NR2F2 regulates bone marrow-derived mesenchymal stem cell-promoted proliferation of Reh cells

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Abstract. Bone marrow-derived mesenchymal stem cells (BM-MSCs) are pivotal components of the leukemic microenvironment. BM-MSCs have been previously reported to promote the proliferation of leukemic cells. To further understand the molecular mechanisms of BM-MSC-induced proliferation of leukemic cells, the present study co-cultured acute lymphoblastic leukemia (ALL) Reh cells with BM-MSCs. The current study used methods including shRNA, flow cytometry, MTT, reverse transcription-quantitative polymerase chain reaction, ELISA and western blotting. The data of the present study demonstrated that BM-MSCs promote the proliferation of Reh cells and the NR2F2 mRNA and protein levels were elevated in BM-MSCs following co-culture. Additionally, it was demonstrated that shRNA knockdown of NR2F2 inhibited BM-MSC-induced proliferation of Reh cells. Furthermore, following downregulation of NR2F2, vascular endothelial growth factor A (VEGFA) secretion by BM-MSCs was reduced. The present study demonstrated that NR2F2 mediates BM-MSC-induced proliferation of Reh cells, partially via regulation of VEGFA. Disrupting microenvironmental support by targeting NR2F2 may be a potential therapeutic strategy for ALL.

Introduction

Acute lymphoblastic leukemia (ALL) is a malignancy derived from cells of the lymphoid lineage. Despite good clinical outcomes for patients with childhood ALL, adult ALL remains clinically challenging due to a high rate of recurrence (1). It has been previously reported that the overall 5-year survival rate for patients with ALL recurrence is 7% (2). Previous studies have investigated the importance of the bone marrow microenvironment on ALL recurrence (3).

The bone marrow microenvironment is composed of various cell types and abundant extracellular matrix (4). Previous studies have demonstrated that stromal cells promote the proliferation and survival of leukemic cells by secreting soluble chemicals and adhesion molecules, including chemokine (C-X-C motif) ligand 12, IL (interleukin)-7 (5), vascular endothelial growth factor A (VEGFA), platelet derived growth factor (PDGF) (6), and via the integrin β1/cluster of differentiation (CD)44 axis (7). Stromal cell-induced proliferation may be associated with the development of minimal residual disease, which is considered to be an independent prognostic factor in ALL (8). Thus, it is necessary to investigate the mechanisms of bone marrow microenvironment-induced proliferation in leukemic cells. Mesenchymal stem cells (MSCs) are important components of the solid and hematologic tumor microenvironment (9). The present study investigated the bone marrow microenvironment-mediated proliferation of leukemic cells using bone marrow-derived (BM)-MSCs.

Nuclear receptor subfamily 2 group F member 2 (NR2F2) is a member of the nuclear receptor superfamily widely expressed in the mesenchymal compartment of developing organs (10). NR2F2 expression was previously reported to be abundant in MSCs and involved in the modulation of cell differentiation (11). NR2F2 has been predominantly investigated in solid malignancies, including ovarian and prostate cancer (12). Whether NR2F2 is important in the leukemic microenvironment remains to be investigated. Notably, the present study demonstrated that NR2F2 was upregulated in BM-MSCs following co-culture with Reh cells, which is a representative ALL cell line. The current study aimed to investigate the importance of NR2F2 in BM-MSC-induced proliferation of leukemic cells.

Materials and methods

Generation of BM-MSCs. Human bone marrow samples were collected from normal donors by bone marrow aspiration at The First Affiliated Hospital, College of Medicine, Zhejiang University (Hangzhou, China) and mononuclear cells were isolated by density gradient centrifugation at 400 x g at 20°C.
for 25 min. The current study was approved by the First Affiliated Hospital, College of Medicine, Zhejiang University. The cells were cultured in low-glucose Dulbecco’s modified Eagle’s medium (10-014-CVR; Corning Incorporated, Corning, NY, USA) supplemented with 10% fetal bovine serum (FBS; 10099-141; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) at 37°C and 5% CO2 in a humidified incubator. The medium was replaced after the initial 48 h. Subsequently the medium was changed every 3 days. The adherent cells were passaged when 90% confluence was reached. BM-MSCs at passage 2-6 were used in the subsequent assays.

Characteristics of BM-MSCs. BM-MSCs of passages 3-5 were harvested using 0.25% trypsin-ethylenediaminetetraacetic acid solution (EDTA; 25200-056; Gibco; Thermo Fisher Scientific, Inc.). Cells were then incubated with the following anti-human monoclonal antibodies: CD90-fluorescein isothiocyanate (FITC; 1:20; 11-0909), CD105-phycocyanin (PE; 1:20; 12-1057), CD73-APC (allophycocyanin; 1:20; 17-0739), CD45-FITC (1:20; 11-9459), CD34-PE (1:20; 12-0349), CD19-APC (1:20; 17-0199) and CD11b-PE (1:20; 12-0113) antibodies (eBioscience, Inc., San Diego, CA, USA) at 4°C for 30 min. Following fixing with 75% ethanol (Sinopharm Chemical Reagent Co., Ltd.), permeabilisation with permeabilisation solution (CCS012A; Multi Sciences (Lianke) Biotech Co., Ltd., Hangzhou, China) and blocking with Human BD Fc Block (564219; BD Pharmingen, San Diego, CA, USA), cells were detected using an FC 500 MCL flow cytometer (Beckman Coulter, Inc., Brea, CA, USA) with mouse IgGl K isotype control FITC (11-4714), mouse IgGl K isotype control PE (12-4714), mouse IgGl K isotype control APC (17-4714) isotype-matched antibodies (eBioscience, Inc.) applied as controls. BM-MSCs at passages 3-5 were cultured in osteogenic or adipogenic differentiation medium (HUXMA-90021 and -90031, respectively; Cyagen Biosciences, Inc., Santa Clara, CA, USA) for the differentiation assays. Cells were stained with Alizarin Red or Oil red O solution (HUXMA-90031; Cyagen Biosciences, Inc.) to evaluate osteogenic or adipogenic differentiation, respectively. The cells were analyzed using MultiCycle software. Cells were analyzed using CXP Analysis Software 5 (Beckman Coulter, Inc.).

Cell culture and co-culture. The human ALL Reh cells were obtained from the Cell Bank of Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Shanghai, China), and were divided into cells cultured either alone or co-cultured with BM-MSCs. Reh cells were cultured in Iscove’s modified Dulbecco’s medium (SH30228; Hyclone; GE Healthcare Life Sciences, Logan, UT, USA) supplemented with 10% FBS at 37°C and 5% CO2 in a humidified incubator. For co-culture experiments, BM-MSCs were initially seeded on 6- or 12-well plates at 5x10^5 cells/well density. After 4-6 h of culture, Reh cells were added to the adherent MSCs layer at 5x10^5 cells/ml density. After 48 h of co-culture, Reh cells were collected by directly separating suspending cells as described by previous studies (13,14).

Ki-67 detection by flow cytometry. Forkhead Box P3/Transcription Factor Staining Buffer Set (eBioscience, Inc.) was used for intracellular staining with the anti-human monoclonal Ki-67-PE antibody (12-5699; 1:20; eBioscience, Inc.). Reh cells were collected and washed with phosphate-buffered saline (PBS) 2 times. Following incubation with fixation/permeabilization solution at 4°C for 30 min, Reh cells were washed and incubated with 5 µl Ki-67-PE antibody at 4°C for 30 min. Cells were resuspended in 500 µl PBS for flow cytometric detection using an FC 500 MCL flow cytometer. Ki-67 was detectable during the active phases of cell cycle (G1, S, G2, and M) and not during the resting phase (G0), therefore, the positive cells were considered to be proliferating cells (15).

Apoptosis assay. Reh cells were harvested and washed twice with cold PBS. Cells were resuspended in 100 µl 1X binding buffer provided in the Apoptosis Detection kit (559763; BD Pharmingen, San Diego, CA, USA). Following incubation with the Annexin V-PE antibody and 7-aminocinomycin D from the Apoptosis Detection kit (559763; BD Pharmingen) at room temperature for 15 min, Reh cells were analyzed using the FC 500 MCL flow cytometer. Cells that were Annexin V-positive were considered to be apoptotic.

Cell cycle assay. Reh cells were fixed with chilled 75% ethanol for 2 h at -20°C. Following rehydration in PBS for 15 min, cells were washed twice and resuspended in 1 ml propidium iodide (CCS012A; Multi Sciences Biotech Co., Ltd., Hangzhou, China), then incubated at room temperature for 30 min. Samples were analyzed using the FC 500 MCL flow cytometer. Data were analyzed using MultiCycle software, version 3.2 (Beckman Coulter, Inc.).

Knock-down of NR2F2. A previously reported (16), NR2F2-targeting shRNA (5'-AGTAAACGTGATTGATTCAGTATCTTA-3') was cloned into the pGLV3 plasmid (GenePharm, Inc., Sunnyvale, CA, USA). A scrambled sequence (5'-TTTCTCGAACTGTTCAGTCTTTA-3') was also cloned into the pGLV3 as a negative control. shRNAs were obtained from Shanghai GenePharma Co., Ltd. (Shanghai, China). Highly concentrated lentivirus was produced by co-transfecting 293T cells (Cell Bank of Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences) with pMD2.G (Thermo Fisher Scientific, Inc.), psPAX2 (Thermo Fisher Scientific, Inc.) and shRNA plasmids with Lipofectin (Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. The viral suspension was collected, filtered and used to transfect BM-MSCs directly following removal of medium. After 10 h, virus-containing medium was replaced with fresh medium. The positive expression of green fluorescent protein was assessed by fluorescent microscopy (Eclipse-Ti K11301; Nikon Corporation, Tokyo, Japan) and used to evaluate transfection efficiency.

Cell viability assay. Transfected BM-MSCs were seeded onto a 96-well plate at a concentration of 5x10^4 cells/well. Five parallel wells of cells were seeded for each group (knockdown BM-MSCs group and negative control BM-MSCs group). Following incubation for 48 h, 10 µl 3-(4,5-dimethyl-2-thiazolyl) 2,5-diphenyltetrazolium bromide (MTT; MK-MTT001; Hangzhou Lianke Biology Technology Ltd., Co., Hangzhou, China) was added to all wells. Medium was discarded after 4 h. The optical density (OD) values were evaluated by the SpectraMax M5 microplate reader (Molecular Devices, LLC, Sunnyvale, CA, USA) at 570 nm following addition of 100 µl dimethyl sulfoxide (SinoPharm Chemical Reagent Co., Ltd., Shanghai, China).
Enzyme-linked immunosorbent assay (ELISA). The concentration of VEGFA in the co-culture medium was assessed using an ELISA kit (BMS277/2; eBioscience, Inc.). Briefly, co-culture medium supernatant was collected at 24 or 48 h following separation of Reh cells by concentration using centrifugation at 300 x g at 20˚C for 10 min. An adequate volume of sample was incubated with pre-coated plates according to the manufacturer's protocol. The OD value was detected at 450 nm using the SpectraMax M5 microplate reader. Standard curves were produced using the standards of known concentrations supplied in the kit and used to calculate protein concentrations.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Co-cultured BM-MSCs were isolated by thoroughly washing with PBS until all Reh cells were removed. Total cellular RNA was extracted from co-cultured BM-MSCs using TRIzol reagent (15596-026; Thermo Fisher Scientific, Inc.) PrimeScript RT reagent kit with gDNA Eraser (RR047A; Takara Biotechnology Co., Ltd., Dalian, China) was used for RT. The RT protocol was as follows: 37˚C for 15 min; 85˚C for 5 sec; stored at 4˚C. qPCR was performed using SYBR Premix Ex Taq II (RR420A; Takara Biotechnology Co., Ltd.) according to manufacturer’s instructions in a LightCycler system (Roche Diagnostics, Basel, Switzerland). Each reaction was performed in triplicate. The cycling conditions were as follows: Initial denaturation at 95˚C for 5 min, 45 cycles of 95˚C for 5 sec and 60˚C for 20 sec, melting curve analysis at 95˚C for 15 sec, 65˚C for 15 sec and then maintained at 95˚C. The expression of glyceraldehyde 3-phosphosphate dehydrogenase (GAPDH) was used as a reference gene. The primers used were as follows: NR2F2, forward 5’-CAACCAGCCGAC GAGATT-3’ and reverse 5’-ATTGCTCATGACTGAGG AGGACG-3’; VEGFA, forward 5’-ACAGGTACAGGATG AGGACAC-3’ and reverse 5’-AAGCAGGTGAGTAAGC GAAG-3’; GAPDH, forward 5’-AGAGGCTG GGCTC ATTTG-3’ and reverse 5’-AGGGGCATCCACAGTC TCC-3’.

Western blot analysis. The transfected BM-MSCs were harvested by digesting using 0.25% trypsin-EDTA solution. Cells were lysed on ice for 1 h in radioimmunoprecipitation assay lysis buffer (AR0105; Wuhan Boster Biological Technology, Ltd., Wuhan, China) with phenylmethylsulfonyl fluoride solution (AR1179; Wuhan Boster Biological Technology, Ltd.). Cell lysates were centrifuged at 12,000 x g for 5 min at 4˚C. The supernatant was incubated at 100˚C for 10 min following addition of an appropriate volume of loading buffer (5-fold dilution; Hangzhou Fu De Biological Technology, Co., Ltd., Hangzhou, China). Samples were loaded onto a 10% polyacrylamide gels (Hangzhou Fu De Biological Technology, Co., Ltd.), electrophoretically separated (110 V for 100 min) and transferred onto a nitrocellulose membrane. Membranes were blocked at 20˚C for 2 h with 5% non-fat milk then incubated with the primary antibodies at 4˚C overnight, and then incubated with fluorescent IgG IRDye® 650 goat anti-mouse (1:10,000; 926-65010) and goat anti-rabbit (1:10,000; 926-65020) secondary antibodies (LI-COR, Inc., Lincoln, NE, USA) at room temperature for 1 h. Rabbit monoclonal NR2F2 (1:1,000; #6434) and mouse monoclonal GAPDH (1:1,000; #5079) primary antibodies were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA) and Multi Sciences.
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(Lianke) Biotech Co., Ltd., respectively. Immunoreactive bands were visualized using an Odyssey Infrared Imaging System (LI-COR, Inc.). GAPDH was used as a loading control.

Statistical analysis. All independent experiments were repeated ≥3 times. Statistical analysis was performed using SPSS software, version 19.0 (IBM SPSS, Armonk, NY, USA). Differences between mean values were evaluated using Student's two-tailed t-test. Values are presented as the mean ± standard deviation and P<0.05 was considered to indicate a statistically significant difference.

Results

Characteristics of BM-MSCs. BM-MSCs exhibited a spindle-like shape and fibroblast-like morphology. BM-MSCs were CD90- and CD105-positive, and predominantly CD73-positive. However, the cells were negative for CD19 and CD11b, and hematopoietic markers, including CD34 and CD45. The characteristics of BM-MSCs have been described in a previous report (17).

BM-MSCs promote the proliferation of Reh cells. In order to assess whether BM-MSCs promote Reh cell proliferation, Reh cells were isolated and the expression of the Ki-67 antigen was analyzed. As demonstrated in Fig. 1A and B, Reh cells that were co-cultured with BM-MSCs for 48 h exhibited a significantly increased percentage of Ki-67-positive cells compared with Reh cells cultured alone (P<0.05). The present study also assessed whether BM-MSCs affected the apoptosis of Reh cells. Co-culture with BM-MSCs for 48 h had no observable effect on the apoptosis of Reh cells (Fig. 1C and D). Additionally, the cell cycle pattern of Reh cells was analyzed in the co-culture system. When co-cultured with BM-MSCs, the percentage of Reh cells in the G2/M phase was markedly increased compared with Reh cells cultured alone. Whereas the percentages of cells in G0/G1 or S phases were not significantly different (Fig. 2).

NR2F2 regulates the BM-MSCs-promoted proliferation of Reh cells. To analyze the expression levels of NR2F2 in BM-MSCs, BM-MSCs were separated from Reh cells following co-culture for 48 h. The mRNA and protein levels of NR2F2 in BM-MSCs were observed to be upregulated when co-cultured with Reh cells compared with culture alone (Fig. 3A and B). In order to further elucidate whether NR2F2 is involved in mediating the BM-MSC-induced proliferation of Reh cells, NR2F2 expression was measured in BM-MSCs, and was identified to be downregulated by transfection of an NR2F2 antisense shRNA. Western blot analysis demonstrated that the expression of NR2F2 was significantly knocked down by the shRNA (Fig. 3C). As demonstrated in Fig. 4A and B, the percentage of Ki-67-positive Reh cells in the NR2F2 knockdown BM-MSC co-culture group was significantly reduced compared with the negative control group (P<0.05). Furthermore, the percentage of Ki-67-positive Reh cells in the NR2F2 knockdown BM-MSC co-culture group was similar to the percentage observed when Reh cells were...
cultured alone. Additionally, the cell viabilities of BM-MSCs in the knockdown and control groups were compared to exclude the possibility that silencing NR2F2 affected the cell viability. As demonstrated by the MTT assay, no significant difference in cell viability was observed between the two groups (Fig. 4C). These data suggested that NR2F2 is involved in the regulation of BM-MSC-induced proliferation of Reh cells.

**Downregulation of NR2F2 suppresses the expression of VEGFA in BM-MSCs.** As it has been previously reported that VEGFA provides important proliferative cues for ALL cells in a BM-MSC-conditioned system (6), the present study attempted to evaluate whether the effects of NR2F2 on the co-culture system were mediated by VEGFA. BM-MSCs were isolated from the co-culture after 48 h and the mRNA expression levels of VEGFA were measured in NR2F2 knockdown and negative control cells. Following NR2F2 knockdown, VEGFA was significantly downregulated (P<0.05; Fig. 5A). To further investigate the association between NR2F2 and VEGFA, the concentration of VEGFA in the co-culture supernatant was measured by ELISA at 24 and 48 h. A relatively high concentration of VEGFA was detected in the supernatant of BM-MSCs (Fig. 5B). However, the concentration of VEGFA was barely detectable in the supernatant of Reh cells cultured alone (data not shown). The concentration of VEGFA was significantly reduced in the NR2F2-knockdown group compared with the negative control at 24 and 48 h (P<0.05; Fig. 5C). Thus, these results demonstrated that VEGFA is involved in the mediation of the effects of NR2F2 on BM-MSC-induced proliferation of Reh cells.

Figure 4. Downregulation of NR2F2 inhibits BM-MSCs-mediated increased proliferation of Reh cells. (A) Representative graphs of Ki-67 antigen staining detected by flow cytometry in Reh cells cultured alone or with BM-MSCs, including NC group and NR2F2-KD group. (B) Percentage of Ki-67-positive cells are presented as the mean ± standard deviation in the three groups. (C) The OD values at 570 nm in BM-MSC NC and NR2F2-KD groups are presented as the mean ± standard deviation. *P<0.05. FSC, forward scatter; BM-MSC, bone marrow-derived mesenchymal stem cell; NC, negative control; KD, NR2F2 knockdown.

Figure 5. Down regulation of NR2F2 suppresses the expression of VEGFA in BM-MSCs. Reh cells and BM-MSCs were co-cultured for 24 and 48 h, and additionally NR2F2 was knocked down in BM-MSCs. (A) Relative VEGFA mRNA expression levels in the BM-MSC NC and NR2F2-KD groups were measured by reverse transcription-quantitative polymerase chain reaction following 48 h in co-culture with Reh cells. (B) The protein concentration of VEGFA in the co-culture supernatant of untransfected BM-MSCs was measured at 24 and 48 h. (C) VEGFA concentration was detected in the co-culture supernatant of the NC and KD groups at 24 and 48 h. Values are presented as the mean ± standard deviation. *P<0.05. NR2F2, nuclear receptor subfamily 2 group F member 2; VEGFA, vascular endothelial growth factor A; BM-MSC, bone marrow-derived mesenchymal stem cell; NC, negative control; KD, NR2F2 knockdown.
Discussion

The proliferation and survival of ALL cells has been previously reported to be enhanced by stromal cells (6). The present study demonstrated that following co-culture with BM-MSCs, the percentage of Ki-67 antigen-positive Reh cells was increased, demonstrating that BM-MSCs induced Reh cell proliferation. Additionally, in accordance with a previous study (18), apoptosis was measured and no significant differences were observed between Reh cells cultured alone or those co-cultured with BM-MSCs. Yang et al (13) reported that MSCs induce the proliferation of Reh cells and demonstrated that the percentage of Reh cells in the S and G2/M phases was increased in co-culture conditions. The present study only observed an increased percentage of Reh cells in G2/M phase, no observable changes in the percentage of cells in S phase were detected. Although the data of the present study is different to that of a previous study (13), this may be explained by the fact that the cell cycle assay used in the present study did not distinguish between the G0/G1 phases.

Previous studies have demonstrated that stromal cell-mediated increases in proliferation are primarily caused by cytokines produced by stromal cells, including IL-6, VEGFA and PDGF (6). However, the mechanisms involved in producing these cytokines remain unclear. The data of the present study indicated that NR2F2 levels are upregulated in BM-MSCs co-cultured with Reh cells. Furthermore, downregulation of NR2F2 with shRNA attenuated BM-MSC-induced proliferation of Reh cells, which demonstrates that NR2F2 is involved in this process. The present study also demonstrated that knockdown of NR2F2 did not affect the viability of BM-MSCs after 48 h culture. Therefore, the mechanism of NR2F2-regulated BM-MSC-induced proliferation is not associated with effects on cell viability.

NR2F2 has been previously reported to regulate VEGF/VEGF receptor 2 signaling in endothelial cells (19), thus, the present study hypothesized that NR2F2 participates in the regulation of VEGFA in the co-culture system. The results of RT-qPCR and ELISA assays demonstrated that downregulation of NR2F2 in BM-MSCs inhibited the production of VEGFA. Further research is required to explore the specific mechanisms that mediate the effect of NR2F2 on the production of VEGFA in BM-MSCs. The present study additionally demonstrated that VEGFA is secreted by BM-MSCs and detected in the culture supernatant. However, VEGFA was not detected in the supernatant of Reh cells cultured alone. These data suggest that BM-MSC-induced proliferation of Reh cells is partially mediated by the secretion of VEGFA.

In conclusion, the present study demonstrated that BM-MSCs promote the proliferation of Reh cells and that this process was partially mediated by NR2F2 regulation of VEGFA expression. Targeting NR2F2 may be a potential therapeutic strategy to inhibit the proliferation of ALL cells by disrupting the microenvironmental support.

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