

Effect of TGF- β 1/SDF-1/CXCR4 signal on BM-MSCs homing in rat heart of ischemia/perfusion injury

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Abstract. – OBJECTIVE: Heart ischemia/reperfusion (I/R) injury is a common cause of heart failure. However, there is no effective method to treat the disease presently. The present research was to investigate the effects of transforming growth factor- β 1 (TGF- β 1) on homing of bone marrow mesenchymal stem cells (MSC) in heart I/R injury.

MATERIALS AND METHODS: Effects of TGF- β 1 on the expression of CXCR4 [Chemokine (C-X-C Motif) Receptor 4] and chemotactic effect to SDF-1 (stromal cell-derived factor 1) in MSCs were investigated by *in vitro* transmembrane chemotaxis. Anti-TGF- β 1 was incubated with I/R injury's heart tissue of mice. In addition, effects of TGF- β 1 and anti-CXCR4 treatment using MSCs on the expression of SDF-1/CXCR4 in heart tissue and on I/R injury repair were further explored. **RESULTS:** CXCR4 and TGF- β 1 expression were significantly increased after TGF- β 1 treatment in MSCs; TGF- β 1 treatment increased MSCs cell migration, and anti-CXCR4 and anti-TGF- β 1 treatment blocked MSCs/TGF- β 1 cell migration. Expression of TGF- β 1 in the I/R injury's myocardial tissue of mice was increased, and MSCs transplantation could enhance the protein expression of CXCR4 in the I/R injury's myocardial tissue of mice, and the expression of CXCR4 was decreased by the anti-TGF- β 1 and the anti-CXCR4 treatment. TGF- β 1 induced homing of MSCs in the repair of myocardial injury by regulating expression of CXCR4 on the cell membranes. Blue fluorescence of DAPI-positive MSCs cells of myocardial in the I/R+MSC group was enhanced significantly, which was significantly inhibited by anti-TGF- β 1 and anti-CXCR4 antibody, and the inhibitory effect of anti-CXCR4 antibody was more evident than that of anti-TGF- β 1 antibody.

CONCLUSIONS: TGF- β 1 promotes homing of bone marrow (BM) MSCs in I/R injury's myocardial. The study provided useful data on the role of TGF- β 1 in regulating SDF-1/CXCR4 axis-induced MSCs homing.

Key Words:

Transforming growth factor- β 1, Mesenchymal stem cells, Ischemia-reperfusion injury, Chemokine (C-X-C Motif) Receptor 4, Stromal cell-derived factor.

Introduction

The ischemic myocardial injury is a complicated heart disorder in the world, and the main common cause is coronary atherosclerosis caused by occlusion or/and stenosis¹. The reperfusion after ischemic myocardium is a crucial therapeutic strategy to alleviate ischemic symptoms and avoid more extensive injury². However, ischemic/reperfusion (I/R) often causes critical injury to the ischemic myocardium after perfusion. Experimental studies find that 50% of the final myocardial infarct size may occur in I/R injury^{3,4}. Thus, it is essential for us to develop therapeutic strategies for protecting the heart against the detrimental effects of reperfusion injury is of great clinical importance.

SDF-1 (stromal derived factor-1) is a cytokine involved in recruiting stem cells into injured organs. Recent studies have shown that SDF-1 may confer myocardial protection in myocardial infarction through mobilizing the stem cells into the injured myocardial tissue and increasing the local angiogenesis after myocardial infarction⁵. Receptor for SDF-1 is CXCR4, which transduces multiple signals leading to the control of biological functions such as cell chemotaxis, proliferation, apoptosis, survival, and differentiation⁶.

Expression of local CXCR4 plays an important role in promoting stem cell migration, proliferation and injury recovery. Bone marrow (BM) mesenchymal stem cells (MSCs) can migrate directly into the heart after myocardial I/R injury

by SDF-1/CXCR4 axis, but the lower migrating rate restricted the application. CXCR4 is mainly expressed in the cytoplasm of cells, and only a little CXCR4 is expressed on cell membrane⁷. Furthermore, expression of CXCR4 is decreased with prolonged cell culture time⁸.

A variety of cytokines can stimulate the membrane CXCR4 expression⁹⁻¹¹. Chu et al¹² reported that transforming growth factor- β 1 (TGF- β 1) could induce upregulation of CXCR4 expression in human basal cell carcinoma (BCC) cells. Si et al¹³ found that TGF- β 1 could induce CXCR4 expression of MSCs in the homogenate of the acute I/R injured renal tissue. In addition, CXCR4 contributes to the migration of MSCs to SDF-1. Furthermore, neutralization of TGF- β 1 inhibited the migration via down-regulation of the CXCR4 localized to the membrane¹³.

BM-MSCs can play protective roles by homing directly to myocardial, in which SDF-1/CXCR4 axis induced MSCs homing to the ischemic myocardium^{14,15}. Due to the important role of the SDF-1/CXCR4 axis in the regulation of MSCs migration and homing, we suggested that intervention of SDF-1/CXCR4 axis might be a promising way to get better therapeutic outcomes in stem cell therapy.

As a secretory polypeptide signal molecule, TGF- β 1 has extensive biological activity. It is involved in repair of damage and it might be one of the most important mechanisms of repair and protection by the organism itself¹⁶⁻¹⁸. Due to the similar biological characteristics of MSCs, tumor cells and mesenchymal cells and the overlapped molecular mechanism, it is hypothesized that TGF- β 1 may mediate SDF-1/CXCR4 axis-induced MSCs homing.

In the present study, the effects of TGF- β 1 overexpression in MSCs on the expression of CXCR4 and chemotactic effect to SDF-1 were investigated by *in vitro* transmembrane chemotaxis. In addition, the effects of TGF- β 1 and anti-CXCR4 antibody on the SDF-1/CXCR4 expression and damage repair in the myocardial tissues *in vivo* were further explored.

Materials and Methods

Animals

Specific pathogen free (SPF) male, Sprague-Dawley (SD) rats with a bodyweight of about 180-200 g were purchased from Experimental Animal Center of Chinese Academy of Medical

Sciences (CAMS, Beijing, China). For experiments involving animals, approval was obtained from the Institutional Review Board of CAMS, Beijing, China.

Isolation and Culture of Bone Marrow MSCs

Under aseptic conditions, femurs and tibias obtained from healthy male SD rats, were dissected and bone marrow flushed with an 18-gauge needle and syringe containing α -MEM+ GlutaMAX (Gibco, Shanghai, China) as described previously¹⁹. Three days later, the medium was collected by centrifugation at 1,500 g for 5 min in 15 mL sterile tubes, and resuspended in 2.0 mL of Dulbecco's modified eagle's medium (DMEM). The resuspended cells were inoculated into 6-well plates (Corning, NY, USA) containing different media and incubated at 37°C in a humidified atmosphere containing 95% air and 5% (V/V) carbon dioxide (CO₂). The cell suspension was discarded, and the cell culture medium was changed every other day. When these primary cultures reached ~80% confluence, the cells were subcultured with the same medium used in primary cultures. Adherent cells were detached and suspended in ice-cold FACS buffer at 10⁶ cells/ml, and incubated at 4 °C for 30 minutes with monoclonal antibodies against surface markers such as CD29, CD34, CD45 and CD105 (these antibody, such as anti-CD34-FITC, anti-CD29-FITC, anti-CD45-FITC and anti-CD105-FITC were from Bioled).

MSCs Treatment with hr-TGF- β 1

Human recombinant TGF- β 1 (hr-TGF- β 1) was obtained from Cell Signaling Technology (Danvers, MA, USA) and dissolved in 4 mM HCl, with 1 mg/ml BSA (bovine serum albumin). MSCs cells were plated in 8-well chamber slides and allowed to adhere overnight. After treatment with TGF- β 1 (100 pM) or vehicle only for 24 h, expression of TGF- β 1 in MSCs was detected by Western blot.

Cell Migration Assays *in vitro*

Transwell migration was conducted as described previously²⁰. Briefly, the lower chamber of the transwell plates was filled with 600 μ l IMDM medium containing 10% fetal bovine serum (FBS). 1 \times 10⁵ MSCs cells were detached from the tissue culture plates and resuspended in IMDM medium containing 1% FBS and then loaded to the upper side of the chamber (200

μ l/well). After 24-h incubation at 37°C, TGF- β 1 (Santa Cruz Biotechnology, Beijing, China) (100 pM) or vehicle were added to both top and bottom chambers (TGF- β 1 were prepared with serum-free IMDM medium). The cells were placed in incubators at 37°C for 24 h. The filter inserts were then removed from the wells. Cells on the upper surface of the filter were removed using cotton swabs. The cells that had migrated to the underside of the inserts were fixed and stained using Diff-Quik staining kit. Cells that migrated or invaded were counted in five random fields of each filter under a microscope at 200 \times magnification.

For the anti-CXCR4 antibody (Santa Cruz Biotechnology, Beijing, China) blocking group, 10 μ g/mL anti-CXCR4 monoclonal antibody was mixed with MSCs-TGF- β 1 or MSCs/control in the 5% CO₂ incubator 37°C for 2 h, and then the cell migration assays were conducted as described above.

Preparation of Ischemia/Reperfusion Injury Heart Rat Models

SPF male SD rats with a bodyweight of about 180-200 g underwent ischemia/reperfusion (I/R) injury as previously described¹⁸. Briefly, the mice were anesthetized and ventilated; then, a lateral thoracotomy was performed. We used a 7-0 suture to loop under the left descending coronary artery for 30 min, followed by 24 h of reperfusion. The hearts were then removed, and cut into pieces in the clean workbench.

I/R Grouping

The present study included the following groups: 1) I/R+saline; 2) I/R + MSCs; 3) I/R+MSCs+anti-TGF- β 1; 4) I/R+ MSCs+anti-CXCR4.

Western Blot Assay

Expression of CXCR4 and TGF- β 1 in MSCs was detected by Western blot assay in various groups according to the manufacture's instruction.

MSCs Homing Determination

For determination of MSCs homing, heart tissues were immersed in 30% sucrose, for dehydration at 4°C for 24 h. The heart tissues were then embedded in Tissue-Tek OCT, and frozen sections were prepared. Fluorescence expression of sections was used to conduct quantitative analysis, to observe migration and colonization of MSCs under fluorescence microscopy

Infarct Size Analysis

Infarct size was determined by triphenyl tetrazolium chloride (TTC) staining²¹. In brief, at the end of the experimental protocol hearts were infused with 1% TTC in PBS (pH 7.4) for 10 min prior to snap freezing at -20°C. While frozen, the hearts were sliced perpendicular to the long-axis of the heart at 1 mm intervals and de-stained in 10% formaldehyde solution to increase the contrast between necrotic and viable myocardium. The heart slices were then digitally photographed for planimetry using NIH Image 1.62. The area at risk was expressed as a percentage of the total ventricular surface, and the infarcted area was expressed as a percentage of the area at risk.

Statistical Analysis

SPSS 11.0 statistical software (SPSS Inc., Chicago, IL, USA) was used for statistical analysis. The data obtained were presented as mean \pm SD. The relationship between two factors was analyzed by Pearson correlation analysis. A $p < 0.05$ indicated that the difference was statistically significant.

Results

Effect of hr-TGF- β 1 on TGF- β 1 and CXCR4 Expression in MSCs

MSCs were treated with hr-TGF- β 1 (100 pM) or vehicle (neu) only for 48 h. TGF- β 1 and CXCR4 expression were significantly increased by Western blot assay (Figure 1).

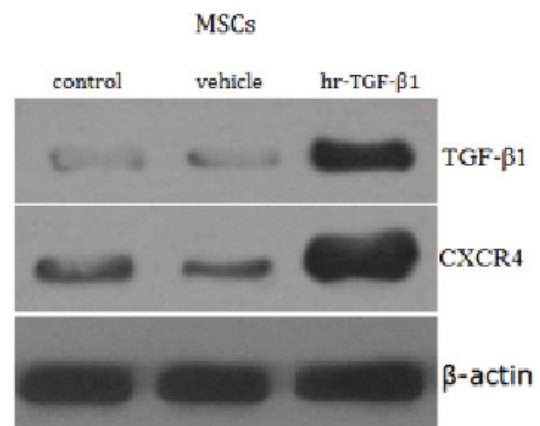


Figure 1. Effect of hr-TGF- β 1 on TGF- β 1 and CXCR4 expression by Western blot assay. TGF- β 1 and CXCR4 expression was increased after hr-TGF- β 1 transfection.

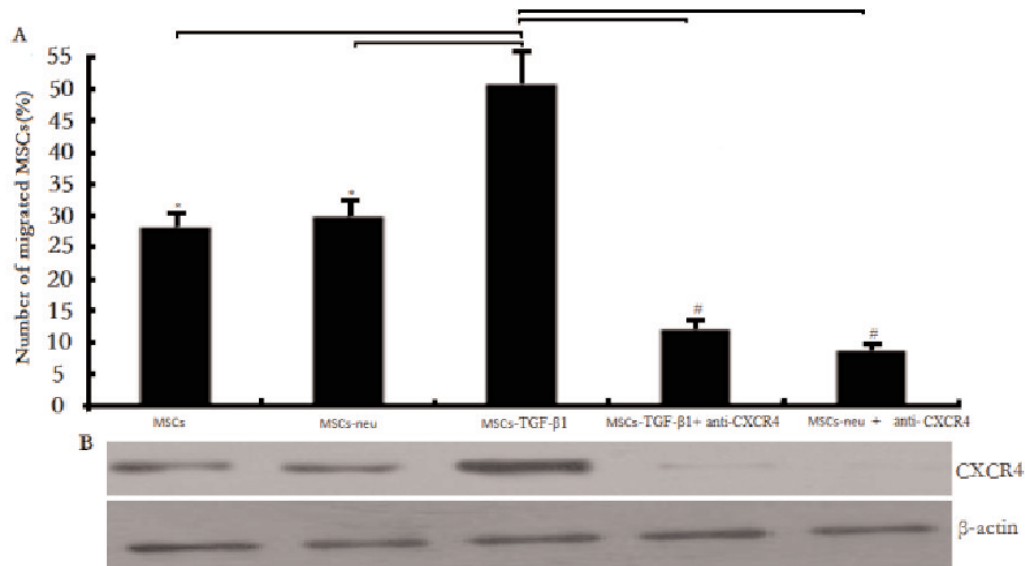


Figure 2. TGF- β 1 enhance MSC migration by upregulating CXCR4. 1×10^5 MSCs cells were treated with TGF- β 1 (100 pM) or vehicle for 24 h, then treated with $10 \mu\text{g/mL}$ anti-CXCR4 monoclonal antibody for 24 h. A, MSC trans-Matrigel migration rate was detected. * $p < 0.05$; # $p < 0.01$. B, CXCR4 expression was detected by Western blot assay.

Effect of hr-TGF- β 1 on MSCs Migration

1×10^5 MSCs cells were treated with hr-TGF- β 1 (100 pM) or vehicle for 24 h. The migration rate was $(53.4 \pm 5.3)\%$ in MSCs-TGF- β 1 groups, which was significantly higher than that of in control MSCs-neu groups $(31.6 \pm 2.4)\%$ and blank controls $(29.8 \pm 2.2)\%$, respectively (Figure 2A, $p < 0.01$). When the MSCs-TGF- β 1 or MSCs-neo cells were treated with $10 \mu\text{g/mL}$ anti-CXCR4 monoclonal antibody for 24 h, both of the migration rates in MSCs-TGF- β 1 or MSCs-neo cells were decreased. Western blot assay showed that the treatment with hr-TGF- β 1 increased CXCR4 expression in MSCs (Figure 1). When treatment with anti-CXCR4 monoclonal antibody for 24 h in MSCs-TGF- β 1 or MSCs-neo cells, CXCR4 expression was significantly decreased (Figure 2B).

TGF- β 1 and CXCR4 is Activated in Myocardial Tissues after Ischemia Reperfusion (I/R)

Mice were subjected to 30 min of ischemia and a subsequent 24 h of reperfusion, Western blot assay was used to detect the protein of TGF- β 1 and CXCR4 in the I/R groups. As shown in Figure 3, the expression of TGF- β 1 was upregulated. TGF- β 1 can be inhibited markedly by anti-TGF- β 1 treatment. CXCR4 expression in the I/R groups was also upregulated significantly, and it was inhibited dramatically by anti-CXCR4 treatment.

MSCs transplantation could enhance CXCR4 expression, which can be markedly inhibited by both anti-TGF- β 1 and anti-CXCR4 treatment in the I/R group in a time-dependent manner (Figure 3). These results suggested that TGF- β 1 induced homing of MSCs in the repair of heart ischemic reperfusion injury by regulating CXCR4 expression, which can be inhibited by the anti-CXCR4 antibody.

Damage Scoring

As shown in Figure 4, the infarct size was $57.3.0\% \pm 5.6\%$ in the I/R group. However, the infarct size in MSCs-I/R groups was markedly de-

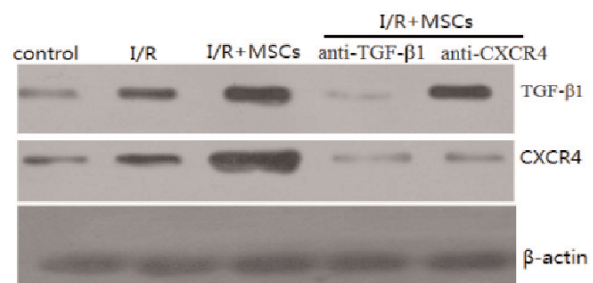


Figure 3. Expression of TGF- β 1 and CXCR4 in heart tissues by Western-blotting assay. A, Controls; B, I/R; C, I/R+MSC; D, I/R+MSC+anti-TGF- β ; E, I/R+MSC+anti-CXCR4. Expression of TGF- β 1 and CXCR4 increased significantly I/R groups, which could be inhibited by anti-TGF- β 1 and the anti-CXCR4.

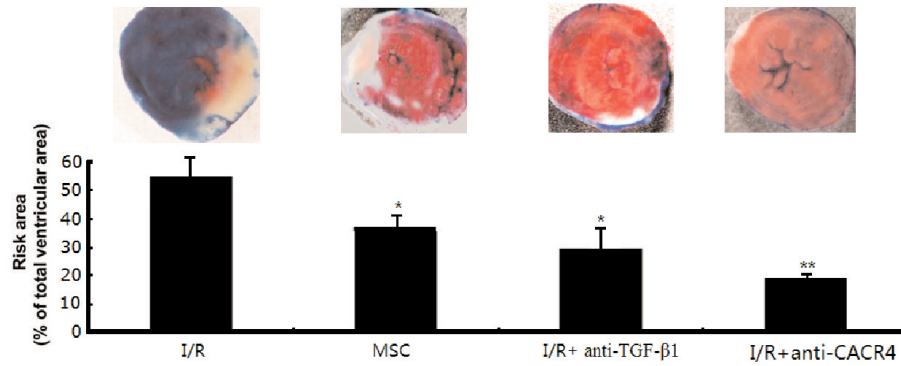


Figure 4. Infarct size is increased in mice after I/R. Quantification of areas at risk and infarct areas after *in vivo* I/R. Values are expressed as mean+SEM, n=10 vs. I/R groups, * p <0.05; ** p <0.01.

creased ($36.4\% \pm 4.5\%$, $p < 0.05$, $n = 10$). Furthermore, the infarct size was also greatly decreased by both anti-TGF- β 1 ($30.5\% \pm 4.5\%$, $p < 0.05$, $n = 10$) and anti-CXCR4 ($19.3.0\% \pm 3.7\%$, $p < 0.01$, $n = 10$) treatment in I/R groups.

MSCs Homing

Less blue fluorescence phenomenon was showed in the I/R group (Figure 5A). Blue fluorescence in the I/R+MSC group was significantly increased (Figure 5B), suggesting that MSCs migration was markedly increased. However, blue fluorescence was significantly decreased when treated with both anti-TGF- β 1 (Figure 5C) and anti-CXCR4 (Figure 5D) in the I/R+MSC groups. Furthermore, the inhibitory effect of anti-CXCR4 was more evident than that of anti-TGF- β 1 antibody.

Discussion

Although perfusion is the standard treatments for patients suffering from ischemic myocardial

disease, ischemia-perfusion (I/R)-induced cell injury, cardiac dysfunction and ventricular arrhythmias have become a complicated obstacle for effective therapy²²⁻²⁴. There is no effective method for the obstacle treatment in the clinical applications, presently.

In the present research, we first investigated the effect of TGF- β 1 on CXCR4 expression in MSCs, and its chemotactic effect to SDF-1 *in vitro* transmembrane chemotaxis. Then, we observed the effect of anti-TGF- β 1 incubation on I/R injury's mice heart. Also, we explored the effect of TGF- β 1 and anti-CXCR4 on both SDF-1/CXCR4 axis and heart damage repair *in vivo*.

The BM-MSCs are non-hematopoietic origin cells that may differentiate into several cell lineages of mesenchymal tissues²⁵. These cells have been showed²⁶ to take part in tissue homeostasis and repair under the influence of the stromal-cell-derived factor (SDF)-1. The experimental mice models have been associated with reduced migratory response of BM MSCs to local SDF-1²⁷. In addition, BM-MSCs has also been reported to repair the kidney injury²⁸. MSC has been

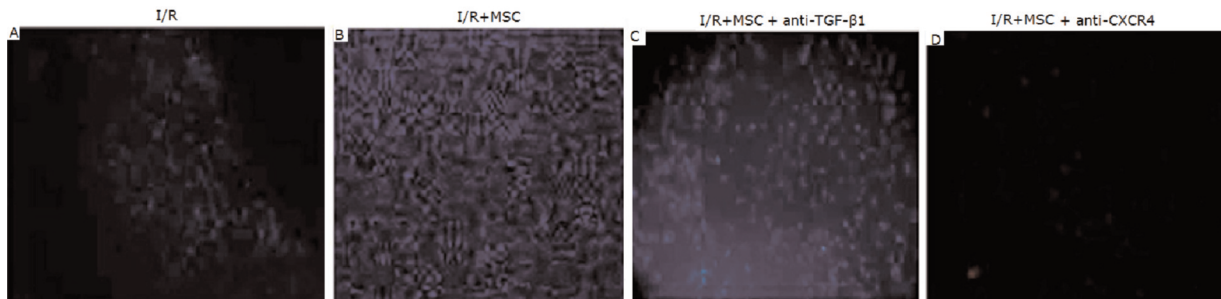


Figure 5. MSCs homing determination. **A**, I/R group; **B**, I/R+MSC group; **C**, I/R+MSC+anti-TGF- β group; **D**, I/R+MSC+anti-CXCR4 group. Blue fluorescence in the I/R+MSC group was significantly increased.

reported²⁹ to produce a variety of chemokines and cytokines which play a role in the regulation of migratory cell properties. In this respect, MSCs have been demonstrated to express a restricted pattern of chemokine receptors, including CXCR4, which allows them to migrate to tissues upon specific chemotactic triggers³⁰.

Activation of TGF- β 1 showed strong chemotaxis on monocytes, neutrophils, fiber cells, and mesenchymal cells. These cells can secrete more product of TGF- β 1. TGF- β 1, on the one hand, acted on the tissue damage's fiber cells, and on the other hand, enhanced large scale synthesis, such as collagen I, III, fibronectin, elastin proteins, integrins, proteoglycan and extracellular matrix components. Cho et al³¹ and Wobus et al³² confirmed that TGF- β 1 could induce and chemotaxis MSCs, and enhancement of TGF- β 1 on MSCs migration. In our study we found that CXCR4 was overexpressed in MSCs when TGF- β 1 was transfected into the MSCs cells, suggesting possible upregulation of this chemokine receptor in an attempt to respond to TGF- β 1. We also found increased levels of CXCR4 in BM MSCs after TGF- β 1 transfection increased the MSCs' migrated ability. However, when the MSCs-TGF- β 1 cells were treated with 10 μ g/mL anti-CXCR4 for 24 h, the migration rate in MSCs-TGF- β 1 cells was decreased. It was indicated TGF- β 1 enhanced the migrated ability of MSCs by CXCR4 regulation.

Many studies found that TGF- β 1 could regulate SDF-1/CXCR4 expression in mRNA level and protein level of hematopoietic stem cells, which then affected hematopoietic stem cell migration and adhesion. It was indicated that TGF- β 1 and SDF-1/CXCR4 axis have similar chemotaxis in the migration and homing of MSCs. However, whether TGF- β 1 induces homing of MSCs by SDF-1/CXCR4 in I/R injury's heart remains unclear.

The current paper demonstrated that I/R injury induced TGF- β 1 and CXCR4 expression in heart tissues of mice, and MSCs transplantation could enhance CXCR4 expression in I/R injury's mice heart tissues. TGF- β 1 induced MSCs homing and repairing the I/R injury's heart by regulating CXCR4 expression.

Blue fluorescence in heart tissue of the I/R+MSC group was significantly increased. The blue fluorescence above could be inhibited by anti-TGF- β 1 and anti-CXCR4, and the inhibitory effect of anti-CXCR4 was more obvious than that of the anti-TGF- β 1 antibody. We, therefore,

hypothesize that TGF- β 1 influence BM-MSCs homing in injured heart tissues by regulation of SDF-1/CXCR4 signal.

Conclusions

TGF- β 1 regulates SDF-1/CXCR4 axis, by which to promote homing of BM-MSCs in I/R injury's heart.

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Conflict of Interest

The Authors declare that they have no conflict of interests.

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