

CXCR4 expression is associated with time-course permanent and temporary myocardial infarction in rats

Ali Asghar Kiani ¹, Fereshteh Babaei ², Mehrnoosh Sedighi ², Azam Soleimani ³, Kolsum Ahmadi ⁴, Somayeh Shahrokhi ⁵, Khatereh Anbari ⁶, Afshin Nazari ^{7*}

¹ Razi Herbal Medicines Research Center and School of Allied Medical Sciences, Department of Hematology and Blood Transfusion, Lorestan University of Medical Sciences, Khorramabad, Iran

² Student Research Committee, Lorestan University of Medical Sciences, Khorramabad, Iran

³ Department of Biology, Science and Research Branch, Islamic Azad University, Tehran, Iran

⁴ Razi Herbal Medicines Research Center, Lorestan University of Medical Sciences, Khorramabad, Iran

⁵ Department of Immunology, Lorestan University of Medical Sciences, Khorramabad, Iran

⁶ Department of Social Medicine, Lorestan University of Medical Sciences, Khorramabad, Iran

⁷ Razi Herbal Medicines Research Center, Department of Physiology, Lorestan University of Medical Sciences, Khorramabad, Iran

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ABSTRACT

Objective(s): Experimental myocardial infarction triggers secretion of Stromal cell-derived factor1 and lead to increase in the expression of its receptor "CXCR4" on the surface of various cells. The aim of this study was to evaluate the expression pattern of CXCR4 in peripheral blood cells following time-course permanent and temporary ischemia in rats.

Materials and Methods: Fourteen male Wistar rats were divided into two groups of seven and were placed under permanent and transient ischemia. Peripheral blood mononuclear cells were isolated at different time points, RNAs extracted and applied to qRT-PCR analysis of the CXCR4 gene.

Results: Based on repeated measures analysis of variance, the differences in the expression levels of the gene in each of the groups were statistically significant over time (the effect of time) ($P < 0.001$). Additionally, the difference in gene expression between the two groups was statistically significant (the effect of group), such that at all times, the expression levels of the gene were significantly higher in the permanent ischemia than in the transient ischemia group ($P < 0.001$). Moreover, the interactive effect of time-group on gene expression was statistically significant ($P < 0.001$).

Conclusion: CXCR4 is modulated in an induced ischemia context implying a possible association with myocardial infarction. Checking of CXCR4 expression in the ischemic changes shows that damage to the heart tissue trigger the secretion of inflammatory chemokine SDF, followed by it CXCR4 expression in blood cells. These observations suggest that changes in the expression of CXCR4 may be considered a valuable marker for monitoring myocardial infarction.

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Introduction

Myocardial infarction (MI) is the most common cause of heart failure and the most common cardiovascular disease worldwide. Obesity, low physical activity, and stresses, mostly known to be attributes of modern life styles, are the major contributors to cardiovascular diseases (1).

From a pathological standpoint, necrosis in the heart tissue is the major sequel of an MI depending on whether transient or permanent ischemia occurs in this tissue (2). Notably, necrotic cell death of heart myocytes triggers an inflammatory response in the tissue due to release of pro-inflammatory cytokines and activation of the innate immune system (3).

C-X-C chemokine receptor type 4 (CXCR4) is the receptor of stromal cell-derived factor 1 (SDF-1) and a critical factor for stem cell migration, implantation and survival (4, 5). SDF-1 is one of the main chemokines that is released in response to hypoxia and acts as a chemo-attracting factor to use of CXCR4-expressing cells to the site of ischemia (6). In fact, the SDF-1 chemokine and its receptor play an axial role in the development of normal cardiovascular system (7). They are key regulators of host defense pathways and migration of leukocytes (8).

CXCR4 is expressed in many tissues including immune and central nervous systems, and on migrating leukocytes and hematopoietic progenitor

*Corresponding author: Afshin Nazari. Razi Herbal Medicines Research Center, Department of Physiology, Lorestan University of Medical Sciences, Khorramabad, Iran. Tel/Fax: +98-66-33120133; email: nazary257@yahoo.com

cells in response to SDF 1 (9, 10). Some studies have shown that SDF1 and CXCR4 are noticeably upregulated in myocytes shortly after MI and help optimize the situation for stem cell or heart tissue transplantation by improving the viability of cardiac cells (11, 12).

So it is expected that MI can increase the expression of CXCR4 on the surface of peripheral blood leukocytes via increasing the secretion of SDF. Moreover it's expected at various time intervals after ischemia and reperfusion, expression of this receptor on the surface of white blood cells change and measuring the alterations in the expression of CXCR4 may be helpful in monitoring heart disease.

The present study aims to provide new insight into the MI-associated gene alterations by exploring CXCR4 gene expression pattern following induced experimental MI in rats. We think that CXCR4 may be used as a monitoring marker for myocardial infarction.

Materials and Methods

Animals

In this study, 14 male Wistar rats, 6 to 8 weeks old and with weight range of 250 to 300 gr were used. Rats were housed in our rodent's standard laboratory (12 hr photoperiodic cycle and temperature $22\pm 2^{\circ}\text{C}$) and had free access to food and water. This study was conducted in the laboratory of Physiology of Razi Herbal Medicine Research Center, Lorestan University of Medical Sciences in 2015-2016. All animal care and experiments were conducted in accordance with the institutional guidelines of Lorestan University of Medical Sciences with code of ethics number LUMS.REC.1394.54.

Rats were divided into two groups (in each group there were seven) and were placed under permanent and transient ischemia. Both groups survived until the seventh day and blood samples were collected from both groups before and at three hr to 7 days after ischemia.

Surgical procedure

Animals were first anaesthetized using intraperitoneal injection of thiopental sodium (60 mg/kg, IP) and if necessary, anesthesia was repeated every 60-90 min by administering half of the above dose. Then rats, intubated via tracheotomy and placed on a rodent respirator (Small Animal Ventilator, Model 683, Harvard Apparatus, 15 ml/ kg stroke volume and 60-70 breaths/min). An incision was created on the chest in the fourth and fifth left intercostal spaces until the heart was exposed. The dose of thiopental (60 mg/kg-1) was that routinely used to animal study and has minimal cardiovascular and respiratory depression in that dose (13).

Electrocardiogram and the induction of permanent and temporary ischemia for comparing the level of CXCR4 gene expression

Rats were administered heparin (200 IU/kg, IV), and then, the heart of each was exposed through a left thoracotomy between the fourth and fifth ribs (1.5 cm in diameter), and an incision was made into the pericardium. The ligation of the left anterior descending (LAD) coronary artery (close to its origin) was performed using 6-0 silk suture. A standard limb lead-II electrocardiogram (ECG) was continuously monitored and recorded throughout the experiment, using a computerized data acquisition system (ML750 Power Lab/4sp, AD Instruments). Successful ligation of the LAD was confirmed by ST elevation and increase in R-wave amplitude in ECG. Muscle and skin incisions were closed with separate purse-string silk sutures (size 4-0), and the lungs were fully expanded. In transient ischemia group, the ischemic myocardium was reperfed by loosening the ligature after 30 min and in permanent ischemia group, the permanent ligation of the left anterior descending coronary artery is performed. Body temperature was measured by rectal thermometer and maintained at $37\pm 1^{\circ}\text{C}$ (13, 14).

Collection of blood samples and RNA extraction

To evaluate the basic level of expression of CXCR4, blood samples were collected from both groups before ischemia. Three hr after ischemia, 2 ml blood was obtained through the jugular vein catheter (Figure 2) from both groups and then this sample collection was continued every 24 hr until day 7. Samples were kept in tubes containing EDTA. Peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll density gradient centrifugation, and then RNAs were extracted using Trizol solution (15).

Quantitative real time PCR

For expression analysis of CXCR4 receptor mRNA, first strand cDNAs were synthesized using a kit

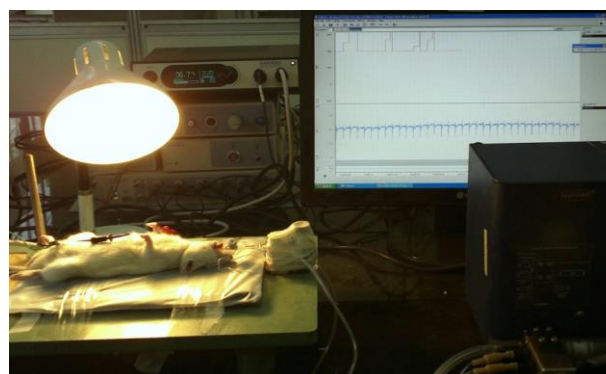


Figure 1. The attachment of leads in preparation for the induction of myocardial infarction

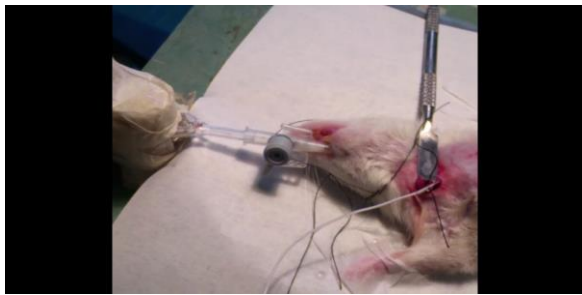


Figure 2. The placement of the catheter in the jugular vein to facilitate the taking of blood samples

following manufacturer's protocol (Fermentas) and applied to SYBR Green-based real time PCR in a 7500 real time PCR machine (Applied Biosystems). Cycle threshold (Ct) values from all conditions were normalized to beta-actin housekeeping gene to calculate relative expression of the CXCR4 gene. Sequences of the primers used in this study are shown in Table 1.

Statistical analysis

All statistical analyses were performed using SPSS version 2. Data were reported as means $\pm$ SEM. Repeated Measurement ANOVA test was used to analyze the differences between the data. $P \leq 0.05$ was considered statistically significant.

Results

Ventricular arrhythmias with its indexes

We recorded ventricular arrhythmias during the 30 min occlusion of LAD in temporary and permanent ischemia. The recorded ventricular arrhythmias following induced ischemia in our model (Figure 1, A-G) are consistent with those from other studies (16). Pattern A indicates ventricular beats in the steady state, QRS, P and T waves are normal. When the vessel is closed with a ligature, the heart is displaced from its axis, and therefore, the QRS complex is recorded as negative, and of course, it becomes positive after a while (17).

In pattern B, the S and T waves have merged together and the segment ST has gone up. In pattern C, single ventricular ectopic beats are observed while other beats are in place indicating that ventricular-ventricular beats rather than atrial-ventricular beats have emerged. In pattern D, two frequently ectopic ventricular beats called couplet are developed. In pattern E, ventricular ectopic beats have been created as alternate with QRS complex, this state is called bigeminy. In pattern F, ventricular beats (QRS complex) have appeared in tandem while P wave has disappeared giving rise to a condition called ventricular tachycardia. In pattern G, QRS ventricular fibrillation is irregular and low, direction of flows is uncertain and wandering. Ventricular contractility has been lost and QRS waves are not recognizable.

Table 1. Sequences of sweep primers of CXCR4 and Beta actin genes

Primer name	Sequence	Fragment length
CXCR4-F	5'- GCTGGAGAGCGAGCATTG - 3'	145 bp
CXCR4-R	5'-TAGATGGTGGGCAGGAAGATCC -3'	
Beta actin-F	5'-CGCGAGTACAACCTTCTTGC - 3'	252 bp
Beta actin-R	5'-GATGCCTCTCTTGTCTGGGC -3'	

CXCR4 gene expression changes was time-dependently during permanent or temporary ischemia

Regarding the fact that CXCR4 gene plays an important role in homeostatic maintenance of myocytes following MI (9), we were prompted to examine whether this gene is also modulated in our experimental model. As shown in Figure 1, three hr after permanent and temporary ischemia, CXCR4 expression level significantly increased compared to the basal level and this increasing trend went on continuously until the second day after ischemia.

Given Figure 4, in transient ischemia, in the 3rd and 24th hr the expression levels of CXCR4 increased to 1.4 ± 0.064 and 1.67 ± 0.057 as compared with the baseline levels and showed statistically significant differences with the baseline ($P < 0.001$). The expression levels of CXCR4 were 1.77 ± 0.095 , 1.75 ± 0.067 , 1.46 ± 0.076 , 1.25 ± 0.081 , 1.17 ± 0.079 , 1.11 ± 0.082 during the second to seventh days, respectively.

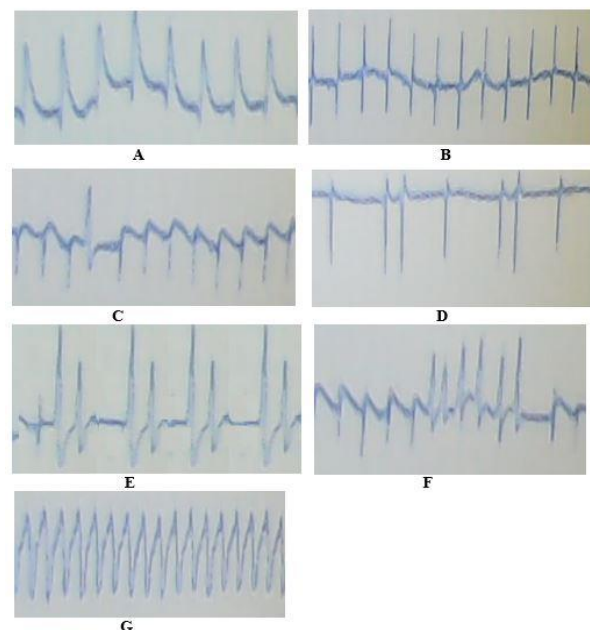


Figure 3. Electrocardiogram recorded during the base (A), Segment ST elevation during the induction of ischemia (B), VEBs single (C), couplet (D), bigeminy (E), ventricular tachycardia (VT) (F), ventricular fibrillation VF (G). A standard limb lead-II electrocardiogram (ECG) were continuously recorded

Table 2. Comparison of the mean and standard deviation of CXCR4 mRNA expression during different time in the study groups

Group	Day 7	Day 6	Day 5	Day 4	Day 3	Day 2	Day 1	3 hr	Time-group interaction effect*	Group effect	Time effect
	SD±μ	SD±μ	SD±μ	SD±μ	SD±μ	SD±μ	SD±μ	SD±μ			
Transient Ischemia	082/0±11/1	079/0±17/1	081/0±25/1	076/0±46/1	067/0±75/1	095/0±77/1	057/0±67/1	064/0±44/1	F=36/716 P<0/001	F**=09/669 P<0/001	F=475/78 P<0/001
Permanent Ischemia	079/0±7/1	1/79±0/09	1/83±0/063	1/87±0/079	1/93±0/07	1/95±0/049	1/79±0/099	1/44±0/027			

In the analysis of repeated measures data, to compare the expression of the gene in the two studied groups during various times, given that the compound symmetry postulate was not established, and given the results of the Mauchly's test ($P=0.044$), the results of the Greenhouse-Geisser corrections were used to analyze the data. Given the results of this correction, the difference in the expression levels of the gene over time in each of the groups was statistically significant (the effect of time) ($P<0.001$). In each group, the gene expression level showed an increasing trend up to the second day, and after this day, during the third to the seventh days, a decreasing trend was observed. These variations in gene expression in each group over time were significant. Moreover, the difference in gene expression between the two groups was statistically significant (the effect of group), i.e. at all times, the expression level of the gene was higher in the transient ischemia group than in the permanent ischemia group ($P<0.001$). Additionally, the interactive effect of time-group on gene expression was statistically significant, i.e. the difference in the expression of the CXCR4 mRNA gene between the two groups over time was statistically significant when the interactive effect of time-group was taken into account ($P<0.001$).

F: The statistic of the repeated measures ANOVA

μSD: Mean standard deviation

Time effect: Assessment of variations in gene expression over time disregarding the study groups

Group effect: Intra-group variations or differences in gene expression between the two groups

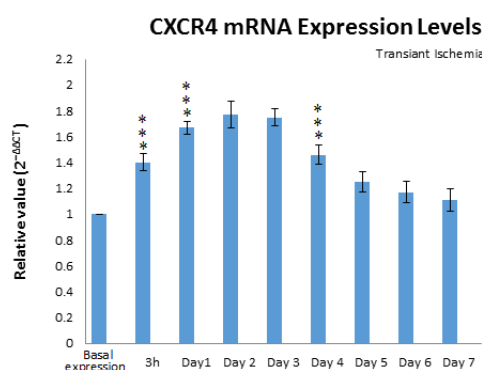


Figure 4. The variations in the expression of CXCR4 in the rat blood cells in the 3rd and 24th hr and in the second and seventh days after the transient infarction. It can be observed that the expression levels of CXCR4 significantly increase from the 3rd to the 24th hr ($P<0.001$). Additionally, a significant decrease can be observed in the expression of the gene on the surface of the blood cells from the fourth day

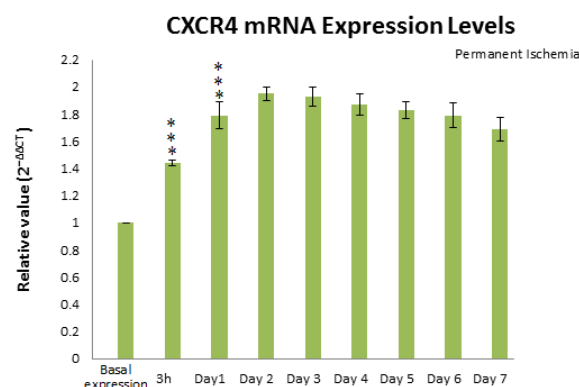


Figure 5. Variations in the expression of CXCR4 in the rat blood cells in the 3rd and 24th hr and also during the second to the seventh days after the infarction. The expression levels of CXCR4 significantly increase from the 3rd to the 24th hr ($P<0.001$). No significant decrease could be observed in the expression of the CXCR4 gene after the second day

The expression levels had a decreasing trend after the second day and a significant decrease in expression was observed in the fourth day as compared with the third day ($P<0.001$). This decreasing expression trend continued to the seventh day, however, the differences were not significant. Furthermore, the increased expression levels of CXCR4 in the 24th hr showed a significant difference with the levels of expression of the 3rd hr ($P<0.001$).

In permanent ischemia, the expression level of CXCR4 was 1.44 ± 0.027 in the 3rd hr and 1.79 ± 0.99 in the 24th hr, which were significantly different with the baseline levels of expression ($P<0.001$). Additionally, the expression levels were 1.95 ± 0.049 , 1.93 ± 0.07 , 1.87 ± 0.079 , 1.83 ± 0.063 , 1.79 ± 0.09 , and 1.7 ± 0.079 during the second to the seventh days, respectively. The expression level of CXCR4 began to decrease from the fourth day.

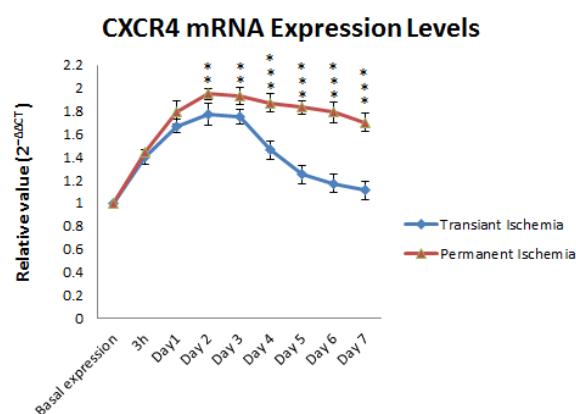


Figure 6. Comparison of expression levels of CXCR4 mRNA in permanent and temporary ischemia in the steady state, the early hr and the first to seventh days

Discussion

Our results of CXCR4 mRNA expression rate comparison in permanent and temporary ischemia (30 min) at steady state, early hr and first to seventh days showed that three hr after permanent and temporary ischemia, CXCR4 expression level in comparison to basal expression have significantly increased and this process of increasing of expression was continued until the two days after ischemia.

C-X-C motif chemokine receptor 4 (CXCR4) is a G protein-coupled chemokine receptor, which is expressed in a countless number of cells and its specific ligand is CDF-1A [18]. The local expression of CXCR4 plays an important part in the triggering of stem cell migration, cellular multiplication, and the repairing of injuries. Mesenchymal stem cells (MSC) of the bone marrow (BM) can directly migrate to the heart via the signaling pathway of SDF-1/CXCR4 after myocardial ischemic/reperfusion damage.

The CXCR4 expression changes compatibility in our study with SDF-1 α expression changes, in studies of other researchers confirms that CXCR4 is specific receptor for SDF-1 α ligand at the signal axis of CXCR4 - SDF-1 α . Studies have shown that SDF-1 α by binding to its receptor (CXCR4) exerts several protective effect such as: Survival, improve of cardiac remodeling, use of Productive endothelial cells and making vessels for improving the heart function (19, 20). Thus, inhibition of its receptor (CXCR4) after MI lead to recruitment of stem cells derived from bone marrow in myocardium of rats (21).

Chu *et al.* in a study on the effect of transplantation of mesenchymal stem cells expressing CXCR4 on fractured bone of rats have confirmed that this implantation can stimulate the increasing cell movement to bone in mice which can be helpful for bone repair (22).

Also in the study of Luo *et al.* start the process of over expression of CXCR4 (three hr after the ischemia and the peak of its expression during the first 24 to 48 hr) in temporary and permanent ischemia have been reported (23). Also it has been found that acute hypoxia (one percent oxygen) lead to over expression of CXCR4 in mesenchymal cells in 4, 8 and 16-hr timescales, and 48 hr after hypoxia, expression has reduced (24, 25). This increasing and decreasing timeframe also approved in our study because by closing the LAD and generation hypoxia, CXCR4 has increased. Lack of oxygen effect on CXCR4 expression also in Semenza *et al.* was well approved because HIF-1 factor highly express in hypoxia and has regulatory effect on CXCR4 and SDF-1 expression (26).

Therefore, it can be said that variations in the expression of CXCR4 observed in the present study is related to the hypoxic effects created during the induced ischemia.

According to these findings, oxygen concentration and duration of its deficiency have essential role in CXCR4 gene expression in mesenchymal stem cells derived from human bone marrow. So we can attributed the difference of timescale initial start (three hr after the ischemia) of CXCR4 expression in this study compared to some other studies to LAD occlusion in 30 min and the applied oxygen concentration.

In total, there is a consensus in our study and other study all about increasing trend timeframe of CXCR4 expression (24 to 48 hr) and reach to basal expression (after 7 days) during permanent and temporary ischemia. Differences observed in the timeframe initial start (Between 3 to 16 early hr) of CXCR4 expression can attribute to different conditions of each experiment especially evaluation of ventricular arrhythmias characteristic that were evaluated during 30 min occlusion of LAD in transient and permanent ischemia.

The findings of laboratory studies have shown that 50% of the size of the final cardiac infarct can occur because of the ischemic / reperfusion injury (27, 28). Therefore, it is of great clinical importance to attempt to develop treatment strategies for the protection of the heart against the deleterious effects of reperfusion injury.

As was previously mentioned, CXCR4-SDF plays an important role in cell migration and the substitution of transplanted cells in the injured sites. Given the results of preclinical studies and clinical data, there is some hope that stem-cell-based cell therapy can provide a method for repairing the irreversible and widespread injury to cardiac cells after acute myocardial infarction (29-34).

In this regard, our study has provided new insight into the MI-associated gene alterations by exploring CXCR4 gene expression pattern following induced experimental MI in rats.

Therefore, the correlation between the variations in the expression of CXCR4 with the induced ischemic conditions in this study shows that CXCR4 can be effectively used both as a monitoring marker for myocardial infarction and as a potential target for the improvement of cell therapy.

Among the limitations of this study, the investigation of the specific ligand of CXCR4, i.e. SDF-1 α , can be mentioned. If the SDF-1 α -CXCR4 axis is simultaneously investigated, valuable information can definitely be obtained regarding downstream signaling mechanisms.

Conclusion

Expression patterns of CXCR4 during permanent and transient ischemias indicate that the inflammatory response in the permanent ischemia is high due to broad damage leading to sustained upregulation of CXCR4. On the contrary, during transient ischemia

the inflammatory response is low due to less damage hence less upregulation of CXCR4, which is restored faster to baseline after ischemia removal. Therefore, alteration in CXCR4 expression can be suggested as a biomarker for diagnosis and prognosis of the damage and for monitoring the therapy trend. This enables us to control the inflammation process and prevent inappropriate cardiac regeneration in a well-managed timely manner. It can be said that SDF1 and its specific receptor CXCR4 play an important role in regulation of infarction repair through post-ischemic recruitment of endothelial progenitor cells to ischemia site. This supports the substantial notion that understanding the molecular mechanisms behind increase or decrease of chemokines and their specific receptors will help identify therapeutic targets to improve cardiac regeneration. In conclusion, our findings can promote insight into clinical applications of chemokines and their specific receptors.

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