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ORIGINAL ARTICLE

Atorvastatin Pretreatment Ameliorates Mesenchymal Stem Cell Migration through miR-146a/CXCR4 Signaling

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Abstract

BACKGROUND: We previously found that atorvastatin (ATV) enhanced mesenchymal stem cells (MSCs) migration, by a yet unknown mechanism. CXC chemokine receptor 4 (CXCR4) is critical to cell migration and regulated by microRNA-146a (miR-146a). Therefore, this study aimed to assess whether ATV ameliorates MSCs migration through miR-146a/CXCR4 signaling.

METHODS: Expression of CXCR4 was evaluated by flow cytometry. Expression of miR-146a was examined by reverse transcription-quantitative polymerase chain reaction. A transwell system was used to assess the migration ability of MSCs. Recruitment of systematically delivered MSCs to the infarcted heart was evaluated in Sprague–Dawley rats with acute myocardial infarction (AMI). Mimics of miR-146a were used *in vitro*, and miR-146a overexpression lentivirus was used *in vivo*, to assess the role of miR-146a in the migration ability of MSCs.

RESULTS: The results showed that ATV pretreatment *in vitro* upregulated CXCR4 and induced MSCs migration. In addition, flow cytometry demonstrated that miR-146a mimics suppressed CXCR4, and ATV pretreatment no longer ameliorated MSCs migration because of decreased CXCR4. In the AMI model, miR-146a-overexpressing MSCs increased infarct size and fibrosis.

CONCLUSION: The miR-146a/CXCR4 signaling pathway contributes to MSCs migration and homing induced by ATV pretreatment. miR-146a may be a novel therapeutic target for stimulating MSCs migration to the ischemic tissue for improved repair.

Keywords Acute myocardial infarction · Atorvastatin · Mesenchymal stem cells · Cell migration · MicroRNA-146a

1 Introduction

Stem cell therapy represents a novel tool for treating ischemia-associated heart pathologies [1–4]. Of all cell types assessed so far, bone marrow-derived mesenchymal stem cells (MSCs), which show multi-directional differentiation potential, reduced immunogenicity, high portability and anti-inflammation, could constitute ideal seed cells for repairing the injured myocardium [5–7]. However, poor viability of MSCs upon exposure to unfriendly engraftment conditions in acute myocardial infarction (AMI) hampers their use [8, 9]. Hence, increasing MSCs survival is a major hurdle in this setting.

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Statins, a group of 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors, exert multiple biological effects beside lowering lipid levels, including antithrombotic, anti-inflammatory and antioxidant activities [10–13]. In animals, for stem and progenitor cells, statins have been demonstrated to affect their biology and function, such as improving cells mobilization, proliferation and regenerative potential [14, 15]. In porcine MSCs cultured under hypoxia and serum-free conditions, atorvastatin reduced their apoptosis [16]. Moreover, statins can improve the hostile microenvironment in the post-infarct myocardium, thus enhance the regenerative potential of transplanted cells [17]. In patients, statins obviously reduce morbidity and mortality from coronary artery disease [18, 19]. Compared with regular statin, intensive statin treatment enhances the survival and angiogenesis of transplanted bone marrow mononuclear cells, and produces greater therapeutic benefits in patients suffering from anterior ST-elevated myocardial infarction [20].

We previously found that atorvastatin (ATV) pretreatment enhanced MSCs migration and ameliorates heart function in a rat AMI model [21], by a yet unknown mechanism. It is admitted that CXC chemokine receptor 4 (CXCR4) is critical to cell migration and regulated by microRNA-146a (miR-146a) [22, 23]. Therefore, this study aimed to assess whether ATV ameliorates MSCs migration through the miR-146a/CXCR4 signaling pathway.

2 Materials and methods

2.1 Animals

Sprague–Dawley (SD) rats were assessed in this study. Experiments involving animals had approval from the Care of Experimental Animals Committee of Beijing Chaoyang Hospital. MSCs were isolated from male SD (60–80 g), while the AMI model was established in female animals (200–220 g).

2.2 Cell isolation and culture

After bone marrow collection from the tibia and femur of male rats, the specimens were placed in Iscove's Modified Dulbecco's Medium with 10% fetal bovine serum (FBS) and 1% penicillin–streptomycin (Gibco, Grand Island, NY, USA). Cell culture was carried out at 37 °C in a 5% CO_2 environment. At 70–80% confluency, the cells underwent subculture at a ratio of 1:2. Passage three-cells were evaluated in subsequent assays.

2.3 Cell treatment

MSCs were pretreated with ATV at the optimal concentration (1 μM) for 12 h as previously reported [21]. All cells were grown at 37 °C in a 5% CO_2 environment.

2.4 Cell transfection

MSCs were grown in complete medium in six-well plates. For transfection, miR-146a mimics and microRNA negative control (GenePharma, Shanghai, China), respectively, were incubated with MSCs at 70–80% confluency in presence of Lipofectamine^{TM} 2000 (Thermo Fisher Scientific, Waltham, MA, USA), in Opti-MEM R Reduced Serum Medium (Thermo Fisher Scientific). The rno-miR-146a mimics had the following sequences: sense, 5′-UGAGAACUGAAUUCCAUGGGUU-3′; antisense, 5′-CCCAUGGAAUUCAGUUCUCAUU-3′. Next, GFP-labeled lentivirus (GenePharma) transfection was performed: when MSCs reached 50% confluence, miR-146a-overexpressing lentivirus and microRNA negative control, respectively, were transfected into MSCs at 37 °C in a 5% CO_2 environment with the help of polybrene.

2.5 Flow cytometry

To assess cell surface CXCR4 amounts in MSCs, rabbit polyclonal antibodies targeting rat CXCR4 (Alomone labs, Jerusalem, Israel) were used as directed by the manufacturer. In brief, washed MSCs underwent staining with 1 mg/mL CXCR4 for 20 min at ambient. After another washing step, 300 μL PBS was used for cell resuspension. Isotype controls were assessed in parallel. Analysis was carried out on a FACSCalibur flow cytometer with the Cell Quest software (BD Biosciences, Bedford, MA, USA).

2.6 Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

MiR-146a was reverse transcribed using TaqMan MicroRNA Reverse Transcript Kit (Invitrogen, Carlsbad, CA, USA), and quantitated by PCR with TaqMan MicroRNA Assays Kit (Invitrogen) on an ABI 7500 RT-qPCR instrument (Applied Biosystems, Foster City, CA, USA). Specific primers for miR-146a and U6 were obtained from Qiagen. The $2^{-\triangle\triangle CT}$ method was utilized for data analysis.

2.7 Transwell migration assay

Cell migration was assessed in 24-wellpolycarbonate transwell plates (8-μm pores; Corning, Corning, NY, USA). MSCs (100 μL) at 10^6 cells/mL in IMEM were added to the upper chamber, with 600 μL medium containing 50 ng/

mL of stromal cell-derived factor 1 (SDF-1) (Peprotech, Cranbury, NJ, USA) in the lower chamber. Incubation was carried out at 37 °C and 5% CO_2 for 6 h, and non-migrated cells were removed from the upper surface of the membrane. After PBS wash, 4% formalin fixation was performed (15 min) followed by 0.5% crystal violet staining (20 min). Migrated cells were counted in 5 randomly selected high-power fields at × 200 under a Leica microscope (Leica, Wetzlar, Germany). Quadruplicate experiments were carried out.

2.8 AMI model establishment and cell transplantation

Female SD rats were used for AMI model establishment [24, 25]. In brief, permanent left anterior descending coronary artery (LAD) ligation was carried out. The sham group was operated by passing a suture around the LAD with no ligation. The animals were assigned to the Sham (sham operation), PBS (AMI + PBS), MSCs (AMI + MSCs), ATV-MSCs (AMI + ATV-pretreated-MSCs), ATV + LV-miR-146a (AMI + ATV-pretreated-lentivirus transfected-miRNA-146a overexpressing-MSCs) and ATV + LV-NC (AMI + ATV pretreated-lentivirus transfected-miRNA-146a negative control-MSCs) groups. Totally 24 h after AMI, 2.0×10^6 MSCs in 500 μL PBS or 500 μL PBS were administered by tail vein injection.

2.9 Echocardiographic assessment

Transthoracic echocardiography was carried out on a Sonos 7500 (Phillips) with a 12-MHz phased-array transducer to measure cardiac function and ventricular dimensions at 3 (baseline) and 30 (endpoint) days following cell transplantation [24]. The hearts (n = 5/group) were assessed in 2D and M-mode from the parasternal long-axis view at the papillary muscle level. Left ventricular end-diastolic dimension (LVEDd) and end-systolic dimension (LVESd) were assessed for ≥ 3 successive cardiac cycles. Left ventricular fractional shortening (LVFS) was derived as [(LVEDd-LVESd)/LVEDd] × 100%, and left ventricular ejection fraction (LVEF) was determined as $[(LVEDd)^3-(LVESd)^3]/(LVEDd)^3] \times 100\%$. An experienced sonographer carried out all measurements in a blinded manner.

2.10 Histological assessment

Animal sacrifice was carried out 30 days following cell transplantation. Heart specimens underwent fixation with 10% formalin, paraffin embedding, sectioning at 4 μm (mid-LV level), and staining with Masson's Trichrome and hematoxylin–eosin (H&E). Image-Pro Plus 6.0 (Media Cybernetics Inc., Rockville, MD, USA proportion of fibrotic areas was determined as (fibrotic) was utilized for quantitation. The proportion of fibrotic areas was determined as (fibrotic length/LV circumference) × 100%. Triplicate assays were performed.

2.11 Statistical analysis

SPSS 23.0 was utilized for data analysis. Data are mean ± SD. One-way ANOVA (multiple groups) and Student's t test (group pairs) were carried out for comparisons. Two-sided $p < 0.05$ indicated statistical significance.

3 Results

3.1 ATV pretreatment increases CXCR4 amounts and migration in MSCs

CXCR4 expression was significantly increased by ATV pretreatment as assessed by flow cytometry (21.98 ± 2.38% vs. 2.09 ± 0.30%, $p < 0.001$) (Fig. 1), which was consistent with our previous findings [21]. Moreover, MSCs after ATV pretreatment had increased migration in comparison with untreated MSCs (22.05 ± 4.28 vs. 10.15 ± 1.95, $p < 0.05$) (Fig. 2).

3.2 ATV pretreatment decreases miR-146a expression in MSCs

To explore the mechanism by which ATV upregulates CXCR4, miR-146a expression in MSCs was examined by RT-qPCR. Previous studies have demonstrated that miR-146a targets CXCR4 mRNA [15]. In comparison with the MSCs group, the ATV pretreatment group showed markedly reduced miR-146a amounts (0.41 ± 0.11 vs. 1.00 ± 0.00, $p < 0.05$) (Fig. 3).

3.3 MiR-146a is involved in CXCR4 upregulation by ATV pretreatment

To examine whether miR-146a contributes to CXCR4 upregulation under ATV pretreatment, mimics were used for upregulating miR-146a in MSCs. MSCs transfection with miR-146a mimics were followed by preconditioning with 1 μM ATV for 12 h. In flow cytometry analysis, miR-146a mimics decreased cell surface CXCR4 amounts compared with the ATV pretreatment group (2.31 ± 0.38% vs. 22.52 ± 3.10%, $p < 0.001$) (Fig. 4). Because of decreased cell surface CXCR4 level, migration in MSCs was inhibited (9.90 ± 1.06 vs. 23.45 ± 4.82, $p < 0.001$) (Fig. 5), indicating that elevated miR-146a amounts blunted ATV-induced migratory potential.

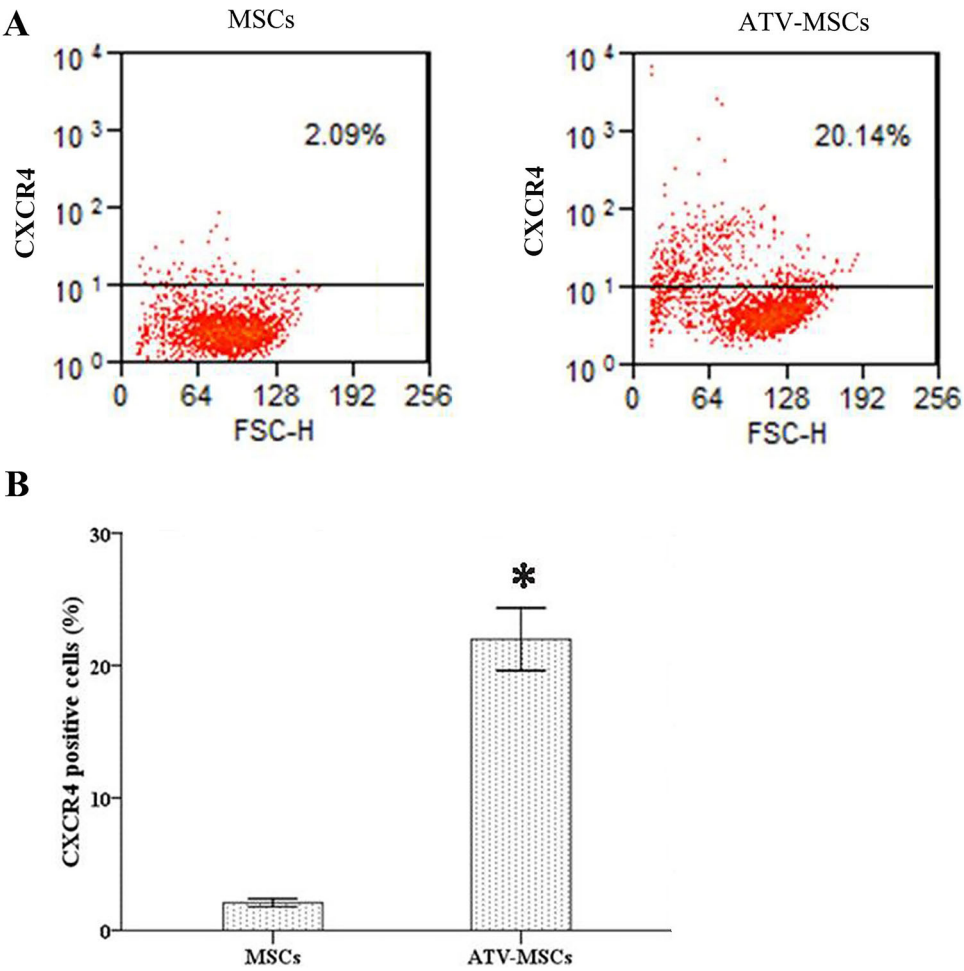

Fig. 1 ATV enhances surface CXCR4 amounts in MSCs as evaluated by flow cytometry. **A** Representative flow-cytograms in both groups. **B** Quantification of flow cytometry results. Data are mean ± SD; $^{*}p < 0.05$ vs. MSCs (n = 4). ATV, atorvastatin; CXCR4, CXC chemokine receptor 4; MSCs, mesenchymal stem cells; ATV-MSCs, atorvastatin-pretreated MSCs

3.4 MiR-146a-overexpressing MSCs increase myocardium infarct size and enhance fibrosis

In this study, Masson's Trichrome staining showed transmural infarction in every group, indicating the success of AMI modeling (Fig. 6A, B). In the MSCs-treatment group, thinner anterior wall, bigger LV chamber size, severer fibrosis and larger infarct size were observed. In the ATV-MSCs group, these effects were markedly improved. However, the beneficial effects of ATV were abrogated by miR-146a overexpression.

The degree of inflammation in the infarcted heart was assessed by H&E staining. As shown in Fig. 6C, reduced inflammatory cells infiltration was found in the ATV-MSCs group in comparison with the remaining groups, and the anti-inflammatory effect of ATV was diminished by miR-146a overexpression.

3.5 MiR-146a-overexpressing MSCs decrease cardiac function

The baseline parameters of cardiac function at 3 days post-stem cell transplantation (4 days following AMI modeling) suggested that the AMI model was successfully and stably established (Table 1).

LVEDd and LVESd were obviously elevated in the PBS group at 30 days (endpoint), indicating an enlargement of the LV chamber. A moderate LV chamber improvement

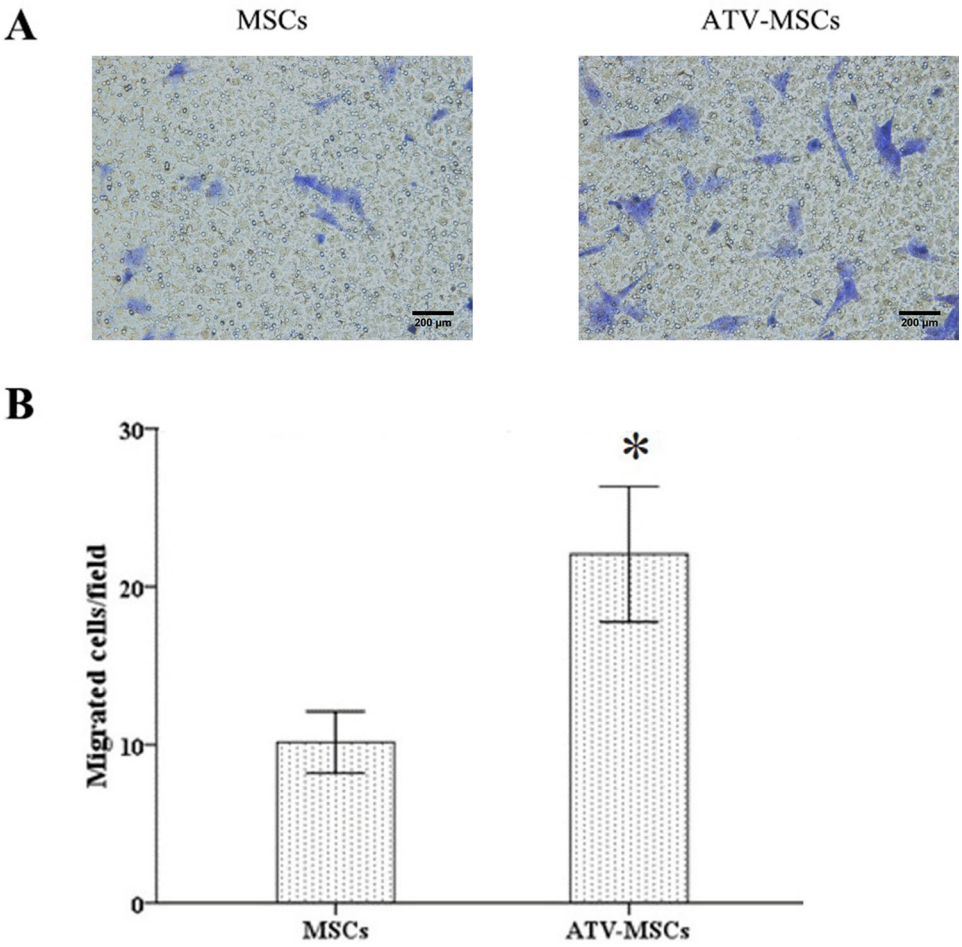

Fig. 2 ATV enhances MSCs migration *in vitro* as assessed by transwell assay. **A** Representative images of migrated MSCs in both groups (magnification × 200). **B** Quantification of transwell images. Data are mean ± SD; $*p < 0.05$ versus MSCs (n = 4). ATV, atorvastatin; MSCs, mesenchymal stem cells; ATV-MSCs, atorvastatin-pretreated MSCs

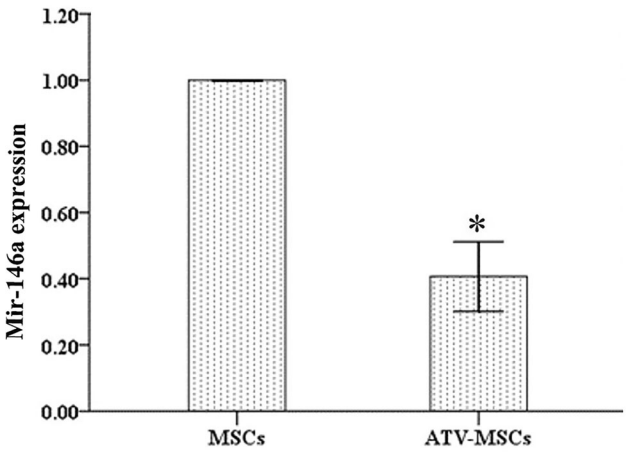

Fig. 3 ATV decreases miR-146a expression in MSCs as analyzed by RT-qPCR. Data are mean ± SD; $*p < 0.05$ versus MSCs (n = 3). ATV, atorvastatin; MSCs, mesenchymal stem cells; ATV-MSCs, atorvastatin-pretreated MSCs

was seen in the MSCs group, and this amelioration was more pronounced in the ATV-MSCs group. Meanwhile, an indicator of cardiac function, LVEF, was markedly elevated in the MSCs group in comparison with the PBS group. LVEF and LVFS were drastically improved in the ATV-MSCs group in comparison with the PBS and MSCs groups. The above findings indicated that ATV-pretreated MSCs starkly promoted cardiac function recovery and suppressed cardiac remodeling, while miR-146a-overexpressing MSCs abrogated the beneficial effects of ATV pretreatment.

4 Discussion

This work confirmed that ATV pretreatment upregulated CXCR4, increased migration and homing ability in MSCs and improved cardiac performance, consistent with our

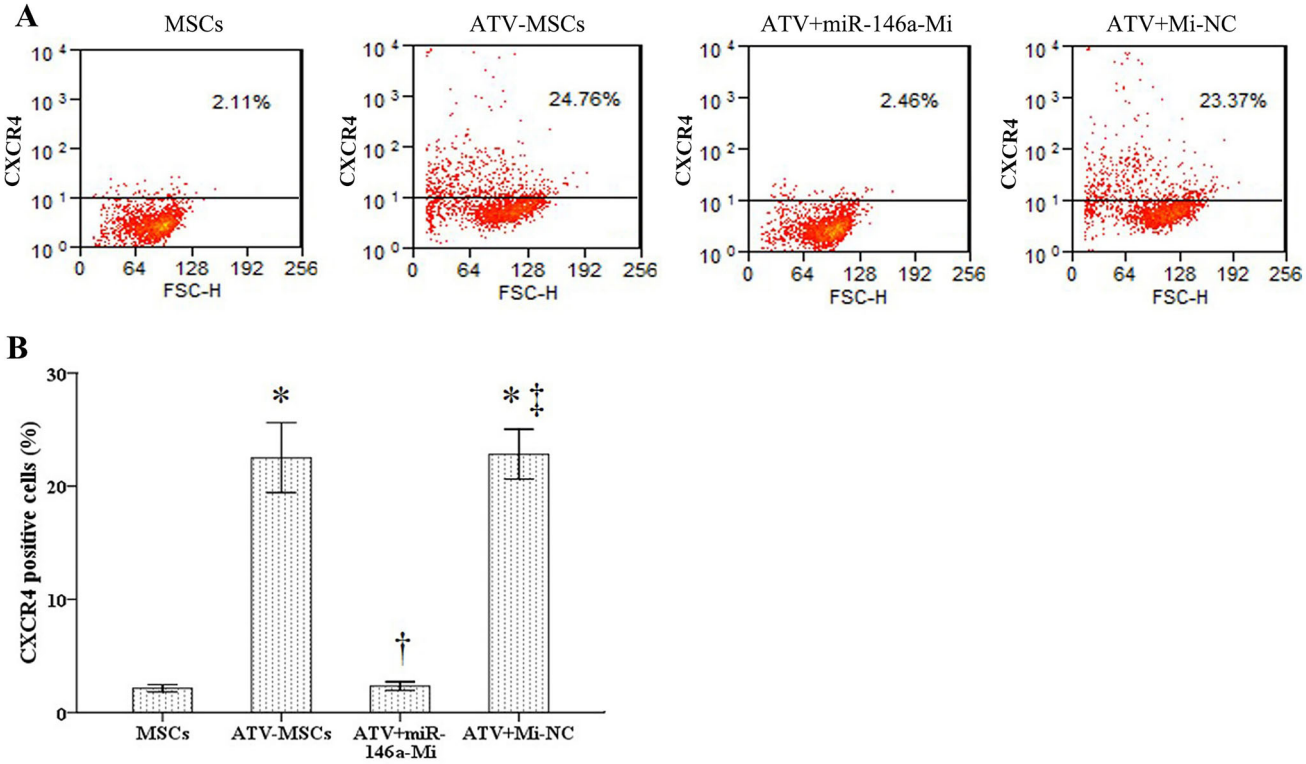

Fig. 4 Overexpression of miR-146a reduces cell surface expression of CXCR4 in MSCs. **A** Representative flow-cytograms in various treatment groups. **B** Quantitation of flow cytometry results. Data are mean ± SD; n = 4/group; $^{*}p < 0.05$ versus MSCs; $^{\dagger}p < 0.05$ versus ATV-MSCs; $^{\ddagger}p < 0.05$ versus ATV + miR-146a-Mi. ATV, atorvastatin; CXCR4, CXC chemokine receptor 4; MSCs, mesenchymal stem cells; ATV-MSCs, atorvastatin-pretreated MSCs; ATV + miR-146a-Mi, ATV + miRNA-146a mimics; ATV + Mi-NC, ATV + miRNA-146a mimics-negative control

previous study [21]. In the present study, the mechanism by which ATV pretreatment enhances MSCs migration was further investigated. The beneficial effect of ATV was abrogated by overexpressing miR-146a, indicating that elevated CXCR4 expression by ATV is attributable to miR-146a downregulation.

MSCs transplantation into the infarcted heart represents a promising new strategy to cure the infarcted myocardium [7, 26]. However, donor MSCs are poorly engrafted, limiting the reparative potential of this treatment. Recruiting a greater number of MSCs to the ischemic myocardium can effectively increase the therapeutic efficacy of engrafted cells. MSCs movement is mostly regulated by SDF-1 and its receptor CXCR4 [27–29].

It is well-known that SDF-1 and CXCR4 constitute major factors in cell mobilization and homing. SDF-1 is highly expressed in the infarcted myocardium, and represents an important mobilization or retention signal to attract CXCR4-positive stem cells for healing [30, 31]. CXCR4 is highly expressed in bone marrow MSCs. However, the level is overtly decreased during ex vivo cell expansion, reducing migration toward the SDF-1 gradient [32, 33]. Methods for enhancing CXCR4 expression have been investigated. Virus transduction constitutes one of the proposed techniques, but seems infeasible in clinical practice currently [34, 35]. Drug pretreatment is a much more simple and feasible method [36]. Among the drugs used, ATV is highly effective. As demonstrated in our previous and present studies, ATV pretreatment increased CXCR4 expression and migratory potential in MSCs, and improved cardiac performance [21]. As a lipid-lowering drug, ATV is widely used in clinical practice, and can be

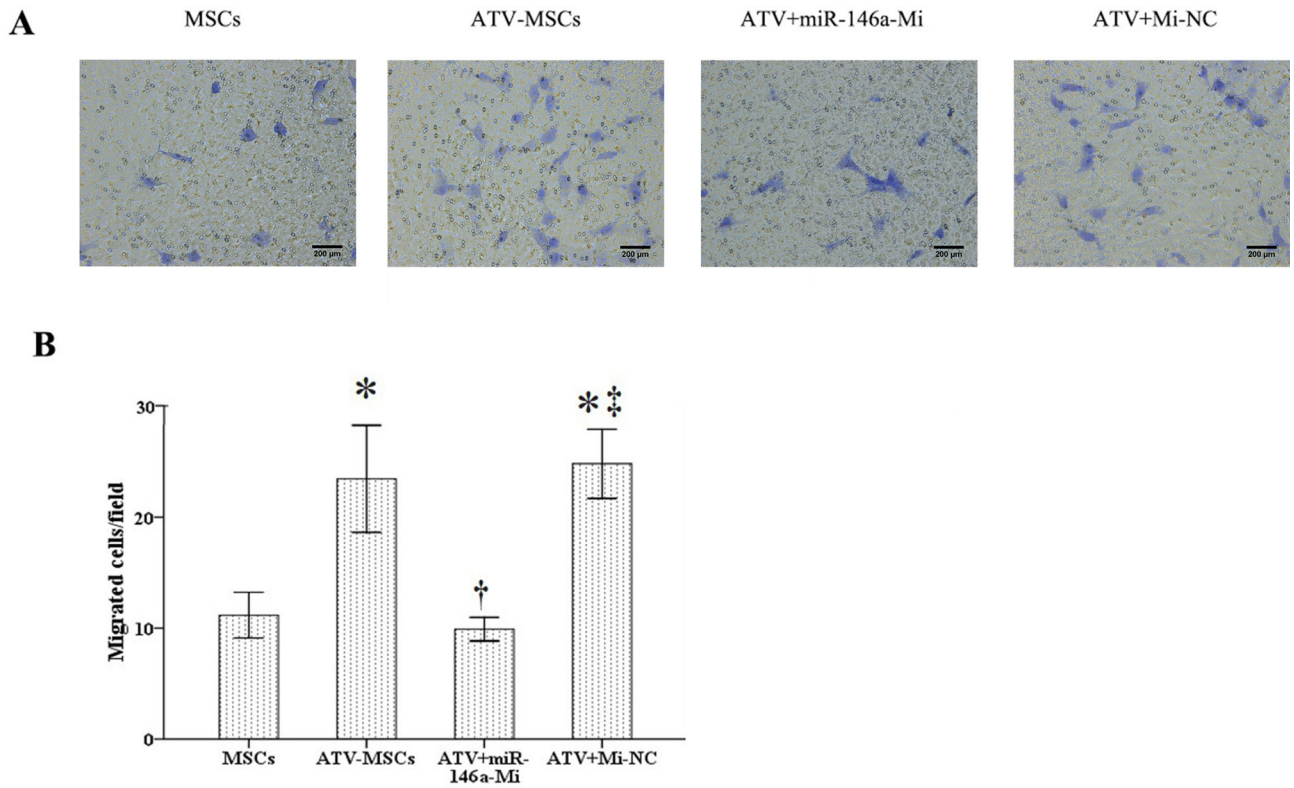

Fig. 5 Overexpression of miR-146a decreases the migratory ability of MSCs. **A** Representative images of migrated MSCs in various groups (magnification × 200). **B** Quantitation of transwell results. Data are mean ± SD; n = 4/group; $^{*}p < 0.05$ versus MSCs; $^{\dagger}p < 0.05$ versus ATV-MSCs; $^{\ddagger}p < 0.05$ versus ATV + miRNA-146a Mi. ATV, atorvastatin; CXCR4, CXC chemokine receptor 4; MSCs, mesenchymal stem cells; ATV-MSCs, atorvastatin-pretreated MSCs; ATV + miR-146a-Mi, ATV + miRNA-146a mimics; ATV + Mi-NC, ATV + miRNA-146a mimics-negative control

obtained easily. Therefore, the current findings have obvious translational implications for treating patients with AMI.

As is well known, statins exert pleiotropic and cholesterol-independent effects on the cardiovascular system [37]. Statins are molecules of fungal origin, and each vary in their lipophilicity, elimination half lives, and potency [38]. Common statins include lovastatin, pravastatin, simvastatin, atorvastatin and so on, which are used to lower the blood cholesterol level and reduce the risk of heart disease [39]. Among them, ATV is the most prescribed one. Experimental studies have revealed that in the ischemic heart ATV increases mobilization of endothelial progenitor cells and myocardial neovascularization, and results in improved heart function [40]. Moreover, in the swine AMI model, ATV treatment improves the post-infarct microenvironment, and facilitates the survival and therapeutic action of implanted stem cells, by significantly reducing oxidative stress and suppressing expression of the inflammatory cytokines in the post-infarct myocardium [17, 41]. The effects of statins on stem cells have also been evaluated in human studies. Treatment of statins further increase stem cells mobilization after AMI [42]. Intensive statin therapy has been demonstrated to lower the risk of recurrence of major cardiac events in patients with acute coronary syndrome [43]. To clarify whether intensive ATV increases the efficacy of transplanted bone marrow mononuclear cells, a study was conducted in patients with anterior ST-elevated myocardial infarction. In comparison with regular ATV, intensive ATV treatment improves the therapeutic efficacy of transplanted cells, probably for that intensive ATV could further improve the hostile

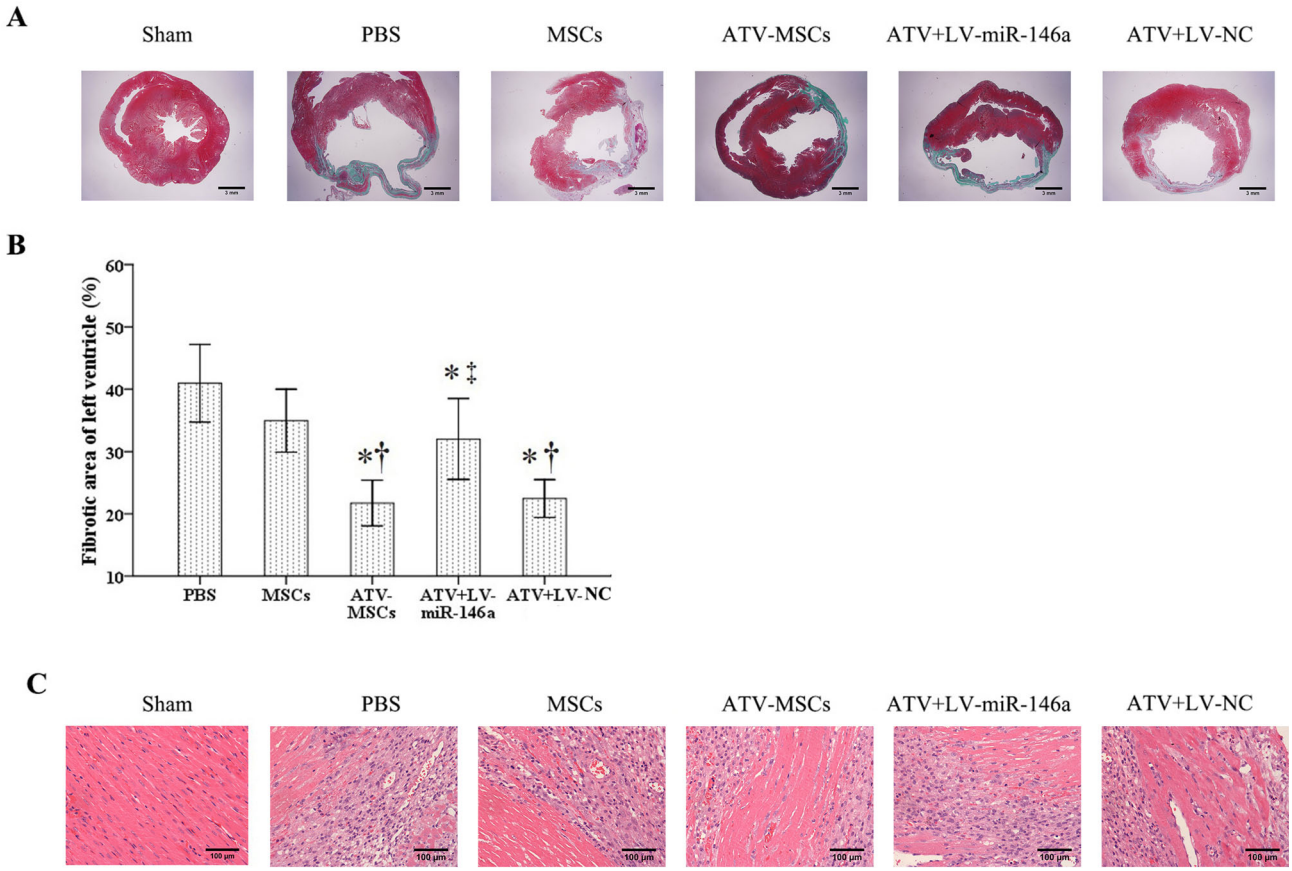

Fig. 6 Masson's trichrome and Hematoxylin–Eosin staining findings. **A** Representative micrographs after Masson's trichrome staining in various groups (magnification × 12.5). **B** Quantitation of left ventricle fibrotic areas. **C** Inflammatory cell infiltration in the heart tissue in peri-infarct areas (magnification × 400). Data are mean ± SD; n = 5/group; $^{*}p < 0.05$ versus PBS; $^{\dagger}p < 0.05$ vs. MSCs; $^{\ddagger}p < 0.05$ versus ATV-MSCs. MSCs, mesenchymal stem cells; ATV-MSCs, atorvastatin-pretreated MSCs; ATV + LV-miR-146a, ATV-pretreated-lentivirus transfected-miRNA-146a overexpressing-MSCs; ATV + LV-NC, ATV pretreated-lentivirus transfected -miRNA-146a negative control-MSCs

microenvironment in the infarcted area and increase implanted cells survival and possible regeneration [20].

MiRNAs, a family of noncoding single-stranded RNAs consisting of approximately 22 nucleotides, regulate gene expression post-transcriptionally via two mechanisms: mRNA clearance and repression of productive translation [44]. They modulate multiple cellular events, including proliferation, differentiation and migration [45, 46]. Previous studies have indicated that CXCR4 is post-transcriptionally regulated by miR-146a [47, 48]. Spinello et al. [49] found that in acute monocytic leukemia, high CXCR4 protein levels are associated with low/absent expression of miR-146a; conversely, miR-146a overexpression in leukemic cells decreases CXCR4 protein amounts. Whether miR-146a controls CXCR4 expression to contribute to MSCs migration was the main focus of this study. As shown above, high CXCR4 protein amounts, induced by miR-146a downregulation with ATV pretreatment, were required for the migration and homing of MSCs. Conversely, miR-146a overexpression downregulated CXCR4 and suppressed MSCs migration and homing, inducing cardiac remodeling and dysfunction.

Overall, miR-146a suppression by ATV pretreatment upregulates CXCR4, promoting MSCs mobilization and migration, and improving the performance of the infarcted heart. These findings suggest that miR-146a/CXCR4 signaling contributes to MSCs migration and homing upon ATV pretreatment. Thus, miR-146a may be a novel therapeutic target for stimulating MSCs mobilization to the ischemic tissue for efficient repair.

Table 1 Left ventricular dimensions and function at baseline and endpoint

Parameter	LVEDd(mm)	LVESd(mm)	LVFS(%)	LVEF(%)
Baseline				
Sham	6.05 ± 0.48	3.79 ± 0.20	37.20 ± 3.06	75.09 ± 3.64
PBS	6.74 ± 0.48*	5.30 ± 0.39*	21.33 ± 4.51*	50.92 ± 8.47*
MSCs	6.80 ± 0.40*	5.40 ± 0.47*	20.68 ± 3.60*	49.84 ± 7.08*
ATV-MSCs	6.91 ± 0.21*	5.41 ± 0.53*	21.83 ± 5.97*	51.58 ± 10.35*
ATV + LV-miR-146a	6.81 ± 0.32*	5.36 ± 0.58*	21.32 ± 5.81*	50.65 ± 11.46*
ATV + LV-NC	6.96 ± 0.21*	5.54 ± 0.31*	20.46 ± 4.14*	49.35 ± 7.85*
Endpoint				
Sham	6.19 ± 0.32	3.87 ± 0.30	37.53 ± 3.50	75.43 ± 4.16
PBS	9.41 ± 1.31*	7.76 ± 0.75*	17.16 ± 3.99*	42.82 ± 8.28*
MSCs	8.29 ± 0.94*†	6.36 ± 0.53*	23.00 ± 4.45*†	53.98 ± 7.68*†
ATV-MSCs	7.37 ± 0.95*†‡	5.22 ± 0.64*†	29.19 ± 3.08*†‡	64.33 ± 4.65*†‡
ATV + LV-miR-146a	8.01 ± 0.84*†*	6.12 ± 0.67*†	23.58 ± 3.61*†*	55.14 ± 6.13*†*
ATV + LV-NC	7.10 ± 1.03*$^{\dagger\ddagger\#}$	4.95 ± 0.72†	30.07 ± 4.42*$^{\dagger\ddagger\#}$	65.48 ± 6.39*$^{\dagger\ddagger\#}$

All values are expressed as mean ± SD (n = 5 in each group)

Baseline = 3 days after stem cell transplantation; Endpoint = 30 days after stem cell transplantation

LVEDd left ventricular end-diastolic dimension, *LVESd* left ventricular end-systolic dimension, *LVEF* left ventricular ejection fraction, *LVFS* left ventricular fractional shortening

*$p < 0.05$ versus sham; $^{\dagger}p < 0.05$ versus PBS; $^{\ddagger}p < 0.05$ versus MSCs; $^{\star}p < 0.05$ versus ATV-MSCs; $^{\#}p < 0.05$ versus ATV + LV-miR-146a

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Compliance with Ethical Standards

Conflict of interest The authors declare that they have no competing interests.

Ethical statement The animal studies were performed after receiving approval from the Care of Experimental Animals Committee of Beijing Chaoyang Hospital (Approval No. 2020-Animal-271).

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