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Fibronectin chorused cohesion between endothelial progenitor cells and mesenchymal stem cells of mouse bone marrow

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Abstract

Endothelial progenitor cells (EPCs) could function as niche cells to promote self-renewal of mesenchymal stem cells (MSCs) in the mouse bone marrow. Cohesion was the basis of the two cells to display their biological functions to each other. In this study, we investigated the mechanism of cohesion between MSCs and EPCs. And demonstrated that fibronectin (FN) in EPCs activated the integrin α5β1 of MSCs and further mediated cell–cell cohesion. Integrin α5β1 and its FN ligand played critical roles not only in single-cell line adhesion, but also in adhesion between stem and niche cells. This novel finding is important to understand the cross-talk between MSCs and their niche cells.

Key words: Endothelial progenitor cells, Mesenchymal stem cells, Cohesion, Stem cell niche, Fibronectin, Integrin.

Introduction

Mesenchymal stem cells (MSCs), which are mononuclear fractions derived from bone marrow (BM), are pluripotent cell lines with a fibroblast-like morphology (1). MSCs can differentiate into several mesodermal cell lineages, such as osteoblasts, chondrocytes, and adipocytes (2). MSCs maintain their multipotent and self-renewing properties by inhabiting close to various cells and substances to form a special microenvironment known as stem cell niche (3,4).

We previously reported that MSCs neighbored to $CD31^+$ endothelial progenitor cells (EPCs) formed cell clusters in the blood sinus of the BM cavity. As possible components of the stem cell niche, EPCs can promote the pluripotency of MSCs, particularly self-renewal (5) and multi-differentiation to osteoblasts, chondrocytes, and adipocytes (data not published). This process influences migration of EMCs and their adhesion to EPCs, but the underlying mechanism remains unclear.

Fibronectin (FN) is a major component of the extracellular matrix (ECM) derived from mature BM and other organs (6). Viability, adhesion, spreading, and migration of various cells are regulated by their interaction with FN. MSCs cultured on FN coating exhibited improved adhesion, spreading, and migration ability (7-9). FN as a coating matrix also improved EPC culture collection and reduced EPC colony appearance (10). In our recent study, MSCs and EPCs secreted FN through the autocrine pathway. Thus, investigation on the effect of FN on cohesion between MSCs and EPCs may contribute to mechanistic understanding of the function of ECM interactions in stem cell–niche adhesion. Given that cell–ECM adhesion is mediated by integrins, which can activate many intracellular signaling pathways (11), we hypothesize that the FN–integrin signaling may be the critical step in the cross-talk between MSCs and the surrounding niche cells. Nevertheless, the ability of FN and integrin to regulate cohesion between MSCs and EPCs in the stem cell niche and the underlying mechanism remain to be elucidated.

Materials and methods

Animal preparation and cell culture

All animals were maintained in the Animal Facility of the Shihezi University. 6-month-old C57BL/6J (wild-type) mice, purchased from Xinjiang Medical University, were used as a cell source. Each cell type was harvested and cultured independently using a similar technique except for the materials and the culture media. All experimental cells were the third generation.

Isolation and culture of murine bone marrow MSCs (BM-MSCs)

BM-MSCs were isolated using a technique reported by our previous study (5). Briefly, bone marrow cells were collected from 6-week-old wild-type male C57BL/6 mice euthanized by cervical dislocation; the cells were cultured with Low Glucose Dulbecco's Modified Eagle Medium (LG-DMEM, gibico, USA.) supplemented with penicillin (100 U/ml, Sigma-Aldrich), streptomycin sulfate (100 μg/ml, Sigma-Aldrich), and 20% lot-selected fetal bovine serum (FBS, Hyclone) at 37°C in a 5% CO_2 humidified incubator. After 72 h of adhesion, non-adherent cells were removed and

adherent cells were cultured an additional 7 d with a single media change. The adherent cells were then retrieved by trypsin digestion. Cells aliquots were incubated for 20 min at 4°C with phycoerythrin (PE)-, fluorescein isothiocyanate (FITC)-, peridinin chlorophyll protein (Per CP)-, and allophycocyanin (APC)- conjugated antibodies against mouse Sca-1, CD29, CD45, and CD11b (Bio-legend). Acquisition was performed on a fluorescence-activated cell sorting (FACS) aria model (BD Biosciences), and analysis was performed using a FACS DIVE software version 6.1.3 (BD Biosciences). The sorted $CD29^+$ $Sca\text{-}1^+$ $CD45^-$ $CD11b^-$ cells were enriched by further culture.

Isolation and characterization of mouse bone marrow EPCs (BM-EPCs)

Bone marrow cells were collected and cultured according to the aforementioned technique. Cell aliquots were incubated for 20 min at 4 °C with phycoerythrin (PE)-, fluorescein isothiocyanate (FITC)-, peridinin chlorophyll protein (Per CP)-, and allophycocyanin (APC)-conjugated antibodies against mouse CD133 (+), CD31 (+), CD144 (+), CD11b (-), CD11b- APC, CD11b- PE (Biolegend CA), CD144-PE (BD), Sca-1-PE (Biolegend CA), CD31-PE/Cy7 (Biolegend CA), and CD133-FITC (eBioscience). Acquisition was performed using a fluorescence-activated cell sorting (FACS) Aria model (BD Biosciences), and analysis was performed using a FACS DIVE software version 6.1.3 (BD Biosciences). The sorted CD133 (+), CD31 (+), CD144 (+), and CD11b (-) cells were enriched by further culture in EBM-2 medium (Lonza, USA).

Labeling of MSCs with DAPI

MSCs were incubated under 4', 6-diamidino-2-phenylindole (DAPI, Sigma) with a concentration of 2ul/ml to get over 99% of DAPI labeled MSCs (MSCs-DAPI), when used to co-culture with EPCs in serum-free α-MEM medium for adhesion assay.

MSCs and EPCs adhesion assay

MSCs-DAPI and EPCs were co-cultured in serum-free LG-DMEM medium for 24 h to observe the cohesion. MSCs-DAPI and no labeled EPCs were seeded in 35mm dishes covered with cover slip (1:1, 5×10^4 cells/dish) at 37 °C and 5% CO_2.

Human FN (BD, USA, 20ng/ml) was used to exam the extent of adhesion.

Elisa analysis of FN expression in the culture medium

MSCs and EPCs (1×10^6 cells/dish) were respectively seeded to 60 mm dish with each 2 ml serum- and factors- free medium and cultured for 24 h at 37 °C and 5% CO_2. And then the culture medium was collected and centrifuged for Elisa assay (Uscn, China) followed by the product protocol.

Fluorescent distribution of FN

The cover slips for adhesion were then fixed with 4% paraformaldehyde for 15 min and blocked in 2% BSA for 30 min. Then the cover slips were incubated with monoclonal rabbit anti-mouse FN antibody (abcam, Inc., 1:100) for 4 h at 37 ℃. Followed by washing for 3 times with PBS, secondary FITC conjugated goat anti rabbit IgG (ZSBIO, Beijing, China, 1:100) for a further 1 h. The cover slips were washed and soaked in ddH_2O for 3 times, and examined by Leica fluorescent microscopy (DMIL-LT_2D). Experiments were performed in duplicate and repeated at least three times.

FN-siRNA transfection assay

The MSCs and EPCs were suspended by treatment with 0.05% trypsin/0.02% EDTA (Gibco), and about 5×10^5 cells in 2ml antibiotics-free medium were seeded into 35mm dish and grown for 24 h to about 90% confluent. All transfection reagents (Lipofectamine 2000 (Invitrogen, USA), opti-MEM reduced serum medium, siRNA (sc-35371) and control siRNA-A (sc-35371) were purchased from Santa Cruz Biotechnology. Inc, USA. FN siRNA were as follows (GALIG HSS 149595, Invitrogen): 50-CUU UCU UCC CAA AUC UGC UGC UGG A-30; 50-UCC AGC AGC AGA UUU GGG AAG AAA G-30. The MSCs and EPCs were transfected by 6.6 nM FN siRNA, the negative siRNA control group was transfected by 6.6 nM control siRNA-A and the mock group was transfected without siRNAs using Lipofectamine 2000 following the instructions from manufacturer to visualize how effectively siRNAs were being delivered to MSCs and EPCs. For transfection carried out in 35mm culture dishes, the medium was 1 ml per dish. 6.6 µl of siRNA or 4 µl of lipofectamine 2000 was added to 100 µl of opti-MEM reduced serum medium (in separate RNase-/DNase- tubes) and allowed to incubate for 5 min at room temperature. The mixtures were then combined and incubated at room temperature for an additional 15 min and then added to the dish for 48 h transfection. After the transfection, mixture was replaced with fresh complete culture medium for another 24 h, and then cells were further performed Real-time-PCR detection of FN mRNA expression. Experiments were performed in duplicate and repeated three times.

Real-Time-PCR (RT-PCR) analysis

The MSCs and EPCs were cultured respectively (1×10^6 cells/dish) in the serum- and factors- free medium for 24 h, and then the medium was collected and filtered (0.22µm, MILLEX) for further application. 1×10^6 MSCs/dish and EPCs/dish were prepared for this step. Both cells lines were devided into two groups: the non-cultured serum- and factors- free medium (Negative) and the cultured, reclaimed and filtered medium (Test). 24 hours after harvest with the above medium at 37°C in a 5% CO_2 humidified incubator.

Total RNA of MSCs and EPCs were extracted using Trizol Reagent (Invitrogen, USA) and the purity of RNA was determined by the ratio of absorbance readings at 260 and 280 nm (A260/A280, Nanodrop 2000, Thermo Fisher) where 1.8–2.0 was considered proper for cDNA synthesis. Total RNA (200 ng) was reverse transcribed using a QuantiTec Reverse Transcription Kit (Qiagen). The levels of mRNA expression were determined by real-time PCR using Biosystems 7300 system SDS software. Data were collected after each annealing step. GAPDH was used as an endogenous control to normalize for differences in the amount of total RNA in each sample. The primer sequences and the sizes of the amplified fragments were as follows: FN (117 bp) 50-ACC ACC CAg AAC TAC gAT gC-30 (sense), 50-CAT gCT

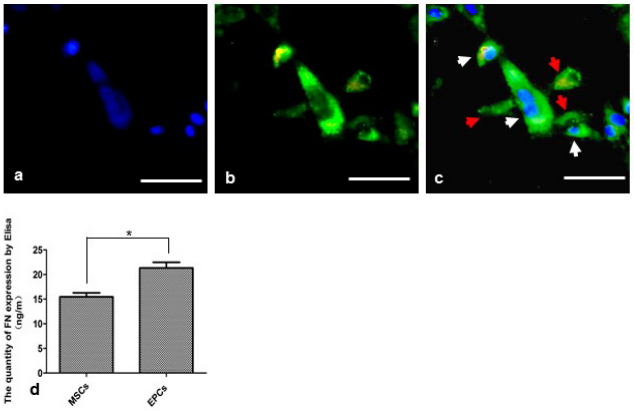

Figure 1. The FN played a role in adhesion between the MSCs and the EPCs in vitro co-culture with serum-free DMEM medium for 24 h. a Fluorescent image of the DAPI staining of MSCs cultured with EPCs in vitro. **b** Fluorescent image of expression of FN in MSCs and EPCs in vitro. The MSCs and EPCs all expressed FN in the cytoplasm and the cytomembrane. **c** Merge of **a** and **b.** The red arrows indicated the no labeled EPCs; the wite arrows indicated the DAPI labeled MSCs. This image showed that the MSCs and EPCs adhered to each other, and both of them expressed FN. *Scale bar*: 100μm. **d** The Elisa result of FN expression of MSCs and EPCs. All of them expressed FN in different levels. The expression difference of MSCs and EPCs showed statistical significance by *t* test (*$p <$ 0.05).

gCT TAT CCC ACT gA-30 (anti-sense); α5β1 (154 bp) 50-gAA Tgg CgA Agg AAA CTC Tg-30 (sense), 50-AAC TgA gAC TgC Tgg gTg-30 (anti-sense); β-actin (23 bp) 50-gTg CTA TgT TgT TgC TCT AgA CTT Cg-30 (sense), 50-ATg CCA CAg gAT TCC ATA CC-30 (anti-sense). Experiments were performed in triplicate and repeated three times.

Western blotting

MSCs and EPCs preparation was the same as the PCR process. Protein samples were extracted from MSCs and EPCs using RIPA with PMSF (Thermo Fisher, USA). The concentration of protein was measured by BCA (Thermo Fisher, USA) method. Samples were run at 6% SDS-PAGE gel, and then transferred to nitrocellulose membranes, and immunoblotted overnight at 4℃ with primary antibody, monoclonal rabbit anti-mouse FN (abcam, Inc. 1:2000) with gentle shaking. After washing, the membrane was incubated with horseradish peroxidase conjugated secondary antibodies and detected by enhanced chemiluminescence assay. GAPDH was used as an endogenous control to normalize for differences. Gel-Pro Analyzer 4.0 was used to analyze the western result and IOD was acquired. Experiments were performed in triplicate and repeated three times.

Statistics

Results are expressed as means ± SEM. Statistical significance was assessed using Student's unpaired t-tests and one-way analysis of variance (ANOVA) followed by *t* test. A probability value of less than 5% was considered significant.

Results

FN expression in adhered MSCs and EPCs in vitro

We previously reported that MSCs and EPCs adhered to each other in the BM cavity and in in vitro culture. Adhesion assay and fluorescence observation were performed to detect whether FN plays a role in cohesion between MSCs and EPCs. The upper panel of Fig. 1 shows that MSCs and EPCs adhered to each other. Both cell lines expressed FN, which is located in the cytoplasm and cytomembrane. We quantified the expression of autocrine FN in MSCs and EPCs cultured in serum- and factor-free media through ELISA. Both MSCs and EPCs expressed FN at different levels (Fig. 1), but FN expression was statistically significantly higher in EPCs than that in MSCs (*t*-test, $P < 0.05$). This result indicates that the two cell lines can secrete FN, which exists because of the autocrine action.

Knock-down of FN mRNA expression by siRNA in MSCs and EPCs

We determined whether FN participates in cohesion between MSCs and EPCs by evaluating FN siRNA. We

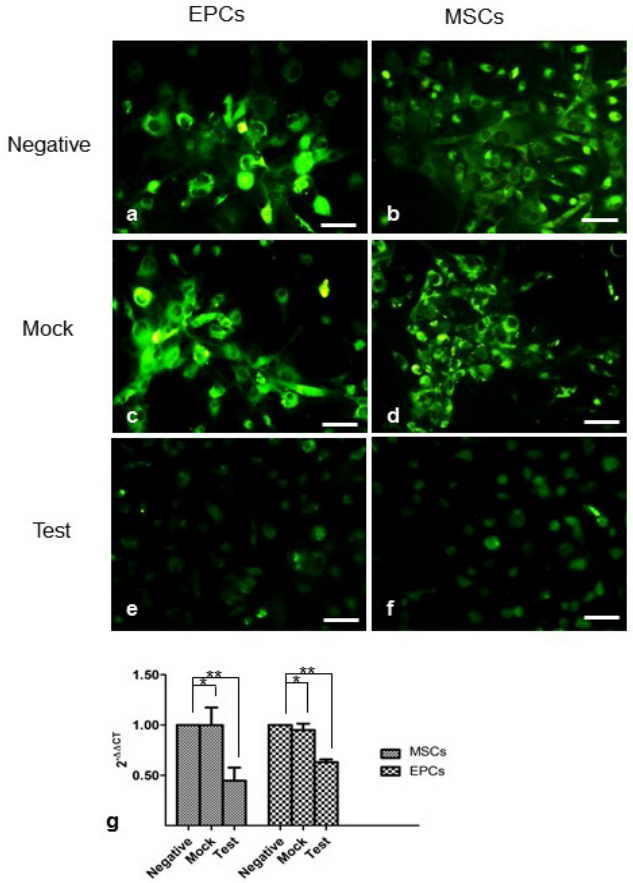

Figure 2. Knock-down of FN expression by siRNA in MSCs and EPCs. a,b the fluorescent images of the FN expression after 24 h of negative siRNA transfected for 48 h, almost of the MSCs and the EPCs were green fluorescent, which meant the FN was normal expressed in both cell lines, and adhesion occured. **c,d** showed the FN expression in the lipofectamine 2000 for 48 h, both MSCs and EPCs were fluorescent, and adhesion occured, whcih indicated that the lipofectamine 2000 did not effect the expression and adhesion for both cells. **e,f** showed that FN expression was significantly reduced after 48 h siRNA transfection. And most importantly, the adhesion of MSCs and the EPCs also inhibited. (Scale bar: *50μm*) **g** RT-PCR results of FN mRNA expression, which indicated that it was highly inhibited by FN siRNA. Differences were statistically significant by one-way analysis of variance (ANOVA) followed by *t* test (** $P <$ 0.05). The FN mRNA expression in the mock group was not severely affected, the difference didn't have significance (* $P > 0.05$).

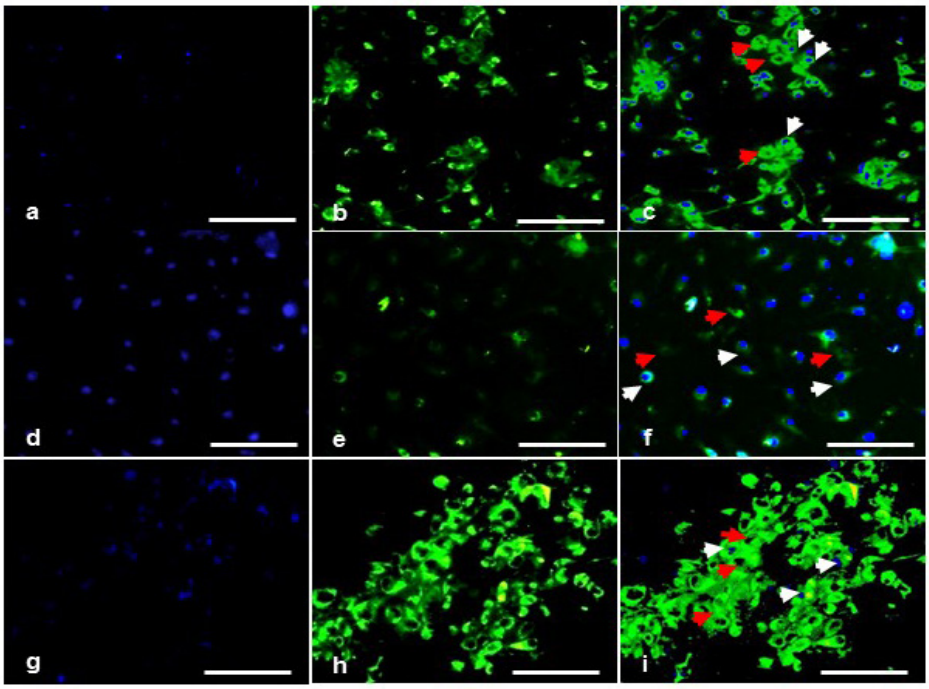

Figure 3. Adhesion phenomena between the DAPI labeled MSCs and the no labeled EPCs in vitro co-cultured for 12 h after FN siRNA transfection for both cell lines. a, b, c Fluorescent image of the DAPI staining of MSCs normally cultured with EPCs in vitro. The red arrows indicated the no labeled EPCs; the wite arrows indicated the DAPI labeled MSCs. This image showed that the MSCs and EPCs adhered to each other, and both cells expressed FN. As seen in the fluorescence intensity of **d, e, f,** the FN expression of MSCs and the EPCs were obviously decreased after 24 h transfection with FN siRNA for 48 h. More importantly, the adhesion between the MSCs and EPCs were highly inhibited, which indicated that the FN may possibly play a role in the adhesion process. **g, h, i** We further added the exogenous FN into the co-culture medium, and found that the adhesion of the MSCs and EPCs was enhanced, though the FN expressions of the MSCs and EPCs were not significantly increased. Scale bar: *200μm.*

knocked down FN expression with siRNA to investigate cohesion of both cell lines. Immunofluorescence and RT-PCR were conducted to detect transfection efficiency. In Figs. 2a and b, the fluorescent images of the negative group showed that most MSCs and EPCs displayed green fluorescence after 48 h of incubation. This result indicates that both cell lines expressed FN and underwent adhesion. As shown in Figs. 2 c and d, FN was expressed in the mock group, MSCs and EPCs were fluorescent, and adhesion occurred, which indicate that Lipofectamine 2000 did not affect the expression and adhesion of both cell lines. After 48 h of transfection and 24 h of normal culture (Fig. 2e and f), FN expression was significantly down-regulated and adhesion of MSCs and EPCs was inhibited in the siRNA-transfected group (test). We further examined the mRNA expression of FN through RT-PCR. As shown in Figs. 2g and h, the FN mRNA expression of both cell lines was inhibited by FN siRNA. Both changes were found to be significant by using one-way ANOVA, followed by *t*-test ($P < 0.05$). Hence, FN expression was successfully knocked down as evidenced by the significant change in the mRNA and protein levels.

FN function in adhesion between MSCs and EPCs in vitro

After knocked down of FN expression in MSCs and EPCs, we assessed adhesion of the two cell lines through adhesion assay. Figs. 3a–c show normal DAPI-labeled MSCs and unlabeled EPCs co-cultured in serum-free LG-DMEM for 24 h. MSCs and EPCs adhered to each other and expressed FN. The fluorescence intensities presented in Figs. 3d–f demonstrated that FN expression in MSCs and EPCs was down-regulated 24 h after transfection with FN siRNA for 48 h. Adhesion between MSCs and EPCs was highly inhibited, indicating the possible participation of FN in the adhesion process. We then added exogenous FN into the co-culture medium and found that adhesion of MSCs and EPCs was enhanced, but FN expression was not significantly up-regulated in both cell lines (Figs. 3g–i).

Upregulation of the mRNA expression of FN in EPCs and integrin α5β1 in MSCs after culture with LG-DMEM and EBM-2, respectively

Cultured/non-cultured serum- and factor-free LG-DMEM and EBM-2, designated as the test and negative groups, respectively, were used to interfere the opposite cells to determine the mutual effects between MSCs and EPCs (Fig. 4). The cells were then prepared for RT-PCR. The mRNA expression of FN in EPCs was significantly up-regulated by LG-DMEM cultured with MSCs compared with that in the non-cultured LG-DMEM ($P < 0.05$, Fig. 4a). The cultured EBM-2 did not up-regulate FN mRNA expression in MSCs ($P < 0.05$, Fig. 4a). The integrin α5β1 mRNA expression in MSCs was also significantly up-regulated by the cultured EBM-2 with EPCs compared with that in the non-cultured EBM-2 ($P < 0.05$, Fig. 4b). The cultured LG-DMEM significantly down-regulated the mRNA expression of α5β1 in EPCs ($P < 0.05$, Fig. 4b).

Up-regulation of FN in EPCs and down-regulation of FN in MSCs cultured in LG-DMEM and EBM-2

Western blot results revealed that FN expression in MSCs incubated in the cultured EBM-2 was down-regulated compared with that in the cultured medium ($P < 0.05$, Fig. 5a). FN protein expression in EPCs was

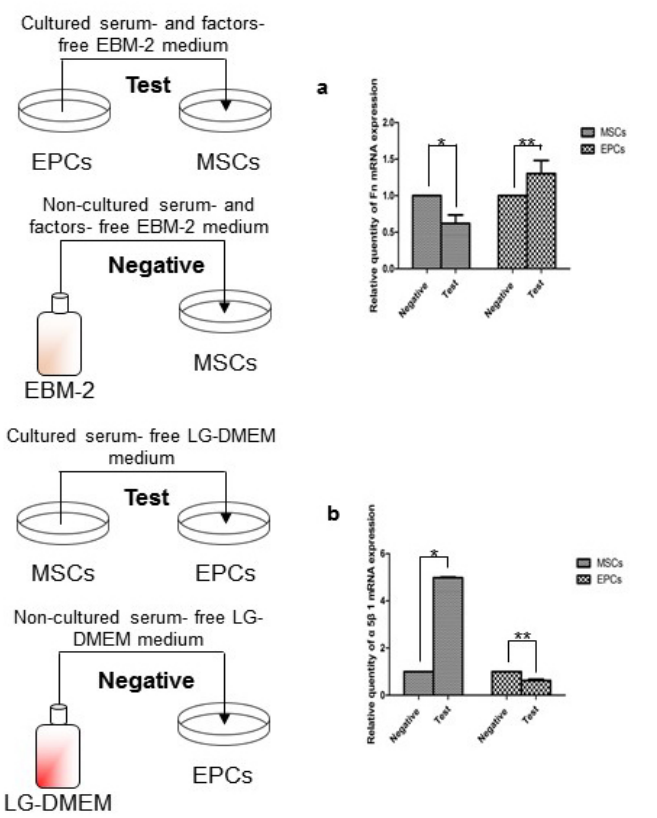

Figure 4. FN of EPCs and integrin α5β1 of MSCs were up-regulated by respective cultured LG-DMEM/EBM-2 mediums. We further applied the serum- and factors-free culture medium to incubate the relative cells, and then the mRNA expression of FN and integrin α5β1 was detected by RT-PCR. **a** The mRNA expression of FN in MSCs and EPCs. The cultured serum- and factors- free EBM-2 medium (Test) did not increase the FN mRNA expression of MSCs (*P < 0.05). The cultured serum- and factors- free LG-DMEM medium (Test) increased FN mRNA expression of EPCs (**P < 0.05). **b** The mRNA expression of the α5β1 in MSCs and EPCs. The cultured serum- and factors- free EBM-2 medium (Test) increased the α5β1 mRNA expression of MSCs (*P < 0.05). The cultured serum- and factors- free LG-DMEM medium (Test) decreased the α5β1 mRNA expression of EPCs (**P < 0.05). All the Test groups were compared with the Negative groups (Non-cultured serum- and factors- free LG-DMEM/EBM-2 medium).

up-regulated in the cultured serum- and factor-free LG-DMEM compared with that in the non-cultured medium (P < 0.05, Fig. 5b).

Discussion

Various populations of stem cells reside in the body and undergo continuous self-renewal and differentiation throughout their lifespan. The complex microenvironment inhabited by stem cells consists of cells, ECMs, and signaling molecules associated with each stem cell lines, and this microenvironment is collectively referred as stem cell niche (12). The stem cell niche is an interactive structural unit that facilitates cell-fate decisions in a spatiotemporal manner. Key signaling and molecular cross-talk events are patterned to occur at the right place and time (13). One of the most convenient, and the most reliable methods is to anchor stem cells in their niche using adhesion molecules. Stem cells present various communication mechanisms with the surrounding niche cells and other molecules through different adhe-

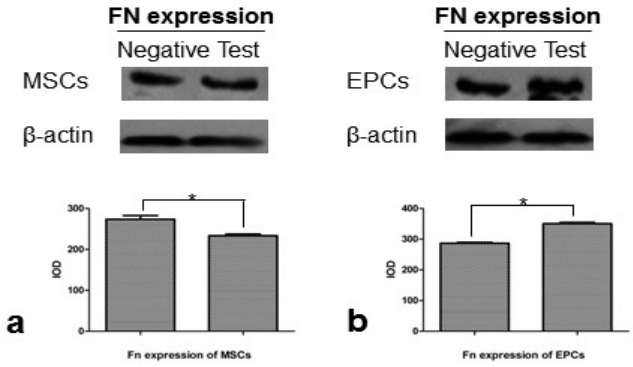

Figure 5. FN of EPCs were up-regulated, and FN of MSCs were down-regulated by respective cultured LG-DMEM/EBM-2 mediums. **a** Western blotting results of FN expression in MSCs incubated with cultured (Test)/non-cultured (Negative) serum- and factors- free EBM-2 medium. The FN protein expression in the Test was decreased than that in the Negative (*P < 0.05). **b** Western blotting results of FN expression in EPCs incubated with cultured (Test)/non-cultured (Negative) serum- and factors- free LG-DMEM medium. The FN protein expression in the Test was increased than that in the Negative (*P < 0.05).

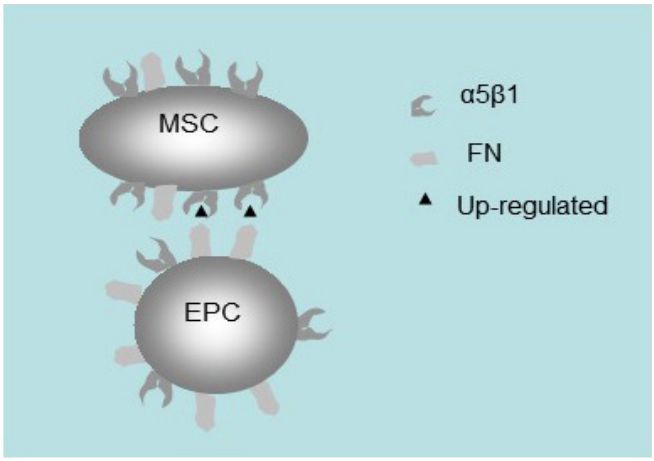

Figure 6. Demonstration of MSCs and EPCs cohesion may through FN of EPCs activated α5β1 of MSCs. Combined with the results of the Elisa, immunofluorescence, RT-PCR and western blotting, it was deduced that the autocrine FN chorused the cohesion of MSCs and EPCs. The autocrine FN of EPCs occupied the main position during this process, and may through the activation of α5β1 in MSCs to accomplish the cohesion, and further to exert their biological functons. The black arrows indicated that the FN of EPCs activated and upregulated the α5β1 of MSCs.

sion proteins and signals (14-18). Given that the two most important molecules are the Cadherin and Integrin families, which are not independent but coefficient.

As a universal component of the ECM, FN connects to cells through integrins and other receptors to regulate cell adhesion, migration, and differentiation (19). FN is secreted as a large dimeric glycoprotein with subunits ranging from 230 kDa to 270 kDa. FN-binding integrins exhibit specificity to one of the two cell-binding sites within FN. Integrin is a transmembrane glycoprotein that forms functional αβ-heterodimeric receptors for ECM ligands or cell membrane-anchored adhesion molecules (20-25). Integrin activation initiates intracellular signaling, which regulates numerous cellular functions, including migration, cell proliferation, survival, and apoptosis (26). Integrin α5β1 is the primary receptor for the ECM protein FN, which is found in most cells (27). Integrin α5β1 and its FN ligand play critical roles

in blood vessel development in mouse embryos (28). Integrinα5β1 and FN also promote polymerization and assembly of FN into the matrix (19). The FN matrix assembly influences tissue adhesion (29-31), and integrin α5β1 and its FN ligand exhibit critical roles in cell adhesion (29).

As we previously found that EPCs could be a niche component of MSCs in the bone marrow, we considered EPCs as niche cells in the present study. We demonstrated that FN mediated cohesion between MSCs and EPCs in the BM stem cell niche. The results are consistent with other studies on FN-integrin signaling in other cell lines. MSCs cultured in LG-DMEM up-regulated FN mRNA expression, and EPCs cultured in EBM-2 up-regulated the integrin α5β1 mRNA expression. From the immunofluorescence results, MSCs and EPCs cohered to each other but this cohesion was inhibited by FN knock-down. Thus, FN plays a role in the cohesion between MSCs and their EPC niche cells. We also deduced that it was the FN of EPCs to activate the integrin α5β1 of MSCs to mediate the cohesion of the two cells. As demonstrated in Fig. 6, FN in EPCs activated the integrin α5β1 of MSCs and further mediated the cohesion. This novel finding is important to understand the cross-talk between MSCs and their niche cells. MSC is a useful tool for developing strategies for regenerative cardiovascular tissue engineering (32-36). In addition to creating cardiac grafts, MSCs have been extensively investigated and used in tissue repair and immune disorder therapy (37-40). This study also provides supplementary information for future "stem cell niche" transplantation to improve the function of MSCs.

FN consists of three different types of modules, namely, types I, II, and III repeats, and each repeat is distinct and contains many domains. The integrin family, which consists of α and β subunits, also comprises several domains. Given that distinguishing the FN domain that corresponds to a specific integrin domain remains challenging, and further investigations must be conducted.

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