |
| ESV (μl)            | 51.8 ± 7.6                     | 18.4 ± 2.6**                           | 21.4 ± 4.0**                 | 20.2 ± 2.2**                  |
| SV (μl)             | 48.0 ± 3.9                     | 38.8 ± 1.2                             | 37.6 ± 1.5*                  | 42.3 ± 1.3                    |
| CO (μl min⁻¹)       | 25021 ± 2364                  | 17291 ± 1287*                          | 15328 ± 907**                | 17043 ± 1666**                |
| EF (%)              | 52.0 ± 3.6                     | 72.6 ± 2.4**                           | 66.8 ± 4.8*                  | 70.5 ± 3.2*                   |
| ESPVR               | 2.5 ± 0.4                      | 3.8 ± 0.5                              | 4.1 ± 0.8                    | 4.5 ± 0.8                     |
| EDPVR               | 0.08 ± 0.01                    | 0.08 ± 0.02                            | 0.08 ± 0.01                  | 0.08 ± 0.03                   |
| PRSW                | 93.9 ± 11.7                    | 97.5 ± 12.9                            | 94.3 ± 9.9                   | 118.6 ± 22.2                  |
| HR (b.p.m.)         | 499 ± 15                      | 445 ± 22                               | 424 ± 19                     | 440 ± 23                      |

Abbreviations: CO, cardiac output; Ea, arterial elastance; EDV, end-diastolic volume; EF, ejection fraction; EDVR, end-diastolic pressure-volume relationship; ESPVR, end-systolic pressure-volume relationship; ESV, end-systolic volume; HR, heart rate; Pes, end-systolic pressure; Ped, end-diastolic pressure; PRSW, preload-recruitable stroke work; SW, stroke volume; SV, stroke volume. Asterisks indicate significance in comparison with the CXCR4-KO + AAV9.LacZ group.

Figure 3. Expression profiling of cardiac genes associated with hypertrophy. (a–c) RNA was isolated from whole ventricular myocardium and the expression of hypertrophy-associated genes, for example, BNP, β-MHC and CXCR7 were assessed. Specific mRNA levels were quantified via qRT–PCR performed in triplicate (n = 3 mice per group *P < 0.05, **P < 0.01, ***P < 0.001). (d) CXCR4 protein expression before and after isoproterenol infusion was quantified by western blot. Representative western blots of CXCR4 from three animals per group are shown in the right panel (n = 3). GAPDH antibody was used as a loading control. Densitometric analysis of data from three different experiments is shown in the left panel. *P < 0.05, **P < 0.01, ***P < 0.001. GAPDH, glycerolaldehyde 3-phosphate dehydrogenase.

myocardial CXCR4. Last, CXCR4 protein expression was quanti
ed in the hearts of the experimental animals by western blot before and after isoproterenol infusion (Figure 3d). Densitometric analysis of data from three different experiments showed significant reduction in CXCR4 protein expression between CXCR4-KO and CXCR4 fl groups treated with AAV9.LacZ and implanted with a saline vehicle pump (Figure 3d, right panel). However, treatment with isoproterenol caused a marked increase in CXCR4 expression in all cohorts when compared with the saline groups. As our model only abolished CXCR4 in cardiomyocytes, this may be due to CXCR4 upregulation in other heart resident cells such as fibroblasts, smooth muscle cells and endothelial cells in response to isoproterenol.

CXCR4-KO mice exhibited increased interstitial fibrosis in vivo following isoproterenol treatment
We used conventional Picrosirius Red and Masson’s Trichrome staining to assess interstitial fibrosis. Histological staining was done on left ventricular sections at mid-papillary level and images
were taken using light microscopy (Figure 4). Cardiac fibrosis was quantified using Masson’s Trichrome staining technique using a composite image of each sample at × 40 magnification (Figure 4a). There was a significant increase in fibrosis in CXCR4 KO groups that was not present in the AAV9.CXCR4 rescued group (Figures 4a and c). CXCR4 overexpression prevented ventricular remodeling in the setting of isoproterenol-induced hypertrophy and failure (Figures 4a and c). Our data also confirms that the AAV9. LacZ overexpression does not have any side effects, as similar levels of fibrosis were observed in isoproterenol-stressed CXCR4 KO mice both with and without AAV9.LacZ gene therapy.

PCR array analysis of CXCR4-KO mice as compared with flox control; CXCR4 gene therapy increases GSK3β activity

Our next step was to assess the possible mechanisms that confer protection and are modulated by CXCR4. RNA was isolated from whole ventricular myocardium and specific mRNA levels were quantified. Using a commercial PCR array, we identified genes that were either upregulated or downregulated in AAV9.CXCR4 rescued mice as compared with CXCR4-KO mice post isoproterenol treatment. Interestingly, many of these genes were associated with cell death pathways (Figure 5a). Our data demonstrates significant upregulation of p53 (tumor suppressor gene) and Bax (Bcl-2-associated X protein) in the CXCR4-KO group, whereas AAV9.CXCR4 gene therapy prevented upregulation and reduced mRNA levels and their protein expression significantly (Figure 5b). p53 and Bax are both pro-apoptotic genes that can be regulated by the glycogen synthase kinase 3 (GSK3) signaling pathway.35

GSK3, of which there are two isoforms, GSK3α and GSK3β, was originally characterized in the context of glycogen metabolism regulation but is now known to regulate many other cellular processes including the promotion of apoptotic signaling. In neural cells, p53 forms a complex with GSK3β that in turn activates the Bax and Caspase 3 pathways.35 In the heart, emphasis has been placed particularly on the GSK3β isoform. Phosphorylation of GSK3α (Ser21) and GSK3β (Ser9) inhibits their protein kinase activity. Hypertrophic agonists such as β-AR ligands increase phosphorylation of GSK3β (Ser9), resulting in a 40–60% decrease in its activity. Immunoblot analysis (Figure 5c, lower panel) and relative quantification of p-GSK3β (Ser9) (Figure 5c, upper panel) demonstrates a significant decrease in the phosphorylation of GSK3β (Ser9) in CXCR4-KO LacZ-treated mice that were exposed to isoproterenol. This implies that there is increased GSK3β-dependent signaling activity associated with these knockouts. In mice injected with AAV9.CXCR4, phosphorylation (Ser9) was restored and GSK3β activity was diminished suggesting that the SDF-1α/CXCR4 axis utilizes this signaling pathway (Figure 5c).

Mitochondrial-related gene expression changes

Mitochondria are the main intracellular location for fuel generation and it is generally accepted that mitochondrial dysfunction develops in the failing heart.36 The purpose of this section was to outline changes in mitochondrial function, oxidative stress and biogenesis in CXCR4-KO mice and to evaluate whether these

Figure 4. CXCR4 knockout mice showed increased perivascular and interstitial fibrosis compared with rescued animals and controls. (a) Ventricular cross sections were taken at the mid-papillary level and stained using Masson’s Trichrome. Comparative images shown are composites of images taken at × 40. Panel shown at × 200. (b) Quantification of fibrosis was calculated over the entire section at × 40 magnification. The significance of each group is shown as compared with knockouts injected with AAV9.LacZ (**P < 0.05, ***P < 0.01). (c) Sirius red-stained sections at × 200. Quantification of fibrosis was calculated on sections obtained from CXCR4-KO and control group (CXCR4-f/f) subjected to isoproterenol treatment without any gene therapy.
intracellular antioxidant enzymes in mitochondria that have an (superoxide dismutase 2) are among the major families of reactive oxygen species. Glutathione peroxidase (GPX) and SOD2 source of mitochondrial injury is oxidative stress produced by handling genes were also quantified with knockouts injected with AAV9.lacZ (n = 3 per group). (b) mRNA expression of GSK3-activated pro-apoptotic factors; PS3 and Bax (quantified via qRT–PCR) was shown in upper panel (n = 3) and representative western blots of PS3 and Bax protein expression from four animals per group is shown in the lower panel (*P = 0.05). (c) Densitometric analysis for GSK3β phosphorylation/total-GSK3β is shown (*P < 0.05) and a representative western blot of GSK3β phosphorylation on Ser9 is depicted. Western blots were performed on lysates prepared from whole ventricular tissue, and quantification was performed using four animals per group.

To confirm our data the expression of key oxidative stress-handling genes were also quantified using qRT–PCR. A major source of mitochondrial injury is oxidative stress produced by reactive oxygen species. Glutathione peroxidase (GPX) and SOD2 (superoxide dismutase 2) are among the major families of intracellular antioxidant enzymes in mitochondria that have an important role in limiting oxidative burden. The content of oxidative stress handing genes such as GPX, SOD2 and PRDX3 (peroxiredoxin 3) mRNAs were analyzed. Both GPX and PRDX3 were increased in the CXCR4-KO group, whereas CXCR4 overexpression ameliorated the increase in PRDX3 significantly (P < 0.05) and reduced the GPX content to some extent (P = 0.091) (Figures 6d–f). Mitochondrial biogenesis was also assessed between the CXCR4-KO and the AAV9.CXCR4-rescued groups. We did not see any significant changes in the content of genes involved in mitochondrial biogenesis such as, nuclear respiratory factor-1; estrogen-related receptor; transcription factor A mitochondrial; and the transcriptional co-activator pereoxisome proliferator-activated receptor γ co-activator 1a (Figures 6g and h).

Our data collectively suggests that the absence of cardiac-specific CXCR4 exacerbates stress-related injuries, and thus, mitochondrial gene responses can be significantly modulated by the absence of CXCR4 when they are chronically exposed to isoproterenol. Our data also demonstrates an improvement in mitochondrial function by AAV9.CXCR4 overexpression. The relation between a lack of CXCR4 in cardiomyocytes and the changes in mitochondrial function are not well defined and further studies are necessary to elucidate the mechanisms by which SDF-1α/CXCR4 regulates mitochondrial function during certain pathological conditions, for example, catecholamine-induced myocardial fibrosis and oxidative stress.

**DISCUSSION**

Cardiac hypertrophy represents a critical compensatory mechanism to hemodynamic stress and injury. In cardiac hypertrophy, alterations of β-adrenergic signaling, increases in myocardial β-AR density and increased expression of hypertrophy-associated proteins have been reported and thus, it is important to assess whether SDF-1α/CXCR4 can limit this response. In our animal model, CXCR4 expression has been selectively abrogated in cardiomyocytes, allowing for an in-depth analysis of SDF-1α/CXCR4...
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successively in cardiomyocytes allowed us to answer fundamental
CXCR4 activation. The ability to abrogate CXCR4 activity exclu-
β
signaling in relation to β-AR activity on cardiac function without
interference from other cell types. Our data demonstrates that
CXCR4 ablation exacerbates cardiac hypertrophy induced by
chronic infusion of isoproterenol, a β-AR agonist. This data
supports our previous finding that demonstrated a novel anti-
remodeling role of the SDF-1α/CXCR4 chemokine axis following
pressure overload.19 Given our previous observations that CXCR4
activation modulates β-AR activity, 17 it was critical to understand
how β-AR activation affects cardiac function with or without
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tions: what is the physiological relevance of its signaling, what
electrophysiological pathways are utilized by CXCR4 in
hearts that are chronically exposed to isoproterenol? In essence,
we hypothesized that cardiomyocytes without CXCR4 may be
more sensitive to β-AR activation, and indeed our in vivo results
indicate cardiac dysfunction and hypertrophy in hearts lacking
CXCR4. Our isoproterenol heart failure model is associated
with eccentric hypertrophy, which is the result of myocardial
apoptosis, decreased contractility and thus myocyte stretch
due to increases in wall stress. By echocardiography, there
was a slight thinning of the left ventricular septal and posterior
wall thickness and significantly higher LV length in diastole
and systole in the CXCR4-KO group compared with the other
three groups. This reflects significantly higher LV end-diastolic
and end-systolic volumes, and is supported by the significantly
higher HW/BW ratio in the CXCR4-KO despite the increase in
apoptotic markers. Thus the hypertrophy seen in the CXCR4-KO
has to be due to eccentric hypertrophy and not concentric
hypertrophy.

One would have expected that the LV end-diastolic pressure
would have been increased in the CXCR4-KO, which was not the
case despite the increase in myocardial fibrosis. In most models
of heart failure, diastolic dysfunction precedes systolic dysfunction.
However, in some circumstances this is not the case, such as in
chemotherapy-induced cardiotoxicity where the depressed LV
systolic function is not necessarily associated with diastolic
dysfunction. This could be explained by loss of large amount of
cardiomycocytes in a short period of time, but is more reasonably
explained by depressed right ventricle function as well. This
could be the case in the isoproterenol heart failure model where the
damage involves both the LV as well as the right ventricle. Thus
the decreases in right ventricle function will decrease the preload
of the LV and will falsely contribute to normal LV EDP in the
CXCR4-KO group.

We managed to rescue this phenotype and improve cardiac
function by AAV9.CXCR4 gene therapy. This suggests that
observed significant differences in CXCR4-KO groups are indeed
regulated by SDF-1α/CXCR4 axis. Given that mice lacking global
CXCR4 (conventional knockout) exhibit hematopoietic and cardiac
defects, 27 it was interesting to see that cardiomyocyte CXCR4
expression is not required for normal heart development. We
believe, however, that the axis has an important role in regulating
cardiac myocyte function particularly in response to stress. Our
data is not in agreement with the recently published study by
Dong et al. demonstrating that there is no phenotypic difference
between control and cardiomyocytes CXCR4-null mouse following
acute myocardial infarction. 39 There are some fundamental
differences between their model and ours that might explain
the contrasting results. Notably, their model uses acute myocardial

Figure 6. Transcription profiling reveals alterations in genes regulating mitochondrial function and oxidative stress response. RNA was isolated from whole ventricular tissues of mice subjected to isoproterenol infusion (CXCR4-KO AAV9.lacZ, CXCR4-KO AAV9.CXCR4, CXCR4-f/f-AAV9.LacZ and CXCR4-f/f-AAV9.CXCR4), mRNA levels of genes that are associated with mitochondrial function (a–c), mitochondrial oxidative stress (d–f), and mitochondrial biogenesis (g and h) were quantified via qRT–PCR performed in triplicate (n = 3 mice per group *P < 0.05, **P < 0.01).
infarction, which is associated with an inflammatory reaction that leads to healing and scar formation. SDF-1α and CXCR4 are potent chemotactic proteins and have an important role in regulating stem cells recruitment, inflammation and inflammation-mediated injury. In our model of isoproterenol-induced hypertrophy, inflammation or the recruitment of stem cells by SDF-1α/CXCR4 are not having major roles, which might contribute to the contrasting results that were observed.

The heart is capable of cellular and ventricular chamber remodeling to adapt against pathologic and physiologic stimulation. Although the sources of stress are distinguished, they share results that were observed. Not having major roles, which might contribute to the contrasting

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MATERIALS AND METHODS

The investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health.

Generation of CXCR4 cardiac-specific knockouts

We have C57Bl/6 cardiac myocyte-specific CXCR4-null mice (CXCR4-KO) that were obtained by cross-breeding CXCR4loxP mice with 2 mice carrying a transgene for Cre-recombinase under the control of the cardiac myocyte-specific α-myosin heavy chain promoter (αMHCcre) (courtesy of Dr Michael Schneider, Baylor College of Medicine). Ox/ox (f/f) controls) (25–30 g males) with or without gene transfer of AAV9.CXCR4 or AAV9.LacZ, slightly posterior to the aorta. To obtain maximal remodeling, mice were sacrificed at three weeks post mini pump implantation. We gave an extra week post injection to allow for fibrosis accumulation. All experimental procedures followed the regulations of and were approved by the animal care and use committee of Icahn School of Medicine at Mount Sinai.

Echocardiography and in vivo hemodynamics

Mice were anesthetized with intraperitoneal ketamine (100 μg g−1) for echocardiographic analysis. Two-dimensional images and M-mode tracings were recorded on the short axis at the level of the papillary muscle and the long axis to determine percent FS and ventricular dimensions (GE Vivid 7 Vision, i13L transducer, Wauwatosa, WI, USA). In vivo hemodynamics was studied using a 1.2-Fr pressure-volume (PV) conductance catheter (Sciensence, London, ON, Canada). Mice were anesthetized with an intraperitoneal injection mixture of urethane (1 mg g−1), etomidate (10 μg g−1), morphine (1 μg−1) and were then intubated via a tracheostomy and mechanically ventilated at 7 μg−1 tidal volume and 125 respirations per minute. The PV catheter was placed in the LV via an apical stab approach as previously described. Pressure-volume data were analyzed using IOK2 software (EMKA Technologies, Falls Church, VA, USA). All procedures were approved by and performed in accordance with the Institutional Animal Care and Use Committee of the Mount Sinai School of Medicine. The investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

Staining procedures for intersitial fibrosis in hypertrophic hearts

At the end time point, hearts were perfused with 30 ml of cold phosphate-buffered saline with 0.1% of triton X-100. LV was harvested, embedded in OCT (optimal cutting temperature), frozen and stored at −80 °C. OCT heart sample was sectioned at 6–8 μm. Masson’s Trichrome was conducted according to the guideline of the commercially available kit (Sigma). Frozen slides were stained with Masson’s trichrome for collagen detection and Masson’s trichrome for myofibers detection. For Masson’s trichrome staining, blue/purple collagen fibers and red myocytes were observed in the tissues collected from hypertrophic hearts. In the Picosiris Red staining method, collagen appears red and myocytes are pale yellow.

Protein preparation and immunoblot analysis

Membrane and tissue homogenates were prepared as previously described. Briefly, proteins were extracted by using total protein extraction kit (Millipore, Darmstadt, Germany). Protein concentration was determined using a Bradford assay. In all, 30 μg of proteins were then run on 10% SDS–polyacrylamide gel electrophoresis gels and transferred on
polyvinylidene difluoride membrane. Following primary antibodies were used for immunoblotting: GSK3β, GSK3β-Phospho(ser9), p53, Bax, CXCR4, SDF-1 and GAPDH. Proteins were visualized using HRP-conjugated secondary antibodies and exposed using chemiluminescent HRP substrate (Millipore, Billerica, MA, USA).

Real-time quantitative reverse transcription–PCR assays
Whole ventricular tissues were minced and total RNA extracted using Trizol reagent (Gibco BRL, Carlsbad, CA, USA) according to manufacturer’s instructions. mRNA levels were determined by qRT-PCR using a kit according to manufacturer instructions (Applied Biosystems, Foster City, CA, USA). mRNA levels were determined by qRT-PCR using a kit according to manufacturer instructions (Millipore, Billerica, MA, USA). Statistical analyses
Numeric data are presented as mean ± s.e.m. One-way analysis of variance (ANOVA) with Tukey’s post hoc test was performed between all groups at each time point. Student’s t-test were also utilized where it was applicable with P-values < 0.05 considered statistically significant.

CONFLICT OF INTEREST
The authors have no conflicts of interest to declare.

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