

Original Article

Estrogen receptor ESR1 promotes BMSCs cell proliferation and migration via regulation of SDF-1/CXCR4 signaling

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Received July 16, 2016; Accepted September 5, 2016; Epub November 15, 2016; Published November 30, 2016

Abstract: Stromal cell-derived factor-1 (SDF-1) and its membrane receptor C-X-C chemokine receptor type 4 (CXCR4) are involved in the proliferation and migration of multiple stem cell types which are essential for healthy endometrium. Herein, we investigated the mechanisms of ESR1 to promote cell proliferation and migration of bone marrow-derived mesenchymal stem cells (BMSCs). The lentivirus-transfected ESR1-overexpressed BMSCs were constructed for the mechanism study in the effects of ESR1 on the proliferation and migration of BMSCs. We found that overexpression of ESR1 increased cell proliferation in BMSCs. In addition, it was also observed that ESR1 overexpression elevated expressions of SDF1, CXCR4. Activation of ESR1 further led to cell-cycle G1/S transition, which in turn directly upregulated cell cycle-related factors, such as cyclin D1. Taken together, the results indicated that activation of SDF1/CXCR4 signaling was involved in ESR1 induced cell proliferation and migration of BMSCs. In summary, these findings may better our understanding of the role ESR1 plays in the prevention of thin endometrium through affecting the proliferation and migration of BMSCs and promote the development of advanced treatments for series of diseases, including thin endometrium.

Keywords: BMSCs, ESR1, SDF-1, CXCR1, proliferation, migration

Introduction

Bone marrow mesenchymal stem cells (BMSCs) are a major type of multipotent mesenchymal stem cells (MSCs) that are capable of differentiating into kinds of cells [1-4], and they are immune-privileged, moreover, they also could be expanded to a great extent in vitro [5-8]. Thus, BMSCs have been considered to be a strong candidate tool to treat many diseases. Preclinical studies have shown beneficial effects of BMSCs on neurological disorders. The BMSCs facilitate nerve regeneration [9], improve diabetic neuropathy [10], multiple sclerosis [11], and help functional recovery after stroke [12]. Jing Z et al have also reported that transplantation of BMSCs could promote the endometrial cell regeneration, repair endometrial tissues and improve the receptivity of endometrium in rats carrying thin endometrium [13].

On the other hand, it has been reported that the polymorphisms of estrogen receptor in hypomenorrhea patients and found that there was an obvious correlation between the polymorphisms of ER β and attack of unexplained hypomenorrhea [14]. It also has been reported that the polymorphisms of ER α are also closely related to thin endometrium and the expression levels of ER α mRNA and protein are significantly lower in thin endometrium than normal endometrium [15]. Thus, it is indicated that normal estrogen-estrogen receptor interaction is significant for promoting the proliferation and migration of BMSCs which could help to maintain normal physiological cycles of endometrium and repair damaged endometrium. The low expression level and lack of normal responses to estrogen of estrogen receptors (ESRs) are against the BMSCs function. In addition, Stromal cell-derived factor-1 (SDF-1) and its membrane receptor C-X-C chemokine receptor type 4 (CXCR4) are involved in the proliferation

and migration of multiple stem cell types which are essential for healthy endometrium [16]. Thus, the mechanism underlying the regulation of ESR1 to BMSCs may be related to the SDF-1/CXCR4 axis.

In order to investigate the relationship between the expression level of ESR1 and cell function of BMSCs, BMSCs were isolated, incubated and identified for further studies. A lentivirus vector was successfully constructed to introduce overexpressed ESR1 into rat BMSCs to over-express ESR1. Lentivirus-mediated over-expression of ESR1 obviously promoted the proliferation and migration abilities of BMSCs. Moreover, the expression levels of SDF-1 and CXCR1 in rat BMSCs overexpressing ESR1 were also affected, which means the regulation of BMSCs by ESR1 went through SDF-1/CXCR1 axis.

Material and methods

Rat BMSCs isolation

Healthy 3-4 week SPF grade SD rats weighing 100-120 g were sacrificed by cervical dislocation, soaked in 75% ethanol for 10 min and then the femur and tibia were isolated under sterile condition. Samples were immersed in L-DMEM, 100 U/mL penicillin and 100 lg/mL streptomycin. Joint capsules at the ends of the diaphysis were removed without isolating epiphysis and the diaphysis was then divided. A disposable aseptic syringe was used to draw antibiotic supplemented L-DMEM medium and to repeatedly wash the bone marrow cavity to collect cells in a sterile petri dish. The obtained cell suspension was centrifuged at 2509 g for 5 min at 26°C and rinsed once in L-DMEM containing 10% FBS. Cells were further resuspended in complete medium (90% L-DMEM, 10% FBS, 100 l/mL penicillin and 100 lg/mL streptomycin) and transferred to a 25 cm² plastic culture flask for incubation at 37°C in a 5% CO₂ supplemented incubator. Cells isolated from one rat were cultured in one flask.

Construction of lentiviral vector system and transfection

ESR1 cDNA was amplified and cloned into the lentiviral vector LV5 (GenePharma, Shanghai, China). The coding sequence of rat ESR1 (NM_012689.1) was used to prepare lentiviral

vectors. To prepare lentiviral particles, the lentiviral vector (Lv5-ESR1), pGag/Pol plasmid, pRev plasmid and pVSV-G plasmid (GenePharma, Shanghai, China) were co-transfected into HEK293T cells according to the manufacturer's protocol. After the harvest of packaged lentivirus particles, rat BMSC were incubated in 6-well plates in which the Lv5-ESR1 or Lv5-NC was also added (a MOI of 10) for infection. After the infection of 12 h, the cells were subsequently cultured with the basic medium replacing the medium containing lentivirus for 48 more hours. Successful infection was then detected through observing cells fluorescing green under fluorescence microscope.

Western-blot analysis

After the infection of 4 days, to get BMSC extract for analyzing the proteins, cell lysis was performed by resuspending and incubating the cells in cell lysis buffer containing 100 mM Tris-HCl (pH 6.8), 4% SDS, 10 mM EDTA, and 10% Glycerol. The mixture of the extracted proteins was separated by SDS-PAGE (sodium dodecyl sulphate polyacrylamide gel electrophoresis). The separated proteins were transferred into nitrocellulose membranes with Transfer buffer. Membranes were then probed with 1:1000 diluted rabbit anti-rat-ESR1 primary antibody (Cell Signaling; Catalogue number: 8644), 1:1000 diluted rabbit anti-rat-p21 primary antibody (Cell Signaling; Catalogue number: 2947), 1:1000 diluted rabbit anti-rat-Cyclin D1 primary antibody (Cell Signaling; Catalogue number: 2978), 1:1000 diluted rabbit anti-rat-SDF1 primary antibody (Cell Signaling; Catalogue number: 3530), 1:500 diluted rabbit anti-rat-CXCR-4 primary antibody (Santa; Catalogue number: sc-9046), and 1:5000 diluted GAPDH (Santa Cruz, CA, USA; Catalogue number: SC-32233). Whereafter, the membranes were incubated with 1:1000 diluted horseradish peroxidase-conjugated goat anti-rabbit IgG (Beyotime, Shanghai, China; Catalogue number: A0208) after TBST wash. And the chemiluminescent (ECL) detection reagent (Sangon Biotech, Shanghai, China) was added to help with observation.

CCK-8 cell proliferation assay

To test whether the overexpression of ESR1 could affect the proliferation of rat BMSC, 100 µl of control cells, Lv5-NC or Lv5-ESR1 trans-

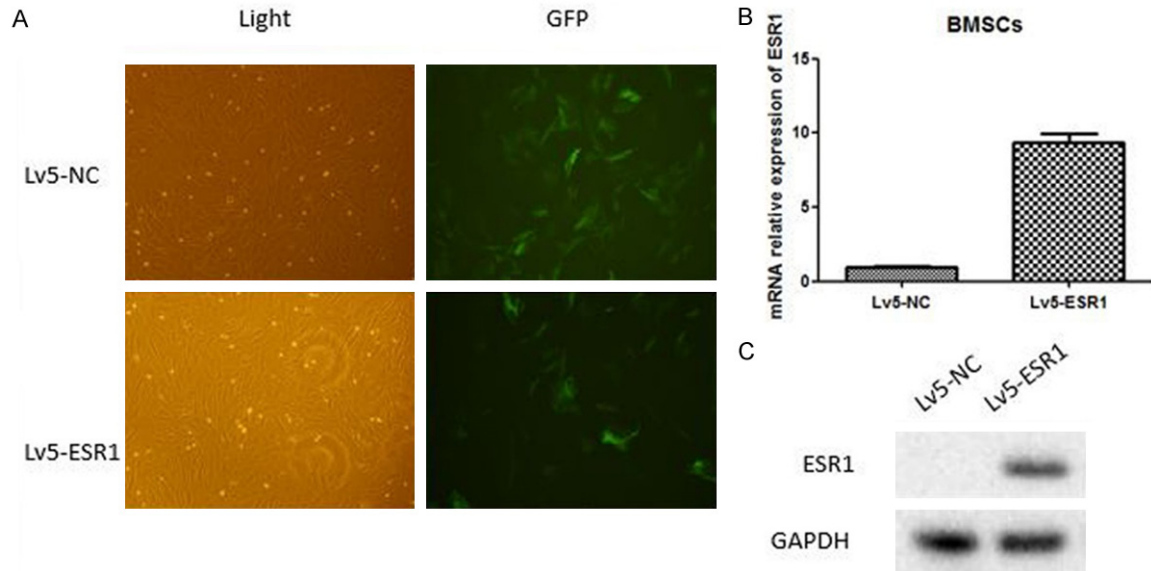


Figure 1. Results of the transfection of Lv5-ESR1 into BMSCs. A. The transfection efficiency was more than 80% which could be observed through the expression of GFP under a fluorescent microscope. B. The relative mRNA level of ESR1 was detected by qPCR to increase dramatically by more than 120%. C. Western blot confirmed the high expression efficiency of Lv5-ESR1 in BMSCs on protein levels.

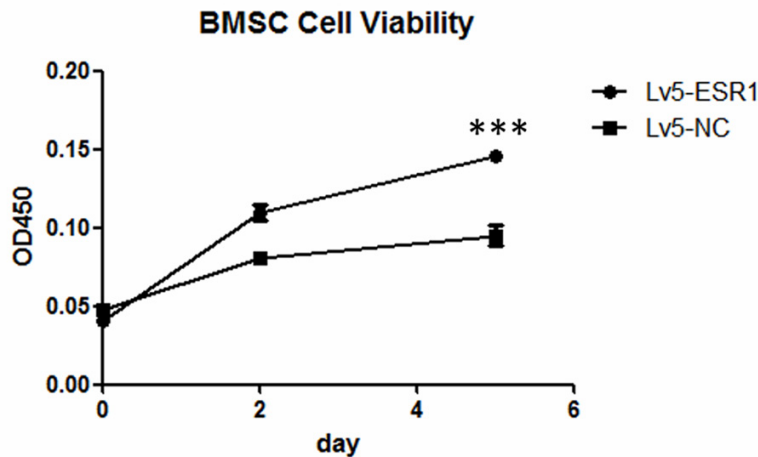


Figure 2. BMSCs infected with Lv5-shESR1 proliferated much faster than the cells infected with Lv5-NC according to CCK-8 assay.

ected cells were added into 96 well plates (1000 cells/well), followed by incubation of 48 h, 96 h and 144 h at 37°C. The cell count kit-8 (CCK-8) (Dojindo, Shanghai, China) was used to detect cell proliferation. After the addition of CCK-8 solution, another 2 hours of incubation was carried out before the measurement of absorbance at 450 nm with a microtiter platereader (Quant BioTek Instruments), and the cell survival rate was expressed as the

absorbance relative to that of the control group. Three more times of the detection of absorbance were also carried out in the next 2, 4 and 6 days respectively. The experiment was repeated for three times under the same conditions.

Analysis of cell cycle progression via flow cytometry

After the infections with Lv5-ESR1 or Lv5-NC, BMSC were seeded in 6-cm dishes with the concentration of 10⁵ cells/dish. The following incubation lasted for 40 h and subsequently the cells were washed with cold PBS on ice. Whereafter, 75% cold ethanol was used to fix the cells at 4°C overnight. The cells were then resuspended in the mixture of 50 mg/ml propidium iodide (PI; Sigma-Aldrich) and 100 mg/ml DNase-free RNase and cultured for half an hour in dark at 37°C. The well stained cells were then collected by filtering the suspension through a 50- μ m nylon mesh and detected by flow cytometry (BD Biosciences). The percentages of cells in the

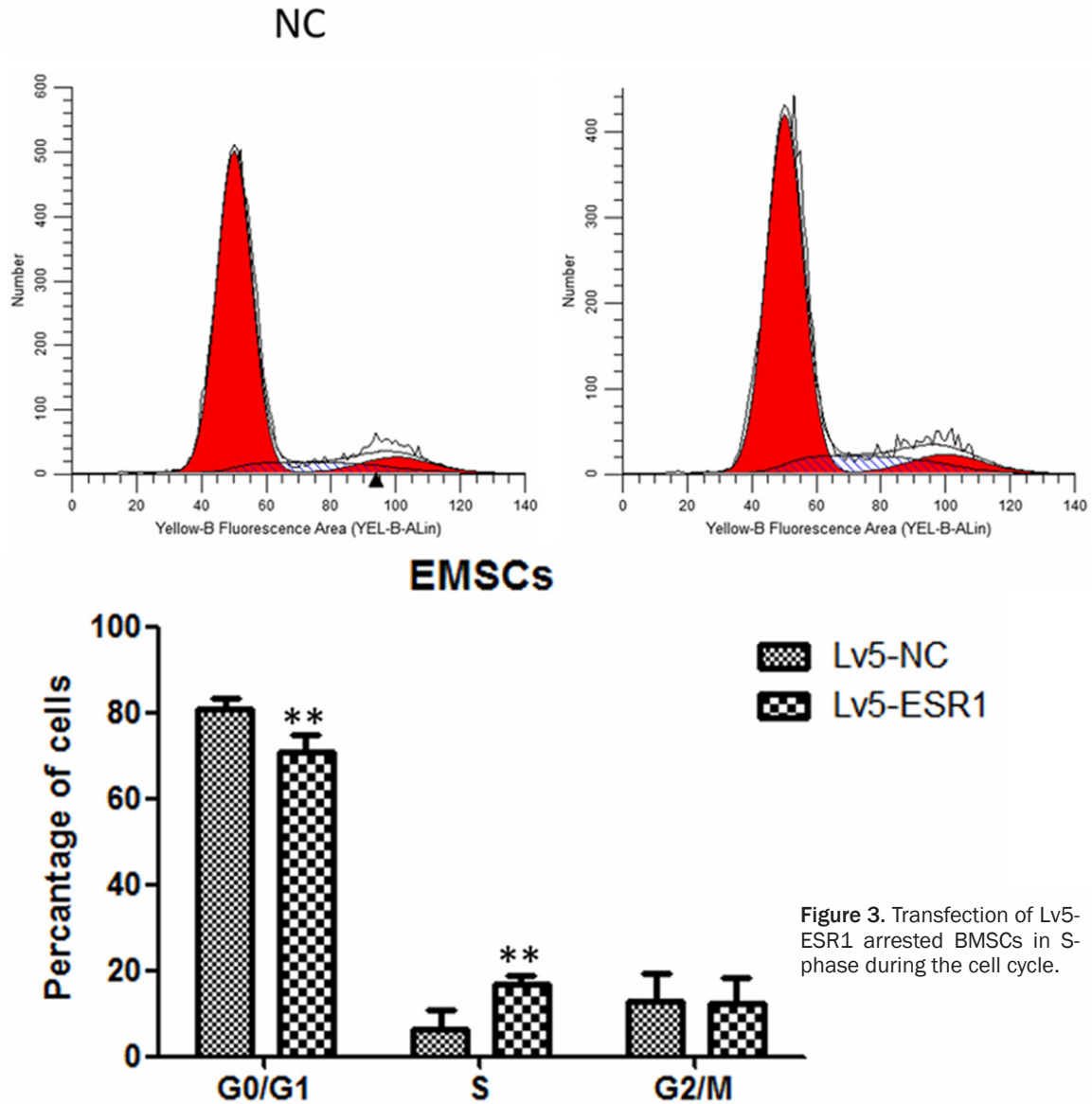


Figure 3. Transfection of Lv5-ESR1 arrested BMSCs in S-phase during the cell cycle.

G0/G1, S, and G2/M phases were also determined respectively via Cell Quest acquisition software (BD Biosciences).

Promotion of the migration and invasion abilities of infected BMSCs

Transwell assay was used to investigate the migration ability of BMSCs overexpressing ESR1. Transwell chambers with polycarbonate membranes of 8.0 μm pore size (Corning Costar Corp., Cambridge, MA, USA) were obtained firstly. Whereafter, BMSCs were suspended in upper transwell chambers containing F-12 solution with 0.2% BSA (serum free) as culture medium. On the other hand, all the upper transwell chambers were put in corre-

sponding lower chambers filled with F-12 supplemented with 10% FBS and cultured at the atmosphere of 37°C with 5% CO₂, overnight. At the end of the incubation, the cells remained in upper chambers were discarded. The crawl over cells on the lower surfaces of the membranes then underwent 10-minute fixation by 4% paraformaldehyde, 30-minute staining by 0.1% crystal violet, and the rinse in PBS before being counted and photographed under a microscope (20 \times objective lens). The experiments were performed in triplicate.

Quantitative real-time PCR

The mRNA expression levels of ESR1 and some selected genes were examined via qRT-PCR

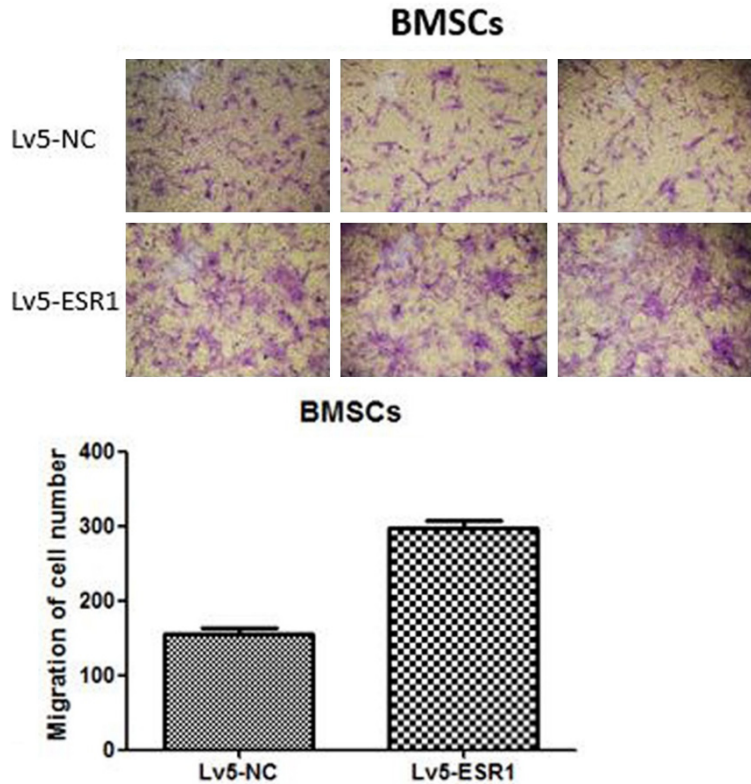


Figure 4. Migration ability of BMSCs was significantly increased by the efficient expression of ESR1 according to transwell assay.

(quantitative real-time polymerase chain reaction). The qRT-PCR was carried out as described previously with some minor modifications [17]. The internal control used in this study was β -actin instead of GAPDH. The forward and reverse primers of STYK1 held the sequences of 5'-CAGTGGGAAGGAGGGACTGA-3' and 5'-TG-CAGCCAGTGAAATTGGA-3' respectively. Whereas the sequences of β -actin primers were 5'-GTGGACATCCGCAAAGAC-3' (sense) and 5'-AAAGGGTGTAAACGCAACTA-3' (antisense).

Results

Lentivirus-mediated overexpression of ESR1 in BMSCs

BMSCs cells from SD rats were isolated to explore the function of ESR1 in BMSCs. The BMSCs were firstly transfected with GFP-tagged-Lv5-NC and GFP-tagged-Lv-ESR1 respectively. The transfection results were observed through the expression of GFP under a fluorescent microscope (**Figure 1A**). The relative mRNA level of ESR1 was increased dramati-

cally by more than 120% according to the qRT-PCR results in BMSCs (**Figure 1B**). Meanwhile, the results of western blot confirmed the high expression efficiency of Lv5-ESR1 in BMSCs on protein levels (**Figure 1C**).

Proliferation ability of BMSCs was enhanced by Lv5-ESR1

Given the high expression efficiency of Lv5-ESR1, the prolificacy of infected BMSCs was further analyzed via CCK-8 assay. In accordance with **Figure 2**, it was indicated that BMSCs infected with Lv5-shESR1 showed much faster proliferation rate comparing with the cells infected with Lv5-NC. This result suggested that proliferation ability of BMSCs were significantly enhanced by Lv5-ESR1 transduction, reflecting the essential role of ESR1 in growth of BMSCs.

Overexpression of ESR1 induced S-phase arrest in BMSCs

Based on the result of CCK-8 assay, further studies were carried out to explore the mechanism ESR1 affect the BMSCs growth. FACS (fluorescence-activated cell sorting) analysis was performed to detect the specific function of ESR1 in BMSCs cell cycle progression. As is shown in **Figure 3**, there was an obvious increase of cell population in S-phase and decrease in G0/G1 stage during the cell cycle (** $P < 0.01$) after the infection of Lv5-ESR1 in BMSCs. This result demonstrated that ESR1 is involved in cell cycle maintenance and regulation and the higher expression ESR1 could promote the proliferation of BMSCs.

The migration ability of BMSCs could be increased by overexpression of ESR1

Since migration is an indispensable feature of BMSCs, the change that overexpression of ESR1 could cause on this ability of BMSCs is necessary to be examined. In this study, tran-

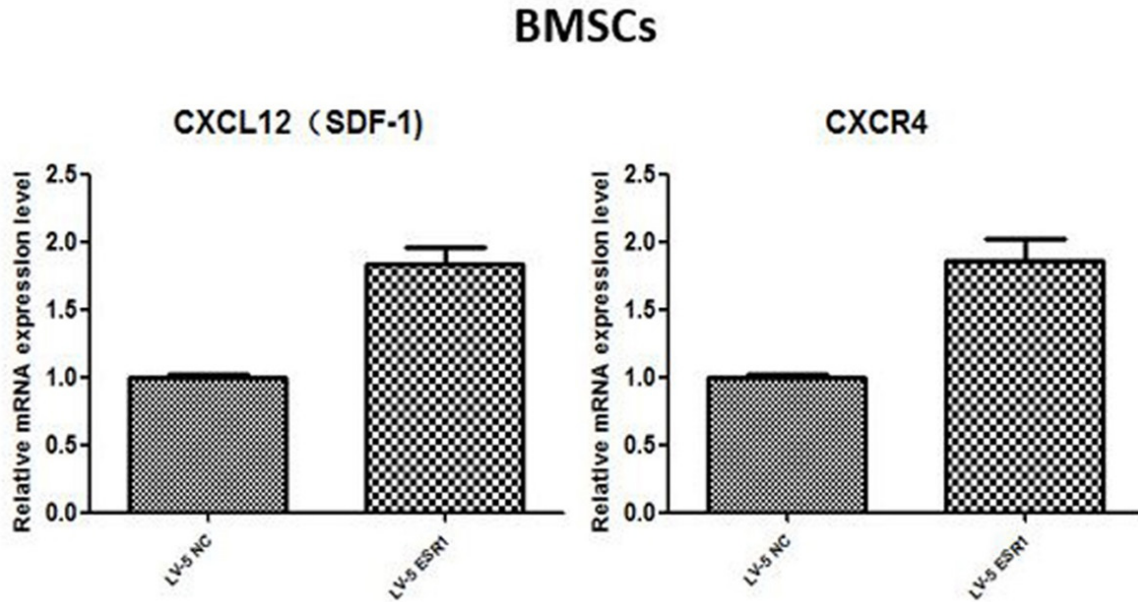


Figure 5. The mRNA expression of both SDF-1 and CXCR4 was significantly increased by the expression of ESR1 in BMSCs.

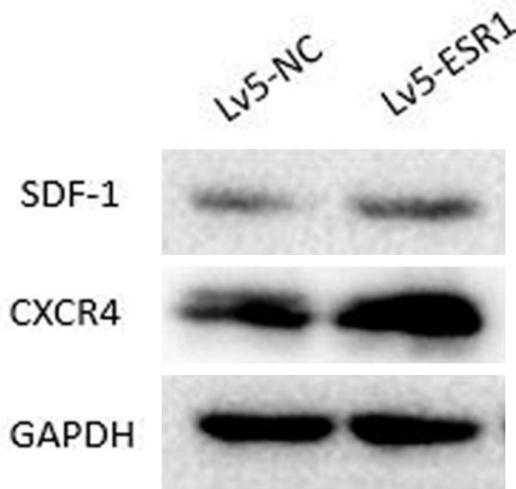


Figure 6. The protein expression of both SDF-1 and CXCR4 was significantly increased by the expression of ESR1 in BMSCs.

swell was used to test the migration ability of the transfected BMSCs. The result was obtained that migration ability of BMSCs was significantly increased by the efficient expression of ESR1 (**Figure 4**, $***P < 0.001$).

The expression levels of SDF-1 and CXCR4 were increased by the expression of ESR1 in BMSCs

The mRNA and protein expression levels of SDF-1 and CXCR4 were examined in both

BMSCs infected with Lv5-NC and Lv5-ESR1. As in shown in **Figures 5** and **6**, the expressions of both SDF-1 and CXCR4 were significantly increased by the expression of ESR1 in BMSCs, which means SDF-1/CXCR4 axis has something to do with the progression of ESR1 regulating the properties of BMSCs.

Discussion

There have been many research studies showing homing of BMSCs and the importance of BMSCs on tissue repair in myocardial infarction [18, 19], neurologic diseases [20, 21], and acute kidney injury [22-24]. Cultured BMSCs also have been infused in humans successfully for treatment of genetic disorders [25, 26], in ischemic cardiomyopathies [27], and in hematological pathologies such as graft-versus-host disease after BMSC transplantation [28]. It also has been reported that transplantation of BMSCs could promote the endometrial cell regeneration, repair endometrial tissues and improve the receptivity of endometrium in rats carrying thin endometrium [13]. At the same time, it was indicated that the polymorphisms of ER α are also closely related to thin endometrium and the expression levels of ER α mRNA and protein are significantly lower in thin endometrium than normal endometrium [15]. Thus, the relationship between the two related facts of thin endometrium drew the interest of us and we investigated their relationship and the

molecular mechanism underlying their correlation.

In order to prove our hypothesis that ESR1 may be a crucial element during the proliferation and migration of BMSCs, a series of experiments were designed and performed and the results are displayed already.

First of all, Rat BMSCs were isolated for further studies in this work. Through the lentivirus-mediated transfection system, ESR1 was significantly expressed in BMSCs on both mRNA and protein levels, which was confirmed by qRT-PCR and western blot and provided a reliable background for the next experiments. Subsequently, the BMSCs infected with Lv5-shNC or Lv5-ESR1 were used to explore the correlation between the expression of ESR1 and cell proliferation. In accordance with the results of CCK-8 proliferation assay that expression of ESR1 could seriously improve the normal proliferation of BMSCs, the important role of ESR1 in BMSCs cell life has been established. Furthermore, the analysis about the relationship between ESR1 and BMSCs cell cycles demonstrated that ESR1 is a pivotal regulator during the cell cycle, which could explain the mechanism ESR1 expression influences cell proliferation to some extent. In order to reveal the pathway which ESR1 regulates the cell cycle through, the expressions of some more key cell cycle proteins in BMSCs transfected with Lv5-shNC or Lv5-shESR1 were detected by western blot and compared.

Stromal cell-derived factor-1 (SDF-1) and its membrane receptor C-X-C chemokine receptor type 4 (CXCR4) are involved in the proliferation and migration of multiple stem cell types which are essential for healthy endometrium. As was indicated that the expression levels of SDF-1 and CXCR4 both increased in BMSCs expressing ESR1. Combining with the functions of these proteins, it could be speculated that expression of ESR1 affected ICC cell growth via upregulating their expressions. In line with the reported information, expression of ESR1 increased the expression of SDF-1 and CXCR4 helping to maintain healthy endometrium which could be reflected from our results that the proliferation ability of BMSCs was promoted.

Meanwhile, the effect of ESR1 on ability of migration was also detected via transwell assay to get the expected results that migration of

BMSCs was enhanced by expression of ESR1 in BMSCs, and this discovery is significant evidence showing that ESR1 could be a molecular target during the therapies through BMSCs.

Taken together, all of our data and results point to one point that ESR1 is crucial in various aspects and stages of BMSCs growth and the expression of STYK1 could help BMSCs develop and migrate to regenerate injured tissues.

Disclosure of conflict of interest

None.

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