

## TNF- $\alpha$ enhances engraftment of mesenchymal stem cells into infarcted myocardium

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## 1. ABSTRACT

TNF- $\alpha$  released from ischemic heart after acute MI increases the production of other cytokines such as interleukin-1 (IL-1), interleukin-6 (IL-6) and adhesion molecules such as intercellular adhesion molecule-1 (ICAM-1). Activation of nuclear factor kappa B (NF- $\kappa$ B) by TNF- $\alpha$ , up-regulates the expression of molecules which are involved in inflammation and cell adhesion. For these reasons, we assessed the extent that treatment of MSC with tumor necrosis factor (TNF)- $\alpha$  modifies the characteristics of MSC, important to their engraftment in experimental myocardial infarct. Here, we show that pre-treatment of MSC prior to transplantation with tumor necrosis factor (TNF)- $\alpha$  increases adhesiveness, and migration of MSC *in vitro* and leads to increased expression of bone morphogenetic protein (BMP)-2 by MSC. Moreover, this treatment increases the rate of engraftment of MSC and improves recovery of cardiac function after myocardial infarction. These insights might provide better strategies for the treatment of myocardial infarction.

## 2. INTRODUCTION

Acute myocardial infarction (MI) results in ventricular remodeling, ventricular dilatation, and may lead to progressive heart failure (1). Several recent reports have shown that mesenchymal stem cells (MSC) exert beneficial effects on functional recovery of ischemic heart and may offer a viable treatment for end-stage cardiovascular disease. However, it remains unclear how MSC lead to the improvement of the heart function. Successful transplantation of stem cells depends on multiple factors including homing, engraftment, migration, and proliferation of stem cells. One of the major impediments to the application of stem cell therapy is that only a small percentage of the injected cells survive and engraft into the myocardium (2). Factors that play significant roles in the engraftment of stem cells include vascular cell adhesion molecules (VCAM), integrins, and stromal cell-derived factor (SDF)-1. SDF interacts with its cognate receptor, CXCR4, plays an important role in the engraftment of HSPCs (hematopoietic stem/progenitor cells) in the bone

**Table 1.** Sequence of rat primers

Product	Forward	Reverse	Size of amplified product
BMP-2	CGCTAAGCTCAGTGGG	TCAAGCCAAA CACAAACAG	257 bp
MCP-1	TTCACAGTTGCTGCCTGTAGC	TCTGATCTCACTTGGTTCTGG	260 bp
IL-6	GACTGATGTTGTTGACAGCCACTGC	TAGCCACTCCTTCTGTGACT CTAAC	509 bp
TNFRI	AGTGCTGTTGCCCTGGTCAT	TTTTCAGGTGTCGATTTCCTCA	301 bp
TNFRII	AGTGAGAGGCCATAGCTG	AAGGTGCACACGGTGTCT	264 bp
GAPDH	GGCCAAGGTCATCCATGA	TCATGAGCCAGGATG	353 bp

BMP: bone morphogenetic protein, MCP: monocyte chemoattractant protein, TNF: tumor necrosis factor

marrow and regulates the adhesion of HSPCs to the endothelium (3). Inhibiting the expression of integrin CD18 in endothelial progenitor cells reduces both the recruitment of stem cells to the ischemic myocardium and the degree of neovascularization, leading to poor tissue repair (4). These observations suggest that stem cell therapy may be improved by modifying stem cell properties such as their adhesion prior to the transplantation. TNF- $\alpha$  released from ischemic heart after acute MI increases the production of other cytokines such as interleukin-1 (IL-1), interleukin-6 (IL-6) and adhesion molecules such as intercellular adhesion molecule-1 (ICAM-1) (5). Activation of nuclear factor kappa B (NF- $\kappa$ B) by TNF- $\alpha$ , up-regulates the expression of molecules which are involved in inflammation and cell adhesion (6). Such actions likely promote the engraftment and activation of the transplanted MSC in damaged myocardium. Thus, it follows that pretreatment of MSC with TNF- $\alpha$  prior to transplantation may enhance their engraftment into infarcted myocardium and may lead to improvement of cardiac function. Bone morphogenetic protein (BMP)-2 which plays an important role in angiogenesis, and formation of neointima is an upstream regulator of TAZ, a critical transcriptional modulator of differentiation (7-9). For this reason, this cytokine might also be an important mediator of MSC's behavior and differentiation. Interestingly, TNF- $\alpha$  induces BMP-2 and mediates the stabilization of its mRNA *via* p38 kinase (10). Based on these available evidence, we examined whether pretreatment of MSC with TNF- $\alpha$  prior to transplantation enhances engraftment of stem cells into infarcted rat myocardium and improves recovery of cardiac function. We also examined whether BMP-2 is involved in the homing and retention of MSC in the myocardium.

### 3. MATERIALS AND METHODS

#### 3.1. MSC isolation, culture, and labeling

Bone marrow-derived MSC were isolated and cultured as reported previously (11). Femurs of male Sprague-Dawley rats (weight 170–200 grams) were extracted and the distal epiphyses were removed. The marrow suspensions were laid over an equal volume of Lymphoprep (1.077 g/mL, Technoclones, Vienna, Austria) and centrifuged at 800 xg for 30 minutes. Mononuclear cells recovered from the interface gradient were washed twice with PBS at 300 xg for 5 minutes at room temperature, seeded at  $2 \times 10^5/\text{cm}^2$  in Dulbecco's modified Eagle's medium containing glucose (1 gram/L, DMEM-LG, Gibco-BRL, Grand Island, NY) and 10% fetal bovine serum (FBS, Gibco), and cultured at 37°C in air containing 5% CO<sub>2</sub>. Non-adherent cells were removed after 24 hours. The culture medium was changed every 3–4 days and the

cultures were split as needed. When the isolated MSC colonies became apparent, the cells were trypsinized (0.05%) and replated at 8,000/cm<sup>2</sup>. Cell viability was assessed by using the MTT (1-(4,5-dimethylthiazol-2-yl)-3,5-diphenylformazan, Sigma, St. Louis, MO) assay. Before injection, the MSC were trypsinized, washed, and labeled with 4',6-diamidino-2'-phenylindole (DAPI) (Sigma). Briefly, sterile DAPI stock solution was added to the culture medium at a final concentration of 50  $\mu\text{g}/\text{mL}$  for 30 min. After labeling, cells were washed three times with PBS to remove excess unbound DAPI.

#### 3.2. Reverse transcriptase-polymerase chain reaction (RT-PCR) and Western blotting

RT-PCR and Western blotting were performed as previously described (10, 11). The sequence of primers and the size of amplified product generated by RT-PCR are shown in Table 1.

#### 3.3. Analysis of BMP-2 expression by enzyme-linked immunosorbent assay (ELISA)

MSC were seeded on a 24-well plate with  $1 \times 10^5$  cells/well and pretreated with noggin (1  $\mu\text{g}/\text{mL}$ ) for 3 hours, after which TNF- $\alpha$  (10 ng/mL) was added. After stimulation with TNF- $\alpha$  for 24 hours, the cell culture medium was collected and the BMP-2 levels therein were measured by using an ELISA kit according to the manufacturer's protocol (R&D System, Minneapolis, MN).

#### 3.4. Cell adhesion and transmigration assay

To analyze the MSC adhesion capacity, MSC were added to a collagen-coated 24-well plate and incubated for 20 minutes. The cultures were washed twice with PBS to remove the nonadherent MSC and the adherent cells were counted under a microscope. The transmigration of MSC was measured by using a transwell migration apparatus (Corning Life Science, Loswell, MA). The MSC were suspended in DMEM/0.1% BSA at a density of  $2 \times 10^6$  cells/mL and placed into the upper wells of the apparatus. These wells were covered with polyethylene terephthalate membranes that had pores of 8  $\mu\text{m}$  and were coated on both sides with 1% gelatin. After an overnight incubation, TNF- $\alpha$  was added to 0.6 mL DMEM/0.1% BSA at a concentration of 10 ng/mL in lower wells of the chambers. The chambers were incubated for 4 hours at 37°C in 95% air and 5% CO<sub>2</sub>. At the end of the incubation period, the cells were fixed and stained with 0.25% crystal violet. The non-migrating cells on the top of the filters were wiped off, the filters were stained with Crystal violet. Cells that migrated to the lower chambers and were attached to the bottom of the filters were counted in 6 random high-power ( $\times 400$ ) microscopic fields.

### 3.5. Myocardial ischemia-reperfusion (IR) injury in a rat model

Myocardial infarction was produced in male Sprague-Dawley rats (170-200g) by ligation of left anterior descending coronary artery (LAD). Rats were anesthetized by an intraperitoneal injection of xylazine (10 mg/kg) and ketamine (80 mg/kg) and then were ventilated artificially. The heart was exposed *via* a left thoracotomy and the LAD was ligated by a 4-0 MerSilk suture which was removed after 30 minutes. MSC were stained with DAPI for 30 minutes before injection. Immediately after removing the suture,  $1 \times 10^6$  MSC (fourth passage) (MSC group, n=8), or TNF-MSC (TNF-MSC group were treated with TNF- $\alpha$  for 24 hr prior to transplantation, n=8), were suspended of phosphate-buffered saline (PBS) and these were injected into the peri-infarcted region(s). Control rats received 50  $\mu$ L PBS (I/R group, n=8). Injections were carried out in a blinded fashion. Hearts were removed after two weeks, washed, fixed and tissues samples were embedded in paraffin. The number of DAPI stained cells was determined in paraffin sections.

### 3.6. Adenoviral transduction

*E. coli* BJ 5183 cells (a generous gift from Dr. Anthony Rosenzweig, Massachusetts General Hospital, Boston, MA) were transformed with Ad.luc (luciferase), pAdTrackCMV, pAdEasy1 and pAdTrackCMV. Ad-CMV-Fluc (FL) was constructed and amplified, from a replication-defective recombinant adenovirus that harbors the cytomegalovirus (CMV) promoter driving the firefly luciferase reporter gene as described previously (12). MSC were transduced with Ad-Fluc, 24 hours before they were injected into the peri-infarcted region(s) of myocardium (MSC group n=4, TNF-MSC group n=4).

### 3.7. Optical bioluminescence imaging of MSC transplantation

Optical bioluminescence imaging was conducted with a cooled charged-coupled device (CCD) camera (Xenogen, Alameda, CA). After intraperitoneal injection of the D-Luciferin reporter substrate (375 mg/kg body weight), each rat was imaged for 30 minutes by 30 $\times$ 1-minute acquisition scans. Twenty four hours after cell injection, rats were scanned daily until the imaging signals completely disappeared. The bioluminescence signals were quantified in units of photons/second/centimeter<sup>2</sup>/steradian.

### 3.8. Assessment of cardiac function

The function of left ventricle was assessed by transthoracic echocardiography, 2 weeks after surgery with a 7.5-MHz sector scan probe. Rats were anesthetized and placed in the supine position and the chest was shaved. M-mode echocardiograms, guided by two-dimensional long-axis images, were obtained. LV percent fractional shortening (FS) was calculated as  $100 \times (LVEDD - LVESD)/LV$ , and LV ejection fraction (EF) was calculated as  $100 \times (\text{stroke volume}/\text{diastolic volume})$  where LVEDD is the end diastolic diameter of LV and LVESD is end systolic diameter of LV.

The Millar Pressure-volume Catheter system was also utilized to evaluate the cardiac function, two weeks

after the IR injury. The 1.5 Fr micronanometer-tipped catheter was placed in the left ventricle through the apex (Millar Instruments, Houston, TX, USA) and a recording was made during ventilation after a minimum equilibration period of 20 minutes.

### 3.9. Histological examination

Two weeks after cell injection, the hearts were removed, and weighed. Portions of the midventricular heart were fixed in 4% paraformaldehyde, and tissue blocks were embedded in paraffin, and 4- $\mu$ m-thick sections were prepared. The extent of cardiac fibrosis in the left ventricles was assessed by staining collagen in sections with Masson trichrome, digitizing captured images, and quantifying the stained collagen by computerized morphometry (Image-Pro, Media Cybernetics, Inc., USA). For immunohistochemical analysis, formalin-fixed, paraffin-embedded sections were deparaffinized, rehydrated, immersed in 3% H<sub>2</sub>O<sub>2</sub> for 10 minutes, blocked with 3% horse serum in PBS for 1 hour at room temperature, and incubated overnight with primary antibody including antibody to connexin43 (Sigma), CD31 (BD Bioscience, San Jose, CA), and von Willebrand factor (vWF; Sigma.) at 4°C. After incubation with relevant biotinylated secondary antibody for 1 hour at room temperature, the sections were incubated with avidin-biotin-complex (ABC kit, Vector Laboratories, Burlingame, CA), developed in a mixture of diaminobenzidine (DAB)-H<sub>2</sub>O<sub>2</sub> and counterstained with hematoxylin. As a negative control, the primary antibody was omitted.

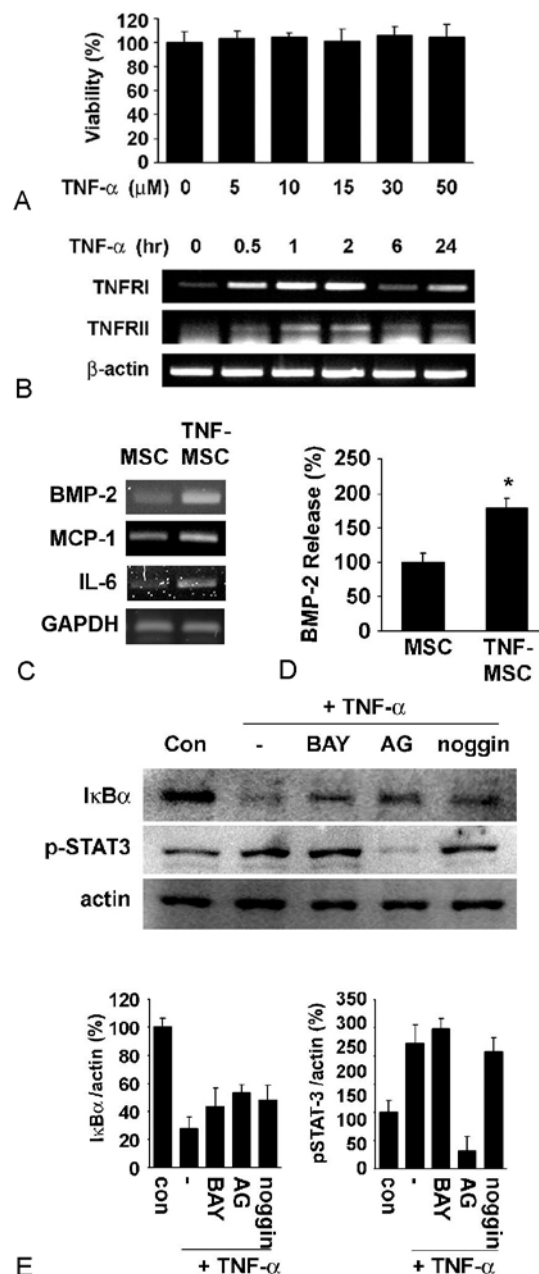
### 3.10. Statistical analysis

Each experiment was performed at least three times. The data are presented as means  $\pm$  SD. Differences were analyzed by ANOVA and were considered statistically significant when  $P < 0.05$ .

## 4. RESULTS

### 4.1. TNF- $\alpha$ enhances MSC adhesion and migration in a BMP-2-, STAT-3-, and NF- $\kappa$ B-dependent manner

First, to rule out the possibility that TNF- $\alpha$  has a toxic effect on MSC, the cells were treated with various concentrations of TNF- $\alpha$  (0 ~ 50  $\mu$ M) for 24 hours. Cell viability was then assessed by using the MTT assay. MSC viability was not changed by TNF- $\alpha$  treatment (Figure 1A). We then used RT-PCR to examine the effect of a 24-hr treatment with 10 ng/mL TNF- $\alpha$  on the expression of TNF- $\alpha$  receptors by MSC. TNF- $\alpha$  elevated the mRNA expression of TNFR I and II within the first 30 minutes of treatment. These TNFR I and II levels were sustained for 6 hours before decreasing (Figure 1B). For 10 ng/mL of TNF- $\alpha$  was sufficient to trigger the induction of its receptors without cytotoxicity, we treated MSC with 10 ng/mL TNF- $\alpha$  in further studies. We also found by RT-PCR analysis that the TNF- $\alpha$  treatment of MSC elevated their mRNA level of BMP-2 as well as MCP-1 and IL-6, known to be induced by TNF- $\alpha$  (Figure 1C). BMP-2 protein release was also increased significantly by TNF- $\alpha$  (Figure 1D). Moreover, TNF- $\alpha$  treatment of MSC increased STAT-3 phosphorylation and induced the degradation of I $\kappa$ B $\alpha$ , which activates NF- $\kappa$ B (Figure 1E). Thus, the STAT3 and



**Figure 1.** Effect of TNF- $\alpha$  on mesenchymal stem cells (MSC). (A) MSC viability after treatment with TNF- $\alpha$ . Viability was assessed by the MTT assay three times, each time in quintuplicate wells. (B) Effect of TNF- $\alpha$  treatment on MSC expression of TNF- $\alpha$  receptor I (TNFRI) and II (TNFRII) mRNAs. MSC were treated with TNF- $\alpha$  (10 ng/mL) for the indicated times and subjected to RT-PCR. The whole process was repeated three times and representative data are shown. (C) Effect of TNF- $\alpha$  treatment on MSC expression of BMP-2, MCP-1, and IL-6. MSC were treated with TNF- $\alpha$  (10 ng/mL) for 24 hours and their BMP-2, MCP-1, and IL-6 mRNA levels were quantified by RTPCR. The whole process was repeated three times and representative data are shown. (D) Effect of TNF- $\alpha$  treatment on BMP-2 protein release by ELISA three times, each time in quintuplicate wells. The process was repeated three times and representative data are shown. (E) Effect of BAY11-7082 (BAY), AG490 (AG), or noggin pretreatment on TNF- $\alpha$ -induced expression by MSC of signaling molecules. MSC were pretreated for 3 hours with BAY (5  $\mu$ M), AG (10  $\mu$ M), or noggin (1  $\mu$ g/mL) and then treated with TNF- $\alpha$  for 30 minutes to detect I $\kappa$ B $\alpha$  or for 2 hours to detect p-STAT3. BAY, AG, and noggin are inhibitors of NF- $\kappa$ B, STAT3, and BMP-2, respectively. The signaling molecules were detected by RT-PCR or Western blot as described in Materials and Methods. Data are expressed as mean $\pm$ S.D. (n=4)

NF- $\kappa$ B signaling molecules, which are known to be involved in the TNF- $\alpha$  pathway, appear to be upregulated in MSC by TNF- $\alpha$  treatment.

To confirm the effect of TNF- $\alpha$  treatment on the NF- $\kappa$ B, STAT-3 and BMP-2 signaling molecules in MSC, we pretreated MSC with their corresponding inhibitors, namely, BAY 11-7082 (BAY, 5  $\mu$ M), AG490 (AG, 10  $\mu$ M), and noggin (1  $\mu$ g/mL) before stimulation with TNF- $\alpha$ . BAY and AG inhibited TNF- $\alpha$ -induced I $\kappa$ B $\alpha$  degradation and STAT3 phosphorylation, respectively. Noggin attenuated the inhibitory effect of BAY but did not change the effect of AG. Examination of BMP-2 protein release by MSC over 24 hours revealed that this was increased by TNF- $\alpha$  treatment and that this increase was inhibited by pretreatment with noggin, BAY, and AG (Figure 2A). Moreover, the adhesiveness of MSC was significantly increased by TNF- $\alpha$  (to 193%) and this increase was attenuated by noggin, BAY, and AG pretreatment (Figure 2B). Improvement of MSC adhesion and transmigration processes could be required for the effective MSC transplantation. Finally, MSC transmigration was accelerated by TNF- $\alpha$  treatment and this effect was attenuated by noggin, BAY, and AG pretreatment (Figure 2C and 2D).

#### 4.2. TNF- $\alpha$ increases MSC adhesion and survival of ischemic myocardium

Experimental MI was induced in rats and the peri-infarct site was injected with DAPI-labeled MSC in three groups. MSC group, TNF-MSC group, and noggin-TNF-MSC group. Two weeks later, each heart was fixed in buffered formalin, and embedded in paraffin. Fluorescence microscopy revealed that the DAPI-labeled MSC were located in the border zone of infarcted ventricular myocardium. Measured area of the DAPI-MSC in the heart revealed the engraftment was expressed by % of MSC group. MSC retention on myocardium increased to  $182.43 \pm 18.42\%$  in TNF-MSC group compared with MSC group, which was blunted to  $134.29 \pm 28.80\%$  by noggin pretreatment prior to TNF- $\alpha$  treatment (Figure 3A). These observations indicate that TNF- $\alpha$  treatment prolongs the ability of MSC to adhere to the damaged myocardium for at least 2 weeks.

To compare the viability of MSC and TNF-MSC on the myocardium, we transplanted Fluc reporter-expressing TNF- $\alpha$ -treated MSC ( $1 \times 10^6$ ) and untreated MSC ( $1 \times 10^6$ ) and imaged them repeatedly over a 2-week period. We observed that the FL cardiac signals were sustained over 2 weeks (data not shown). Representative images are shown in Figure 3B. The expression of MSC-Fluc and TNF-MSC-Fluc was detected in the left thoracic region, and the intensity of photon emission was significantly higher in TNF-MSC injected rat than MSC injected rat. This result suggested that TNF-MSC survived longer than MSC in infarct myocardium.

#### 4.3. Treatment of MSC with TNF- $\alpha$ reduces collagen deposition and enhances recovery of cardiac function

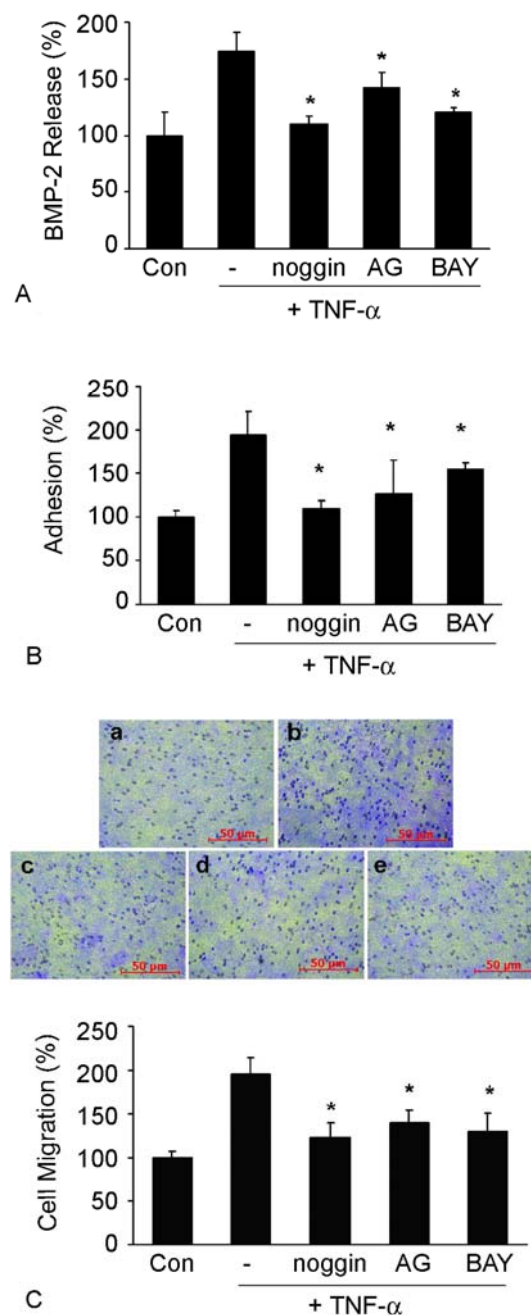
By day fourteen post-MI, collagen deposition was reduced in MSC group by  $6.2\% \pm 3\%$  compared to the I/R group, and reduced in TNF-MSC even further by  $12.5 \pm 3\%$  ( $P < 0.05$  vs. MSC group) (Figure 4A). We then stained the heart sections with antibodies specific for connexin43, CD31, and vWF (Figure 4B). Connexin43 expression was increased both in MSC group and TNF-MSC group compared with in I/R group. Connexin43 expression was more abundant and more organized in TNF-MSC group than MSC group. On the other hand, there was no significant difference in CD31 or vWF expression between the MSC and TNF-MSC groups.

Echocardiography 2 weeks after MSC injection revealed that both the MSC and TNF-MSC group exhibited wall motion recovery (Figure 5). For a more precise analysis, we used a pressure-volume catheter system. The ratio of heart weight (mg) to body weight (g) was increased in the IR rats (normal rats vs. IR rats,  $3.37 \pm 0.04$  vs.  $4.05 \pm 0.12$ ,  $P < 0.05$ ), and this ratio improved significantly in the TNF-MSC rats (TNF-MSC group vs. IR group,  $3.70 \pm 0.20$  vs.  $4.05 \pm 0.12$ ,  $P < 0.05$ ) but not in the MSC rats (MSC group vs. IR group,  $3.87 \pm 0.48$  vs.  $4.05 \pm 0.12$ ,  $P > 0.05$ ). The IR group also showed decreased ejection fraction (EF) (IR group vs. normal group,  $24.73 \pm 5.48\%$  vs.  $84.90 \pm 12.55\%$ ) that had recovered in both the MSC and TNF-MSC groups (MSC and TNF-MSC groups:  $48.33 \pm 13.79\%$  and  $61.63 \pm 9.34\%$ , respectively;  $P < 0.05$  for both groups vs. IR group [ $24.73 \pm 5.48\%$ ]). While the EF value of the TNF-MSC group was higher than that of the MSC group, this difference did not reach statistical significance. In addition, the IR group exhibited significant increases in end-systolic volume (ESV) (normal group vs. IR group,  $83.74 \pm 13.99$  vs.  $135.50 \pm 38.60$ ,  $P < 0.05$ ) and end-diastolic volume (EDV) (normal group vs. IR group,  $159.69 \pm 29.21$  vs.  $186.87 \pm 4.95$ ,  $P < 0.05$ ) that were significantly reduced in the TNF-MSC group (ESV: TNF-MSC group vs. IR group,  $84.79 \pm 47.09$  vs.  $135.50 \pm 38.60$ ,  $P < 0.05$ ; EDV: TNF-MSC group vs. IR group,  $130.35 \pm 23.34$  vs.  $186.87 \pm 4.95$ ,  $P < 0.05$ ) (Figure 6).

#### 5. DISCUSSION

Our study demonstrated that treatment of MSC with TNF- $\alpha$  not only elevated MSC adhesion and transmigration *in vitro*, it also substantially improved MSC survival and adhesion to ischemic myocardium *in vivo*. Our major findings are: (1) TNF- $\alpha$  treatment increased MSC adhesion and migration *via* a pathway that involves BMP-2, NF- $\kappa$ B, and STAT-3; (2) noggin, an antagonist of BMP-2, abolished the effect of TNF- $\alpha$  treatment; and (3) the TNF-MSC-injected hearts showed significant improvements in LV heart function indices. Thus, this *in vivo* study showed that treatment of MSC with TNF- $\alpha$  enhanced the therapeutic effect of MSC transplantation on ischemic myocardium.

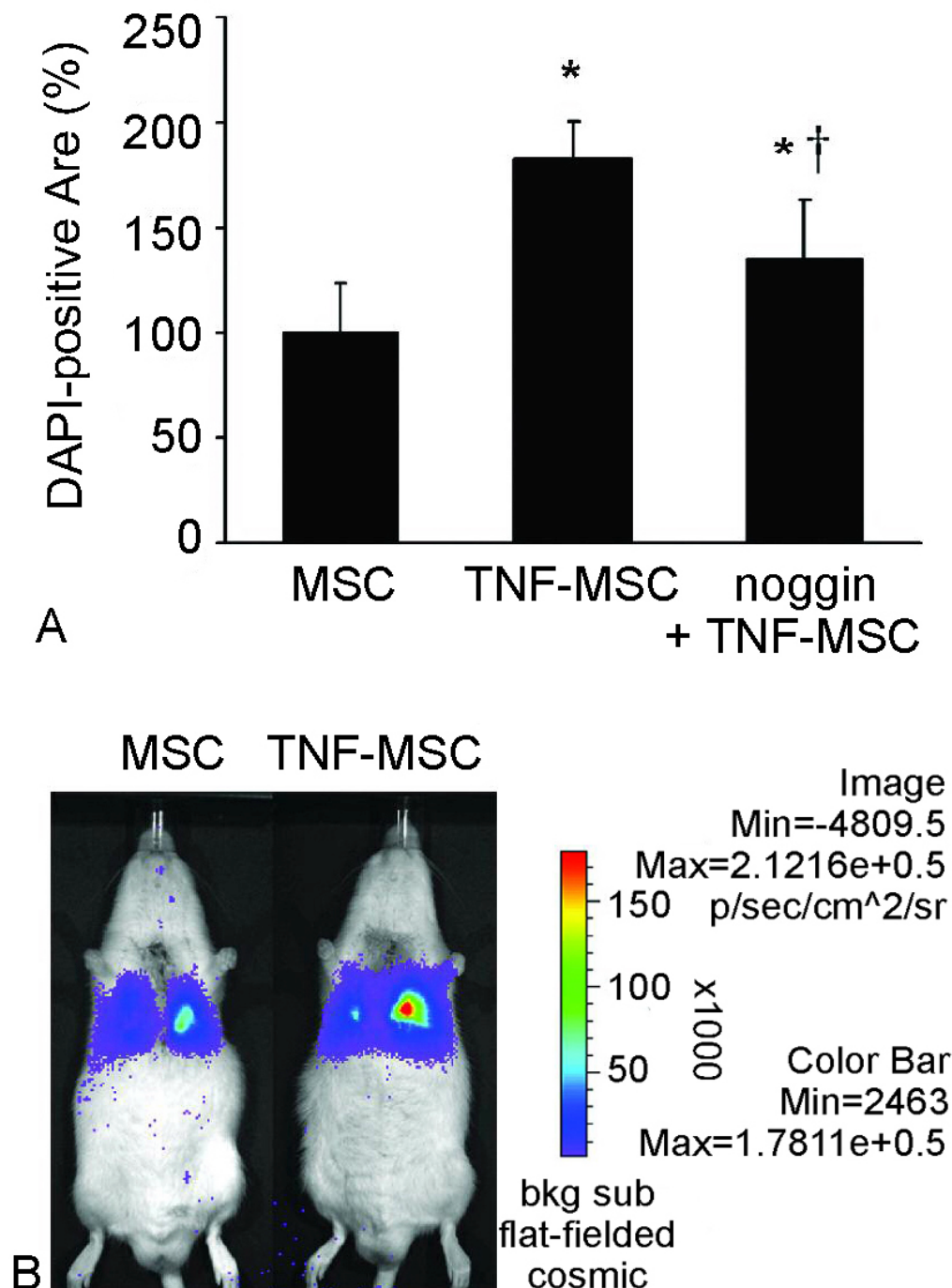
Previous studies have suggested that stem cells can mediate myocardial protection and repair after MI, and that these effects involve a number of mechanisms,



**Figure 2.** Effect of noggin, AG, and BAY pretreatment on TNF- $\alpha$ -induced MSC bone morphogenetic protein (BMP)-2 release (A, n=8, in each group), adhesiveness (B, n=8, in each group), and transmigration (C-D, n=5, in each group). \* $P$ <0.05 relative to MSC that had only been treated with TNF- $\alpha$ . a: untreated MSC; b: TNF- $\alpha$ -treated MSC; c-e: MSC that were pretreated with noggin (c), AG (d), or BAY (e) and then treated with TNF- $\alpha$ .

obstacles that hamper the application of stem cell therapy for cardiac diseases. One of these is poor survival that leads to acute donor stem cell death within four days of engraftment. This problem is thought to have a major negative impact on the ensuing graft size (4). Multiple factors may contribute to the death of graft cells but an major mechanism appears to be local tissue ischemia-induced apoptosis, as recent reports suggest that graft cells

placed in an ischemic environment are dependent on diffusion for the delivery of oxygen and substrates (2, 13). In addition, host inflammatory responses and loss of a survival signal from cell-cell contact also may contribute to cell death (14). One report also suggests that various proapoptotic or cytotoxic factors may promote the death of native or exogenous stem cells in ischemic hearts (15). Another major problem that hampers stem cell therapy for

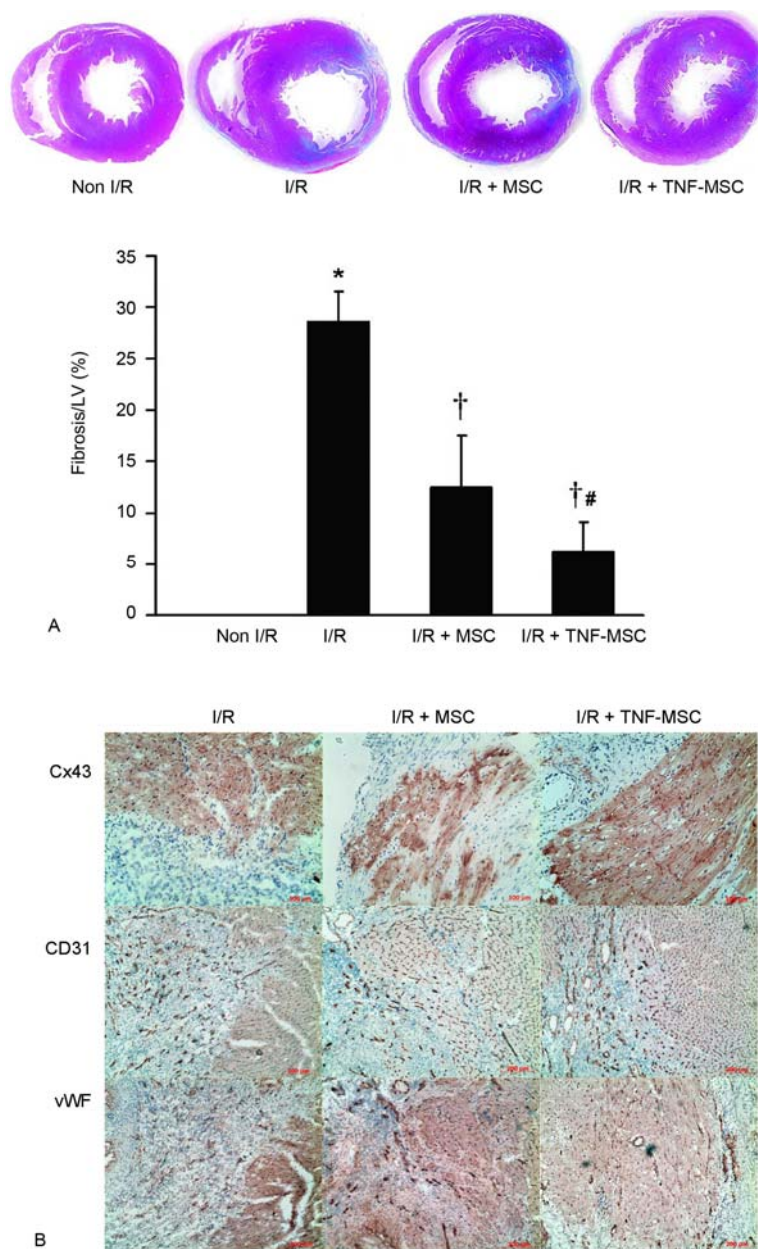


**Figure 3.** Effect of TNF- $\alpha$  on MSC adhesion to myocardium. (A) The DAPI-positive areas were calculated under a low-magnification fluorescent microscope and expressed in a bar graph. \* $P < 0.05$  vs. MSC group, † $P < 0.05$  vs. TNF-MSC group. (B) Bioluminescence optical imaging of MSC and TNF-MSC transplantation in living rats. The anterolateral wall of the myocardium of rats was injected with MSC or TNF-MSC that expressed the firefly luciferase gene. The imaging signal intensity of the TNF-MSC group was significantly higher than that of the MSC group.

cardiac diseases is a low rate of transplanted cell intercalation into the target organ. It is well known that stem cells must be capable of migration and adhesion before they can invade an ischemic tissue. A number of factors have been shown to play important roles in stem

cell migration. For example, SDF-1 plays a crucial role in the mobilization of stem cells, and ICAM/CD18 was recently reported to be critical for endothelial progenitor cell recruitment and repair of the infarcted myocardium (4). To reduce these impediments that hamper stem cell therapy





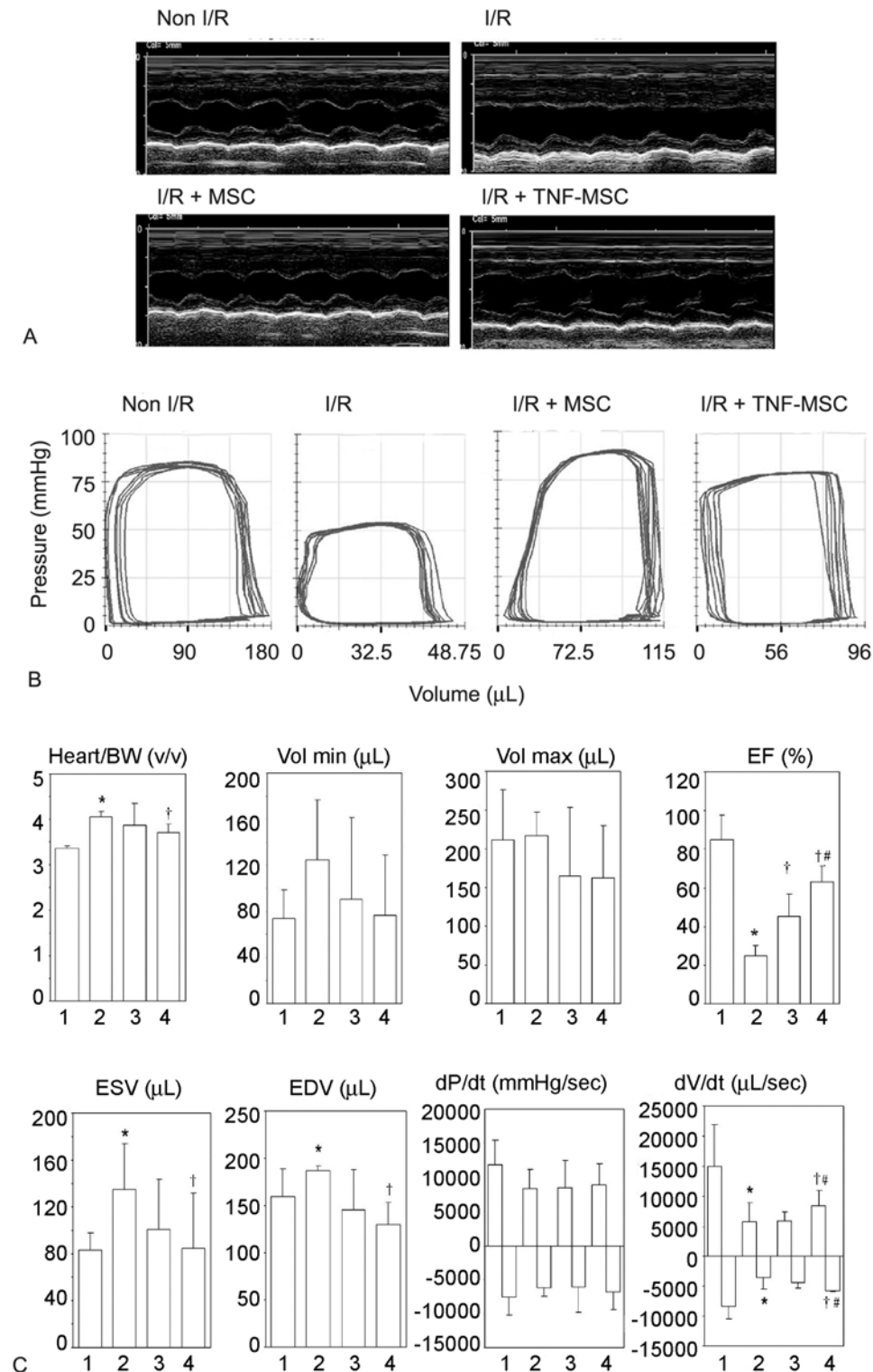
**Figure 4.** Effect of TNF- $\alpha$ -treated and -untreated MSC on cardiac fibrosis and expression of connexin43, CD31, and vWF after IR injury. (A-B) After 2 weeks, cardiac fibrosis was assessed by Masson's Trichrome staining and the areas of blue staining were quantified morphometrically and expressed as a ratio of the entire left ventricular wall area. (C) Expression of connexin43, CD31, and vWF in peri-infarct myocardial tissue was detected by immunohistochemical staining. Connexin43 expression was increased in the MSC group and was even higher in the TNF-MSC group but CD31 and vWF expression was not altered significantly by MSC transplantation, regardless of whether the MSC had been treated with TNF- $\alpha$ . \* $P$ <0.05 relative to the normal group, † $P$ <0.05 relative to the IR group, # $P$ <0.05 relative to the MSC group.

for cardiac diseases, the molecular mechanisms responsible for grafted cell death and poor stem cell migration and myocardial intercalation need to be identified more precisely. This will allow us to devise remedial strategies that prevent graft cell death and promote stem cell migration and homing. For example, we demonstrated previously that the implantation of Akt-overexpressing MSC significantly improved cardiac function in an

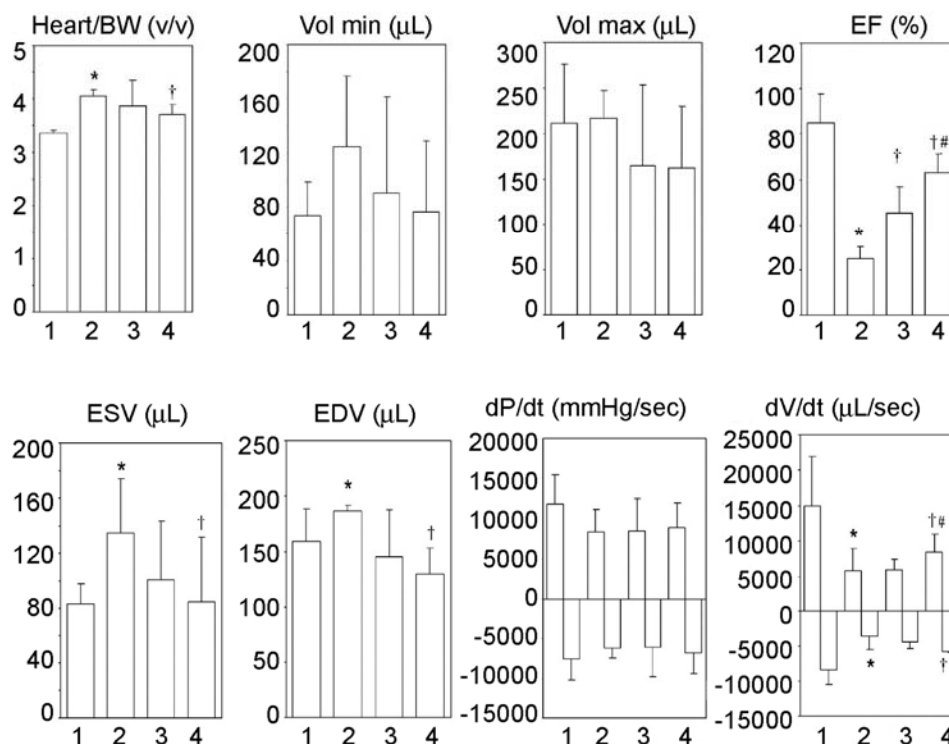
ischemic-reperfusion porcine model because Akt promoted MSC resistance against oxidative stress and apoptosis in the infarcted myocardium (16).

Our study here also suggests that treatment of MSC with TNF- $\alpha$  before injection into rat ischemic myocardium promotes both MSC adhesion and survival on infarcted myocardium and consequently improves cardiac





**Figure 5.** Effect of TNF- $\alpha$ -treated and -untreated MSC on cardiac function after IR injury. (A) Representative echocardiograms were shown. (B) Left ventricular pressure-volume curves were shown. (C) Functional parameters were expressed as graphs. \* $P < 0.05$  relative to the normal group at 14 days, † $P < 0.05$  relative to the IR group at 14 days, # $P < 0.05$  relative to the MSC group at 14 days. BW: body weight; Vol: volume; EF: ejection fraction; ESV: end-systolic volume; EDV: end-diastolic volume. 1: normal group; 2: IR group; 3: MSC group; 4: TNF-MSC group.



**Figure 6.** Effect of TNF- $\alpha$ -treated MSC on hemodynamics. Functional parameters were expressed as graphs. \* $P < 0.05$  relative to the normal group at 14 days, † $P < 0.05$  relative to the IR group at 14 days, # $P < 0.05$  relative to the MSC group at 14 days. BW: body weight; Vol: volume; EF: ejection fraction; ESV: end-systolic volume; EDV: end-diastolic volume. 1: normal group; 2: IR group; 3: MSC group; 4: TNF-MSC group.

function. Upon acute MI, various proinflammatory cytokines and growth factors, including TNF- $\alpha$ , IL-1, IL-6 and VEGF, are expressed by both the infarcted and non-infarcted regions of the heart, and the plasma levels of these cytokines and growth factors are elevated for about 2 weeks. It has also been shown that mechanical overload and ischemic injury induce the myocardial expression of several proinflammatory cytokines, including TNF, IL-1, and IL-6 (17, 18). Thus, TNF- $\alpha$  appears to play an important role in post-MI events. Indeed, it has been shown that one of the effects of TNF- $\alpha$  is cytoprotection of adult cardiac myocytes (19, 20), which supports our observation that transplantation with TNF- $\alpha$ -treated MSC improves cardiac function. We found that this effect of TNF- $\alpha$  may be mediated by improved MSC adhesion and survival, and that this involves the activation by TNF- $\alpha$  of several signaling molecules, namely, BMP-2, STAT3, and NF- $\kappa$ B. First, we observed that TNF- $\alpha$  treatment increased both MSC adhesion activity and BMP-2 translation *in vitro*, and that these effects were both partly suppressed by noggin, an antagonist of BMP-2 (21). That NF- $\kappa$ B is involved in the MSC adhesion-promoting effect of TNF- $\alpha$  was evidenced by the fact that its specific inhibitor, BAY, abrogated TNF- $\alpha$ -induced MSC adhesion. NF- $\kappa$ B is an important transcription factor that mediates the effects of TNF- $\alpha$  in various cell types. NF- $\kappa$ B activation pathways

have been classified a classical (canonical) and alternative (non-canonical) pathways. In the classical pathway, cells stimulated with an agonist such as TNF- $\alpha$  activate IKK $\alpha$ , which phosphorylates I $\kappa$ B proteins, which in turn leads to a predominant NF- $\kappa$ B signaling pathway. In the alternative pathway, NIK is activated *via* the TNF-receptor, which in turn activates IKK $\alpha$ . TNF- $\alpha$  is known to activate the IKK pathway through the TNF receptor-associated factor (TRAF) protein (22). The use of AG, a STAT3 inhibitor, showed that STAT3 is involved in the effect of TNF- $\alpha$  on MSC since it partly suppressed the TNF- $\alpha$ -induced enhancement of MSC adhesion. The STAT factors are a family of cytoplasmic transcription factors that mediate the intracellular signaling that is initiated by cytokine cell surface receptors and are transmitted to the nucleus. STAT-3 has been demonstrated to be a key molecule that acts downstream of gp130, which is activated under various stressful conditions such as pressure-overload and MI (23). It has also been reported that STAT-3, which can protect the myocardium following ischemic injury, may be a survival factor in heart disease (24). AG is a small molecule inhibitor of JAK that also interferes with other JAK pathways. It has been shown that AG interferes with TNF- $\alpha$  signaling as it reduces JNK phosphorylation, IL-6 release, and monocyte adhesion (25). Since we found that AG but not BAY or noggin abrogated TNF- $\alpha$ -induced STAT-3

phosphorylation, and noggin, AG, and BAY all attenuated TNF- $\alpha$ -induced BMP-2 protein release, we speculate that TNF- $\alpha$  treatment of MSC triggers STAT-3 phosphorylation, which activates NF- $\kappa$ B, which in turn upregulates BMP-2 expression and secretion. Since noggin, AG, and BAY attenuated TNF- $\alpha$ -enhanced MSC adhesion and transmembrane migration, it is likely that this sequence of signaling events also participated in TNF- $\alpha$ -enhanced MSC adhesion and migration.

It should be noted that TNF- $\alpha$  induces pleiotropic effects whose complexity is paralleled by the complexity of the ligand-receptor interactions at the molecular and cellular level. Thus, how TNF- $\alpha$  participates in MSC properties such as adhesion and survival remains to be fully elucidated. It is possible that TNF- $\alpha$  also triggers other processes that are crucial for the cardioprotective activities of MSC. For example, Segers *et al.* reported that when an adhesion molecule such as VCAM-1 is blocked by its antibody, the increased *in vitro* adhesiveness of MSC to microvascular endothelial cells due to TNF- $\alpha$  treatment is abrogated (26). While it has not yet been shown that VCAM-1 blockage hampers the therapeutic effect of MSC on cardiac function *in vivo*, these observations suggest that TNF- $\alpha$  may also enhance the expression of adhesion molecules, thereby promoting the efficacy of stem cell therapy for cardiac diseases.

There is currently a debate about whether exogenously-injected and engrafted stem cells become new myocytes or act as paracrine cells that secrete cardioprotective proteins. In either case, the enhanced survival of transplanted MSC leads to improvements in cardiac function. We speculate that treatment of MSC with TNF- $\alpha$  and/or their genetic engineering with gene insertions may protect these cells from ischemia in the short-term and from the anti-ischemic/reperfusion damage in the long-term.

In conclusion, treatment of MSC with TNF- $\alpha$  increased their adhesiveness in a manner that involved NF- $\kappa$ B activation and BMP-2 release. This elevated MSC engraftment into the IR rat myocardium and induced significant recovery of cardiac function. We speculate that TNF- $\alpha$  treatment increased MSC engraftment by elevating BMP-2 production. The activation of other factors such as NF- $\kappa$ B may also promote engraftment. Additional studies are needed to elucidate strategies that will increase MSC survival and resistance to the pathogenic environment and thereby improve stem cell therapy for cardiac diseases.

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