Ablation of CXCR4 expression in cardiomyocytes exacerbates isoproterenol-induced cell death and heart failure

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Abstract. CXCR4 is a seven-transmembrane-spanning Gi-coupled receptor for the SDF-1 chemokine and plays a critical role in cardiovascular development and post-injury repair. However, the specific role of CXCR4 in cardiomyocytes is incompletely understood. It was hypothesized that CXCR4 activation in cardiomyocytes antagonizes β-adrenoceptor/Gs signaling-induced cardiac dysfunction. Cardiomyocyte-specific CXCR4 knockout (CXCR4-^{CM}KO) mice were generated by crossing CXCR4$^{fl/fl}$ and MHC-Cre$^{+/-}$ mice. Their cardiac structure and function in the basal state are equivalent to that of the control MHC-Cre$^{+/-}$ littermates until at least 4 months old. However, following continuous subcutaneous administration of isoproterenol (Iso) via an osmotic mini-pump, the ventricular myocardial contractility, dilation, cardiomyocyte apoptosis, and interstitial fibrosis are worse in CXCR4-^{CM}KO mice than in MHC-Cre$^{+/-}$ littermates. In the cultured H9C2 cardiomyocytes, SDF-1 treatment markedly attenuated Iso-induced apoptosis and reduction in phospho-Akt, and this protective effect was lost by knockdown of CXCR4 or by co-treatment with Gi inhibitors. In conclusion, CXCR4 promotes cardiomyocyte survival and heart function during β-adrenergic stress.

Introduction

The C-X-C chemokine receptor type 4 (CXCR4) is a Gα_i protein-coupled receptor, and its only identified ligand is the CXC chemokine stromal-derived factor-1 (SDF-1). The SDF-1/CXCR4 axis is essential for organogenesis during embryonic development, including hematopoiesis, cardiac septum formation, and neuronal development; genetic deletion of CXCR4 or SDF-1 leads to almost identical developmental defects and embryonic lethality (1-3). In the adult animals, previous evidence has shown that SDF-1/CXCR4 axis plays an indispensable role in the maintenance of stem/progenitor cells in the bone marrow niche, recruitment of stem/progenitor cells to sites of injury, ischemic neovascularization and tissue repair (4-10). These findings have led to the notion of targeting SDF-1/CXCR4 axis for therapy of ischemic heart disease (10-14).

The role of CXCR4 signaling in cardiomyocyte biology, however, is not well understood. Recent evidence suggested that in the developing heart, SDF-1/CXCR4 signaling is crucial not only for epicardia-guided coronary vessel formation but also for the expansion of the second heart field (6,15). In the adult, CXCR4 is expressed in cardiomyocytes across mice, rats and humans (16). Notably, SDF-1 treatment of isolated mouse papillary muscles or rat cardiomyocytes attenuates β-adrenergic agonist Isoproterenol (Iso)-induced calcium mobilization and contractility (17), and cardiac CXCR4 knockout mice develop progressive cardiomyopathy (18). Importantly, CXCR4 is expressed at high levels in the cardiomyocytes of patients with end-stage heart failure (19), the first cuase of death in the developed countries.

Heart failure is featured by reduced cardiac pump function unable to maintain the systemic demand of blood supply, for which the impaired β-adrenergic receptor (β-AR) signaling plays a central role (20). β-ARs, predominantly β_1 (80%) and β_2 (20%) ARs in a normal mammalian heart, primarily couple Gα_s, although β_2 has been shown to also couple Gα_i. Upon binding to ligands (catecholamine hormone epinephrine and neurotransmitter norepinephrine) or agonist (isoproterenol), β-ARs activate Gα_s GTPase, leading to adenylyl cyclase activation, cAMP production, and signaling events including protein kinase A (PKA) activation and phosphorylation (activation) of proteins involved in cellular calcium mobilization and contraction (21). While acute activation of β-ARs increase heart rate (chronotrophy) and contractility (inotrophy) to augment cardiac performance, over or chronic activation results in cardiomyocyte death, adverse cardiac remodeling, and progressive deterioration of function (22-26). Thus, β blockers are one of most widely used treatment and have been shown to increase the long-term survival of patients with chronic heart failure (27,28). Furthermore, evidence over last

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three decades suggested that β_1 AR not β_2 AR plays a causal role in cardiomyocyte apoptosis and heart failure (26,29-31). Mice lacking β_1 and β_2 ARs or overexpressing β_2 ARs have essentially normal basal cardiac function (32,33), whereas mice overexpressing β_1 AR, Gαs, or active-PKA display a similar cardiac phenotype, including initial increased responsiveness to catecholamines and hypertrophy followed by cardiomyocyte death, fibrosis, cardiac dilation and failure (34-36).

In the present study, the role of cardiomyocyte CXCR4 in Iso-induced heart failure in cardiomyocyte-specific CXCR4 knockout (^{CM}KO) mice was investigated. It was found that these mice display normal heart structure and function in the basal state but are more susceptibility to Iso-induced cardiomyocyte apoptosis, fibrosis and heart failure, and that SDF-1/CXCR4 signaling attenuates Iso-induced cardiomyocyte death in a Gα_i dependent manner.

Materials and methods

Mice. Male, 8-10 weeks old mice on C57BL/6J background with body weights between 25 and 27 g were used. CXCR4$^{fl/fl}$ mice and α-MHC-Cre mice were obtained from Jax lab and bred to generate cardiomyocyte-specific CXCR4 knockout (MHC-Cre$^+$; CXCR4$^{f/f}$ or ^{CM}KO) mice. All animal experiments in the present study were approved (IACUC approval no. 3005) by the Animal Care and Use Committee of Huazhong University of Science and Technology (Wuhan, China) and performed in compliance with the 'Guide for the Care and Use of Laboratory Animals' (NIH publication) and relevant ethical regulations for animal testing and research. Animal numbers for the experiments were pre-calculated based on the reported similar studies from the authors and other laboratories with 80% power (37,38). The basic characteristics of our model animals were reported, and physiological and biochemical measurements were performed in double-blinded fashion. After Iso or Saline minipump implantation, the animal health and behaviour were monitored daily for the first 3 days, then every other day until they were euthanized at day 14. Over the course of the study, no experimental animal experienced conditions needed for euthanasia before the end of experiments. For euthanasia, the mice were placed in a designated chamber with continuous introduction of 100% CO_2 at a fill rate of 50% of the chamber volume per min and for a duration of 15 min; the death was verified by loss of consciousness, head sinking, and disappearance of muscle tone. Then the mice were subjected to a secondary method, either vital organ removal (for 30 mice used in histological analyses) or cervical dislocation (for 30 mice used in echocardiographic analyses), to ensure death. For colony maintenance, mice were fed *ad libitum* and maintained at 22-24˚C ambient temperature and 40-60% humidity under a 12:12-h light: dark cycle.

Chemicals and bioagents. All chemicals and bioagents used in the present study, including Iso, AMD3100, Gi inhibitor Pertussis toxin (all from Sigma-Aldrich; Merck KGaA), SDF-1 (R&D Systems, Inc.) were purchased from commercial sources and authenticated by the providers. Their concentrations, Iso (100 μM), SDF-1 (500 ng/ml), and AMD3100 (100 ng/ml), used on H9C2 cells were based on pilot studies by the authors and consistent with a similar study previously reported (39).

Continuous Iso infusion model. ^{CM}KO and Cre mice received Iso (45 mg/kg/day) or saline (control) continuously via Alzet mini-osmotic pumps (model 2004; Durect Corporation) for up to 28 days. Pumps were prepared and subcutaneously implanted in the mice by following the manufacturer's instructions. The surgery was performed under anesthesia by intraperitoneal injection of sterile pentobarbital sodium (50 mg/kg body weight, Sigma-Aldrich; Merck KGaA) and post-operative care was performed by following our approved animal study protocol. For pain management, Metacam was subcutaneously injected (1 mg/kg) at the end of surgery and continued twice daily for 2 days. The heart function was monitored via echocardiography at day 0, 3, 7, and 14. Then the mice were euthanized, and the cardiac tissues were harvested for histological analyses.

Echocardiography. Cardiac function was recorded and analyzed using Vevo 770 high-resolution ultrasound system (VisualSonics, Inc.) as previously described (10). Mice were lightly anesthetized via 2% isoflurane inhalation and secured with surgical tapes to the platform in supine position. Heart rates were recorded. Data were collected after three days of training. Parasternal long-axis view, short-axis view at the papillary muscle level and 2-D guided M-mode images were recorded. Left ventricular ejection fraction, internal diameter and anterior wall thickness during systole and diastole were measured, and the measurements were performed by an individual who was blinded to the treatment assignments.

Histology. Both formalin-fixed paraffin-embedded (FFPE) and fixed frozen (FF) sections were used in the present study. For FFPE sections, cardiac tissues were fixed in 10% formalin overnight, embedded in paraffin, and cut at 5 μm in thickness as previously described (40). For FF sections, tissues were fixed in 4% paraformaldehyde for 4 h, dehydrated in 30% sucrose and embedded in OCT compound followed by cryosectioning into 8 μm in thickness. A total of 3 sections per heart and 6 fields per section were examined. The H&E staining (Thermo Fisher Scientific, Inc.) was performed by following standard procedures. The Sirius Red/Fast Green (Chondrex) staining were performed by following manufacturer's instructions. For TUNEL staining, FF sections or cells growing on slides were fixed in 4% paraformaldehyde for 30 min, then permeabilized with proteinase K solution (20 μg/ml; for tissue) or 0.2% Triton X-100 (for cells) for 5 min at room temperature. After wash with PBS, the sections were stained with TUNEL reagent (Sigma-Aldrich; Merck KGaA) for 60 min at 37˚C. After wash, the nuclei were counter stained with DAPI for 7 min at room temperature. For each section, 4-6 fields were examined under fluorescent microscope. To analyze tissue CXCR4 expression, anti-CXCR4 antibody (Abcam, cat. no. ab124824; 1:200) was used in the histochemical staining, as previously reported by the authors (9). The histological assessments and analyses were performed by an individual blinded to treatment assignments.

Cells. H9C2, a well characterized and widely used cardiomyocyte cell line that was originally derived from rat embryonic heart tissue, was used as the in vitro cardiomyocyte model (41). The cells were purchased from the American Type Culture

Collection (cat. no. CRL-1446), maintained in DMEM with 10% FBS, and used until passage 8.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. The MTT assay was performed to evaluate the viability of H9C2 cells after treatment with test reagents in 96-well plates by using CellTiter 96 Nonradioactive Cell Proliferation Assay kit (Promega Corporation). First, 15 µl of dye solution was added to each well, and the plate was returned to incubator for 4 h at 37˚C. Then, 100 µl of Solubilization/Stop Solution was added to each well. The colored formazan product is stable at 4˚C, and absorbance was recorded at 570 nm using a 96-well plate reader.

Lenti-CXCR4 shRNA. The lentiviral vector that carries CXCR4 shRNA or non-targeting (NT) shRNA was obtained from Sigma-Aldrich; Merck KGaA with the knockdown specificity and efficiency authenticated by the company. The CXCR4-shRNA base sequence was 5'-GGATCAGCA TCGATTCCTTCA-3' and the NT-shRNA base sequence was 5'-TTCTCCGAACGTGTCACGT-3'. The vectors were packaged following the manufacturers' instructions. The multiplicity of infection was 2 for application on H9C2 cells, and the transduced cells were selected in puromycin for 10 days before evaluation for CXCR4 expression and myocyte functions.

Reverse transcription-quantitative (RT-q) PCR. RT-qPCR was performed via standard techniques as previously described by the authors (9). Briefly, total RNA was extracted with RNA Stat-60 (Tel-Test, Inc.), and RNA was reverse transcribed with the Taqman Multiscribe RT kit (Applied Biosystems; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. qPCR was performed in duplicate with cDNA from 10 ng of RNA by using the Lightcycler hybridization Probes Master Mix [Roche Diagnostics (Shanghai) Co., Ltd.]; a negative control (lacking a template) was included for each probe set. Relative gene expression was calculated using the $2^{-\Delta\Delta Cq}$ method (42) and normalized to GAPDH. The primer sequences were as follows: mouse CXCR4 forward, 5'-CCT CGCCTTCTTCCACTGTT-3' and reverse, 5'-CTGGGC AGAGCTTTTGAACTTG-3'; and mouse GAPDH forward, 5'-GGGTCCCAGCTTAGGTTCATC-3' and reverse, 5'-TAC GGCCAAATCCGTTCACA-3'.

Western blot analysis. Western blotting was performed via standard techniques as previously described by the authors (43), using primary antibodies against phosphorylated (p)-Akt, total Akt, β-actin and relevant horseradish peroxidase (HRP)-linked secondary antibodies (all from Cell Signaling Technology, Inc.). Briefly, the membrane was first blotted with anti-p-Akt (cat. no. 9271; 1:1,000) at 4˚C for overnight, then with anti-rabbit IgG (cat. no. 7074; 1:5,000) before application of chemiluminescence detection reagents and image taking. Next, the membrane was placed in the stripping solution [25 mM glycine-HCl, pH 2, 1% (w/v) SDS] with agitation for 30 min at room temperature, followed by the second round of immunoblotting with the use of anti-total-Akt (cat. no. 2920; 1:1,000) and anti-Mouse IgG (cat. no. 7076; 1:5,000). β-actin was immunoblotted in some experiments as loading control by using anti-β-actin (cat. no. 4967; 1:1,000) and then anti-rabbit IgG (cat. no. 7074; 1:5,000).

Statistical analysis. All values are presented as the mean ± SEM. Unpaired two-tailed Student's t-test was used for comparison between two means. One-way or two-way analysis of variance (ANOVA), followed by Bonferroni post-hoc tests, were used in multiple (>2) group comparisons with one or two independent variables, respectively. GraphPad Prism 8 software (GraphPad Software, Inc.) was used for statistical analysis. $P<0.05$ was considered to indicate a statistically significant difference.

Results

CXCR4 is expressed in the adult cardiomyocytes. The present investigation was initiated by assessing the expression of CXCR4 in wild-type mouse hearts. Immunohistological staining revealed that CXCR4 protein is expressed in the cardiomyocytes (Fig. S1) in the pattern consistent with plasmalemma localization as previously reported (16). This result is consistent with previous studies and confirmed that CXCR4 is expressed in cardiomyocytes.

Loss of CXCR4 in cardiomyocyte worsens Iso-induced heart failure. To investigate the role of CXCR4 in cardiomyocyte, a cardiomyocyte-specific CXCR4 knockout (^{CM}KO) mouse line was generated, αMHC-Cre;CXCR4$^{f/f}$, by crossing CXCR4$^{f/f}$ mice (44) with αMHC-Cre mice. The resultant ^{CM}KO mice were born at the expected Mendelian ratio and displayed no overt defect in gross appearance or cardiac structure for at least up to 4 months old, suggesting that CXCR4 expression specifically in the cardiomyocyte lineage is not essential for heart development. To induce heart failure, ^{CM}KO mice and control αMHC-Cre (Cre) littermates (8-10 weeks old) were subjected to a 14-day mini-pump delivery of Iso, a well characterized adrenergic agonist that induces heart failure primarily as the result of activated β1-adrenergic signaling and Gs stress. Serial echocardiography revealed that Iso treatment induced heart failure in both groups of mice; however, in ^{CM}KO mice, the left ventricular contractility was worse, chamber was more dilated, and end-systolic left-ventricular anterior wall thickness was thinner than in Cre control littermates (Fig. 1). In addition, it was observed that ^{CM}KO mice exhibit a higher heart rate than control Cre littermates under the same anesthesia regimen (Fig. S2). These worsened parameters in ^{CM}KO mice suggested that CXCR4 expression in cardiomyocytes plays a protective role in Gs stress-induced heart failure.

Loss of CXCR4 in cardiomyocytes worsens Iso-induced cardiac adverse remodeling and apoptosis. In another set of experiments, mice were administered Iso or saline via subcutaneous mini-pumps and euthanized at day 14, and the hearts were excised. The heart size and heart-weight (H.W.) to body-weight (B.W.) ratio appeared normal and comparable between ^{CM}KO and Cre mice with Saline treatment (Fig. 2A and B). However, with Iso treatment, these measures were significantly elevated in both groups and significantly deteriorated in ^{CM}KO mice compared with Cre mice (Fig. 2A and B). Histological assessments of cardiac tissue

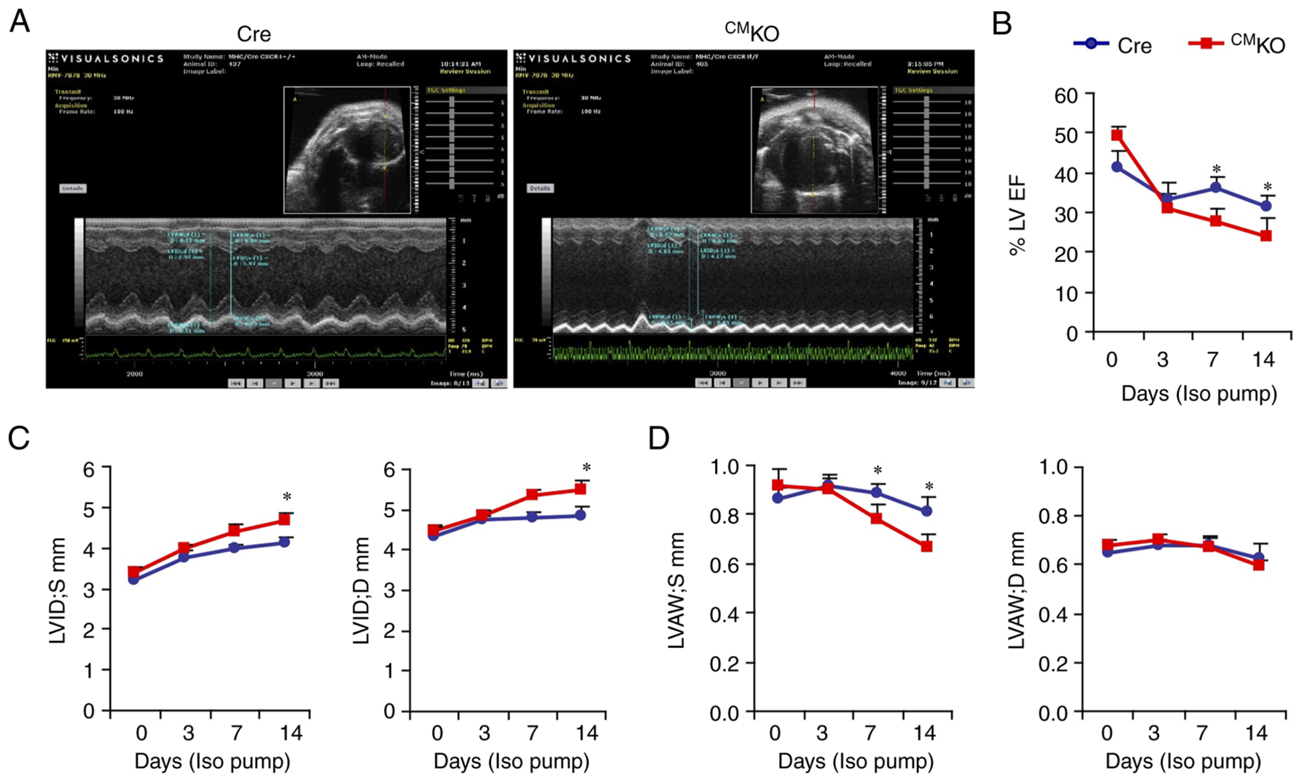

Figure 1. Loss of CXCR4 in cardiomyocytes worsens Iso-induced cardiac dysfunction. ^{CM}KO (αMHC-Cre; CXCR4$^{f/f}$) mice and control αMHC-Cre (Cre) littermates were treated with Iso (45 mg/kg BW/day) or saline via a subcutaneously-implanted mini-osmotic pump for 14 days, then cardiac functions were serially evaluated via echocardiography at day 0 (baseline), 3, 7, and 14. (A) Representative image of echocardiography. (B) LVEF. (C) LVID at end systole (S, left panel) and end diastole (D, right panel). (D) Thickness of LVAW at end systole (S, left panel) and end diastole (D, right panel). n=15 per group. $^{*}P<0.05$ (B-D: One-way ANOVA). Iso, isoproterenol; ^{CM}KO, CXCR4 knockout; LVEF, left ventricular ejection fraction; LVID, left ventricular internal diameter; LVAW, left ventricular anterior wall; Iso, isoproterenol.

revealed that Iso induced a significantly increased interstitial fibrosis (Sirius Red/Fast Green staining; Fig. 2C and D) and apoptotic cardiomyocytes (TUNEL staining; Fig. 2E and F) in ^{CM}KO mice. Thus, it was identified that loss of CXCR4 in cardiomyocytes increases Iso-induced cardiac cell death and adverse remodeling.

SDF-1 treatment abrogates Iso-induced apoptosis in cultured cardiomyocytes. Since CXCR4 is a Gi protein-coupled receptor, and Gi protein-coupled receptor signaling is inhibitory to Gs effects, it was hypothesized that activation of CXCR4 by SDF-1 counters β-AR/Gαs signaling to improve cardiomyocyte survival. In cultured H9C2 cardiomyocytes, treatment with Iso for 24 h significantly reduced cell number and viability (Fig. 3A-C) and increased cell apoptosis (Fig. 3D and E). While treatment with SDF-1 or AMD3100 (CXCR4 antagonist) alone did not show a significant effect, co-treatment with SDF-1 almost completely prevented Iso-induced reduction in cell number and viability (Fig. 3A-C) and significantly attenuated Iso-induced apoptosis (Fig. 3D and E). Consistently, the level of p-Akt, a major downstream effector of Gi signaling-activated PI-3 kinase-β and cell survival, was diminished by Iso treatment (Fig. 3F) however elevated by SDF-1 treatment (Fig. 3G); importantly, co-treatment with SDF-1 attenuated Iso-induced reduction in p-Akt (Fig. 3H). Collectively, these results suggested that CXCR4 protection of Iso-induced cell death is mediated by SDF-1, and that SDF-1/CXCR4 signaling promotes cell survival.

SDF-1/CXCR4 axis promotes cardiomyocyte survival through Gai protein signaling. To understand the role of CXCR4 more definitively, H9C2 cardiomyocytes were transduced with Lenti-CXCR4-shRNA, which knocked down CXCR4 mRNA expression by ~90% (Fig. 4A). While knockdown of CXCR4 alone did not affect cell number and viability, it worsened Iso-induced reduction of cell number and viability and more importantly, abolished SDF-1 mediated protection from Iso-induced reduction in cell number and viability (Fig. 4B and C). Consistently, knockdown of CXCR4 also diminished the ability of SDF-1 to ameliorate Iso-induced p-Akt reduction (Fig. 4D). These results confirmed that SDF-1 mediated cell survival is dependent on CXCR4 expression. To further determine the role of Gi signaling in SDF-1-mediated protection, H9C2 cells were treated with Iso/SDF-1 or Saline (-) in the presence or absence of a Gi-specific inhibitor. Clearly, SDF-1 attenuated the Iso-induced cell number and viability (Fig. 4E and F), and the protection was lost in the presence of Gi inhibitor but not vehicle control, confirming that SDF-1 protection from Iso-induced cell death is mediated by Gi signaling.

Discussion

In the present study, it was identified that loss of CXCR4 in cardiomyocytes renders the mice more vulnerable to Iso-induced heart failure-characterized by worsened LV contractility, greater LV dilation and thinner ventricular wall- and that SDF-1

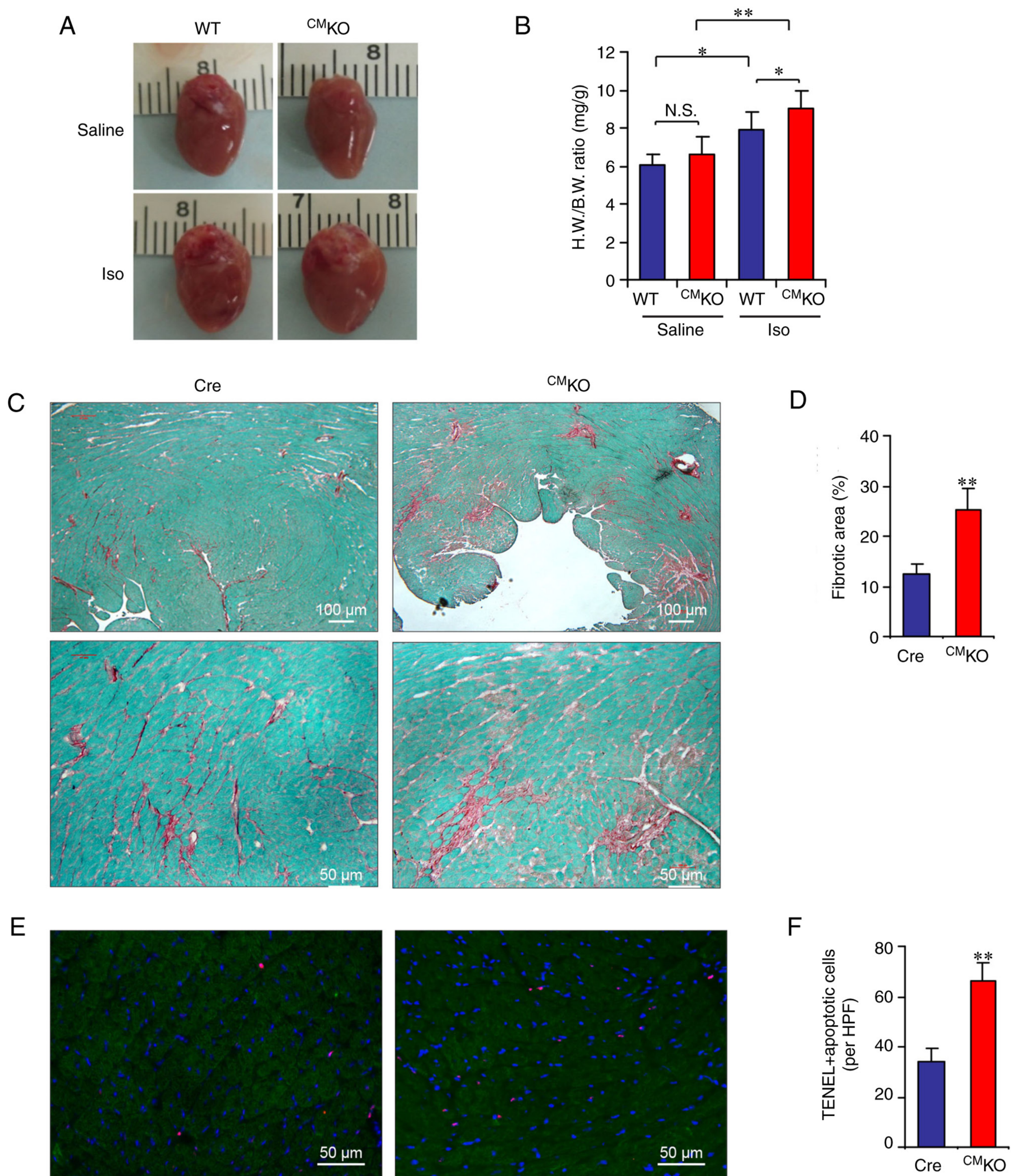

Figure 2. Loss of CXCR4 in cardiomyocytes worsens Iso-induced cardiac adverse remodeling and apoptosis. Mice were administered Iso or Saline via mini-pump implantation and at day 14, euthanized. (A) Representative gross appearance of the mouse hearts. (B) H.W. to B.W. ratio. (C and D) Representative images of Sirius Red/Fast Green staining [C; Original magnifications, x40 for upper panels, x100 for lower panels) and (D) quantification of the interstitial fibrosis areas]. (E and F) Representative images of (E) TUNEL (red) and DAPI (blue) double staining and (F) quantification of the $TUNEL^+$ apoptotic cardiomyocytes. Original magnifications, x100. HPF, high-powered field. n=6 per group. $^*P<0.05$ and $^{**}P<0.01$ (B: Two-way ANOVA; D and F: unpaired two-tailed t-test). Iso, isoproterenol; H.W., heart weight; B.W., body weight; ^{CM}KO, CXCR4 knockout; WT, wild-type; N.S., not significant.

treatment potently protects cardiomyocytes from Iso-induced cell death in a CXCR4 and Gi-dependent manner. These data suggested that SDF-1/CXCR4 signaling plays a protective role in β-adrenergic stress-induced cell death, cardiac adverse remodeling and progression to dilation, and heart failure.

The results of cardiac functional analysis in CXCR4 ^{CM}KO mice are consistent with a previous study (45); however, in the present study direct evidence was provided of Iso-induced cardiomyocyte apoptosis *in vivo* and *in vitro* in the absence or downregulation of CXCR4, respectively. Furthermore, it

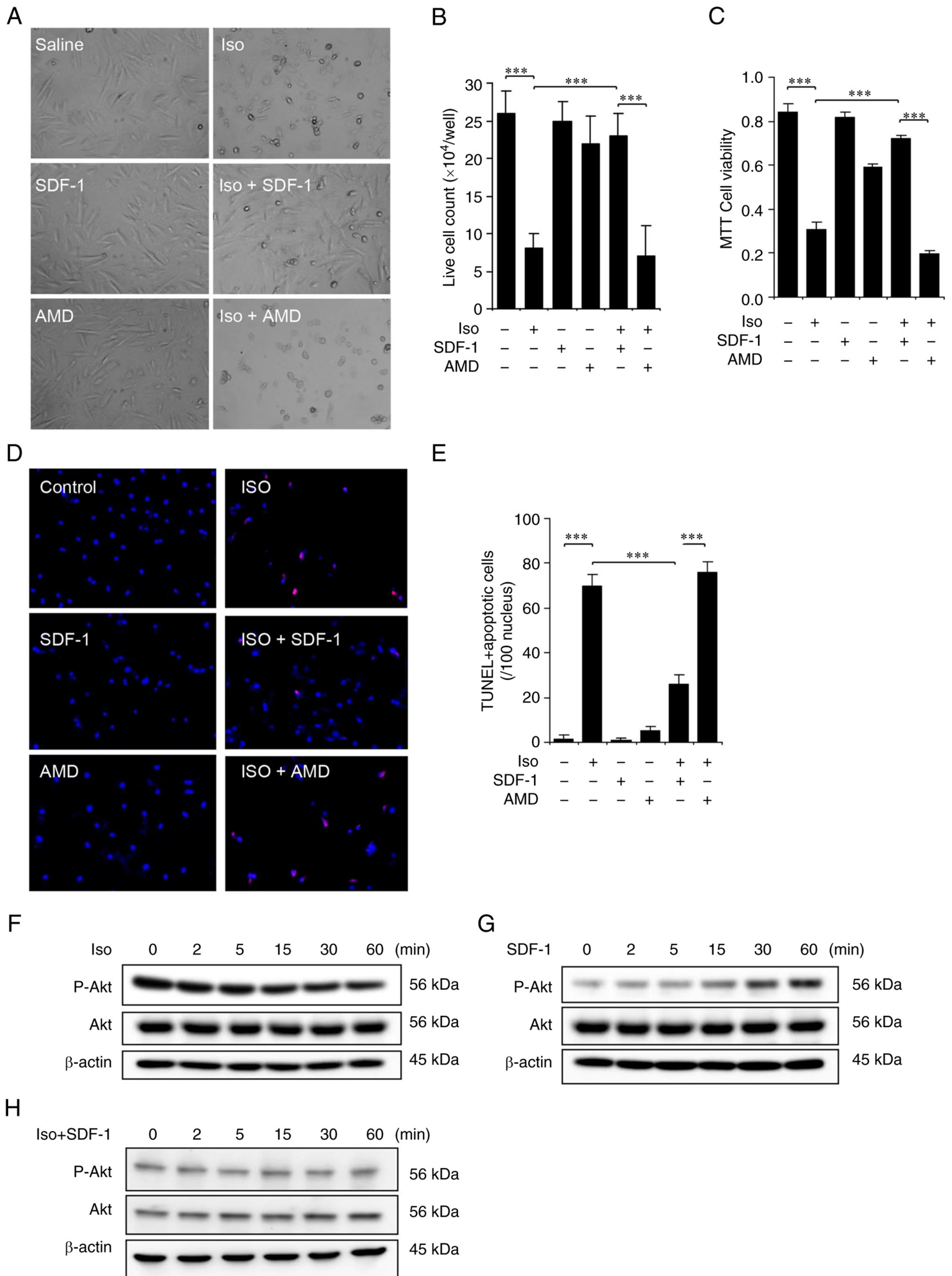

Figure 3. SDF-1/CXCR4 signaling attenuates Iso-induced apoptosis in cardiomyocytes. H9C2 cardiomyocytes were treated for 24 h with Saline, Iso (100 μM), SDF-1 (500 ng/ml), AMD3100 (100 ng/ml), Iso + SDF-1, or Iso + AMD3100, then analyzed. (A) Microphotograph of the treated cells. Original magnification, x400. (B) Manual counts of live cells. (C) MTT assays of viable cells. (D) TUNEL (red) and DAPI (blue) double staining (original magnification, x400). (E) quantification of the TUNEL$^+$ apoptotic cells. (F-H) Western blot analyses of p-Akt and total Akt in cells treated with (F) Iso, (G) SDF-1 and (H) Iso + SDF-1. n=6 per group. ***P<0.001 (B, C and E: One-way ANOVA). Iso, isoproterenol; p-, phosphorylated.

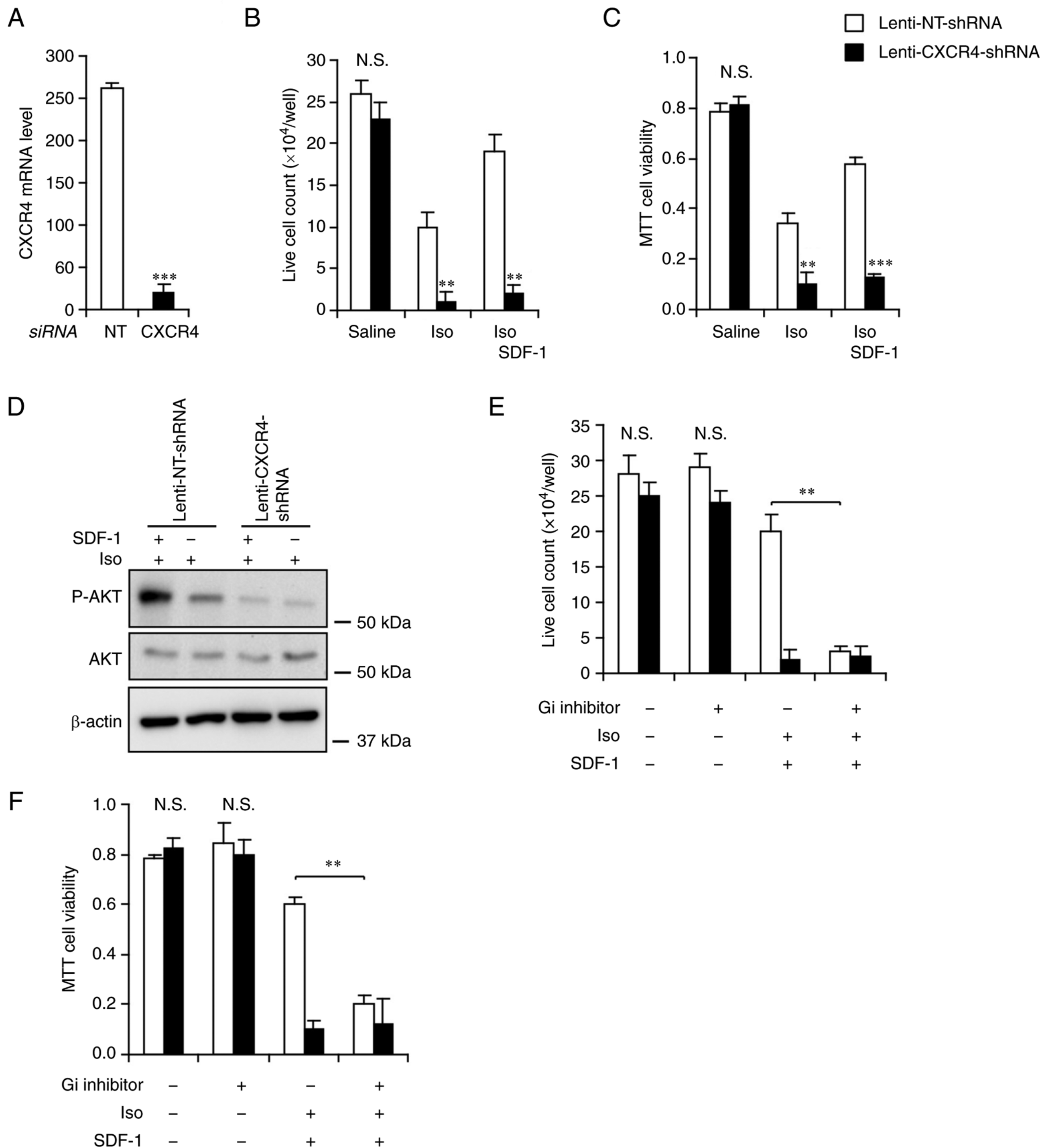

Figure 4. SDF-1/CXCR4 mediates cardiomyocyte survival via Gi signaling. H9C2 cardiomyocytes were transduced with Lenti-CXCR4-shRNA or Lenti-NT-shRNA and subjected to the following experiments: (A) Reverse transcription-quantitative PCR analysis of CXCR4 mRNA levels. (B and C) Treatment with Saline, Iso (100 μM), or Iso + SDF-1 (500 ng/ml) for 24 h, followed by (B) manual counting of cell numbers and (C) MTT viability assays. n=6 per group. (D) Treatment with Iso (+) plus SDF-1 (+) or vehicle (-), followed by western blotting of p-Akt and total Akt. (E and F) Treatment with Saline, Iso, or Iso + SDF1 for 24 h in the presence of Gi inhibitor Pertussis toxin (200 ng/ml) (+) or vehicle (-), followed by (E) manual cell counts and (F) MTT viability assays. n=6 per group. **P<0.01 and ***P<0.001. A, unpaired two-tailed t-test; B and C, E and F, Two-way ANOVA. shRNA, short hairpin RNA; NT, non-targeting; p-, phosphorylated; N.S., not significant.

was demonstrated that this effect is mediated by SDF-1 action and Gi protein signaling. The extent of SDF-1 protection of Iso-induced cardiomyocyte death is particularly striking. Previous studies suggested that SDF-1/CXCR4-mediated cell survival from ischemic stress is linked to Gβγ mediated activation of phosphoinositide 3-kinase (likely PI3Kβ) and Akt (11,13,46). Indeed, it was found that SDF-1 induces a significantly increased p-Akt in cardiomyocytes and also attenuates Iso-induced downregulation of p-Akt. Nevertheless, since sustained activation of Gαs-coupled β-ARs results in apoptotic loss of cardiomyocytes via a cAMP-dependent mechanism (30), SDF-1/CXCR4 signaling, through Gαi activation,

may also enhance cell survival by directly downregulating adenylyl cyclase activity. Further experiments would be needed to confirm this assertion. Furthermore, it was observed that during progression of heart failure, Gai protein expression is increased (20). This increase, previously considered as a compensatory effect from altered β-AR signaling, may in fact play an important protective role by substantiating the effect of CXCR4 and potentially other Gai coupled receptors.

Iso is the prototypical β-AR agonist with well documented profile in positive inotropic/chronotropic effects and induction of apoptosis (47), and continuous infusion is commonly used in rodents to induce phenotypic features of myocardial infarction and heart failure (37). Our *in vivo* analyses were particularly focused on the initial 14 days following Iso treatment, which is considered the best time window for detecting direct effects of adrenergic stress on cardiomyocytes in this model (37). Nevertheless, the detrimental effects are primarily mediated via β_1-AR and $G\alpha_s$ signaling, thus investigation of CXCR4, a $G\alpha_i$-coupled receptor, with Iso stress has a limitation, because heart failure is a complex disease and involves mechanism beyond β-AR and $G\alpha_s$ stress. Thus, examining the role of SDF-1/CXCR4 with additional heart failure model, such as pressure overload, is warranted in future studies.

Notably, it was observed that CXCR4 ^{CM}KO mice display an elevated heart rate associated with progression to heart failure. This is likely due to the dysregulated adrenergic signaling, although detailed electrophysiologic features and underlying mechanisms are unclear at present and remain a subject of our ongoing investigations. Notably, upregulation of CXCR4 expression was observed in patients with atrial fibrillation (AF) a decade ago (48). Recently, a flurry of bioinformatics analyses of clinical high-throughput data sets revealed that CXCR4 is a potent hub gene strongly associated with the pathogenesis of AF (49-52). Previous studies with a mouse model of AF suggested that CXCR4 antagonist AMD3100 can treat AF by ameliorating calcium mishandling and tissue inflammation (50,52). Given that CXCR4 signaling can mediate both cardiomyocyte survival and tissue inflammation, which appear to have contrasting effects on AF, strategies that target CXCR4 for treatment of cardiac arrhythmia would need to be carefully evaluated before clinical application.

One of the limitations to the present study is the limited scope of mechanistic study. Gain-of-function experiments were performed only in cultured cardiomyocytes, but not *in vivo* in animals. While the conclusion-that ablation of CXCR4 expression in cardiomyocytes exacerbates isoproterenol-induced cell death and heart failure-is fully supported by the data, the translational value of the present finding or if CXCR4 signaling can be used for treatment of cardiac adverse remodeling and heart failure remains to be rigorously investigated by using relevant CXCR4 activator/agonists particularly in large animal models with longer term follow-ups.

Lastly, CXCR4-null embryos exhibit dysplasia of the ventricular septum (3). However, a structural or functional defect in our CXCR4 ^{CM}KO mouse line was not observed for at least 4 months after birth, consistent with a previous study from Agarwal *et al* (53). The findings of the present study suggested that CXCR4 is required for septum formation probably through a non-cardiomyocyte lineage or cardiomyocyte progenitor cells prior to expressing α-MHC, which remains a subject of future research by the authors.

In conclusion, the present results suggested that SDF-1/CXCR4 signaling promotes cardiomyocyte survival and attenuates heart failure induced by β-AR overactivation.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

MC conceptualized the study, interpreted the data, and wrote the manuscript. CC, KY, and XL performed the experiments and analyzed the data. ND, QZ and FZ made intellectual contributions and assisted in data interpretation. FZ edited the manuscript. MC and CC confirm the authenticity of all the raw data. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

All animal experiments in the present study were approved (IACUC approval no. 3005) by the Animal Care and Use Committee of Huazhong University of Science and Technology (Wuhan, China) and performed in compliance with the 'Guide for the Care and Use of Laboratory Animals' (NIH publication) and all relevant ethical regulations for animal testing and research.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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