Overexpression of SDF-1 activates the NF-κB pathway to induce epithelial to mesenchymal transition and cancer stem cell-like phenotypes of breast cancer cells

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Abstract. The formation of EMT and EMT-induced CSC-like phenotype is crucial for the metastasis of tumor cells. The stromal cell-derived factor-1 (SDF-1) is upregulated in various human carcinomas, which is closely associated with proliferation, migration, invasion and prognosis of malignancies. However, limited attention has been directed towards the effect of SDF-1 on epithelial to mesenchymal transition (EMT) or cancer stem cell (CSC)-like phenotype formation in breast cancer cells and the related mechanism. In the present study, we screened MCF-7 cells with low SDF-1 expression level for the purpose of evaluating whether SDF-1 is involved in EMT and CSC-like phenotype formation in MCF-7 cells. The pEGFP-N1-SDF-1 plasmid was transfected into MCF-7 cells, and the stably overexpressed SDF-1 in MCF-7 cells was confirmed by real-time PCR and western blot analysis. Colony formation assay, MTT, wound healing assay and Transwell invasion assay demonstrated that overexpression of SDF-1 significantly boosted the proliferation, migration and invasion of MCF-7 cells compared with parental (P<0.05). Flow cytometry analysis revealed a notable increase of CD44+/CD24- subpopulation in SDF-1 overexpressing MCF-7 cells (P<0.001), accompanied by the apparently elevated ALDH activity and the upregulation of the stem cell markers OCT-4, Nanog, and SOX2 compared with parental (P<0.01).

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Abbreviations: SDF-1, stromal cell-derived factor-1; EMT, epithelial to mesenchymal transition; CSC, cancer stem cell

Key words: stromal cell-derived factor-1, MCF-7 cells, breast cancer, epithelial to mesenchymal transition, cancer stem cell-like phenotypes

Besides, western blot analysis and immunofluorescence assay observed the significant decreased expression of E-cadherin and enhanced expression of slug, fibronectin and vimentin in SDF-1 overexpressed MCF-7 cells in comparison with parental (P<0.01). Further study found that overexpression of SDF-1 induced the activation of NF-κB pathway in MCF-7 cells. Conversely, suppressing or silencing p65 expression by antagonist or RNA interference could remarkably increase the expression of E-cadherin in SDF-1 overexpressed MCF-7 cells (P<0.001). Overall, the above results indicated that overexpression of SDF-1 enhanced EMT by activating the NF-κB pathway of MCF-7 cells and further induced the formation of CSC-like phenotypes, ultimately promoting the proliferation and metastasis of MCF-7 cells. Therefore, SDF-1 may further be assessed as a potential target for gene therapy of breast cancer.

Introduction

Breast cancer is one of the most common malignancies in women, with ~1.05 million new cases annually and 3.1% increasing rate (1,2). Currently, surgery, chemotherapy, radiotherapy and endocrine therapy are the mainstream clinical strategies for breast cancer, but 25% mortality rate still remains (3). The deterioration and poor prognosis of breast cancer are attributed to tumor invasion and metastasis (1). Epithelial-mesenchymal transition (EMT) and EMT-induced acquisition of cancer stem cell (CSC)-like phenotypes are crucial for invasion and metastasis of tumor cells (4-7). Hence, finding the key molecules that regulate formation of EMT and CSCs is warranted to reveal the pathological progression of breast cancer and develop novel therapeutic approaches.

The stromal cell-derived factor-1 (SDF-1, CXCL12) is a highly conserved chemoattractant cytokine responsible for regulating diverse biological processes ranging from embryonic development, stem cell movement, angiogenesis and tumor generation (8-10). Clinical research reported that the expression of SDF-1 was elevated significantly in various carcinomas associated with tumor grade, lymph node metastasis, TNM stage and prognosis (11-13). Moreover, the organs

with constitutive SDF-1 secretion, such as liver, lung, lymph nodes, and bone marrow, are also the most common sites for secondary metastasis of breast cancer (14-16). On the contrary, as a potent leukocytic chemokines, CXCL12 also has a potential to promote anticancer immunity by inducing CD8^{+} T cell activity, enhancing cytotoxicity, increasing the number of CD11c^{+} cells in the tumor-draining lymph nodes and reducing the accumulation of myeloid-derived suppressor cells in the spleen (17). *In vitro* studies revealed that SDF-1 was secreted by cancer associated fibroblasts (CAFs) or myofibroblasts and bound to its receptors (CXCR4 and CXCR7) on cancer cell surface to activate downstream intracellular signal pathways that regulate the metastasis, angiogenesis, and drug-resistant of cancer cells (18). Gao *et al* and Jiang *et al* both demonstrated that the proliferation, migration and invasion of pancreatic cancer cells and epithelial ovarian cancer cells were enhanced through SDF-1/CXCR4 axis after treating with certain concentrations of SDF-1 (19,20). Kang *et al* overexpressed SDF-1 in MDA-MB-231 cells to create an autocrine loop of SDF-1/CXCR4 and found that SDF-1 boosted the invasiveness and migration of breast cancer cells (21). Although SDF-1 is crucial for the migration and invasion of cancer cells, little is known about the roles of SDF-1 in EMT or CSC-like phenotype formation in breast cancer and the detailed mechanisms.

In the present study, we established SDF-1 overexpressing MCF-7 cells to investigate the effect of SDF-1 on the proliferation, migration, invasion, EMT, and CSC-like phenotype formation in breast cancer cells and explore the underlying mechanism. The results showed that overexpression of SDF-1 induced EMT of MCF-7 cells through the NF-κB pathway to obtain the CSC-like phenotypes, ultimately facilitating metastasis of breast cancer cells.

Materials and methods

Cell lines and mammosphere culture. MDA-MB-231, MDA-MB-435, and MCF-7 cell lines were purchased from Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences. All cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS, Hyclone, Logan, UT, USA) and streptomycin/penicillin (100 U/ml) at 37˚C in a 5% CO_2 incubator. When cells reached 80-90% confluence, western blot analysis was employed to select a cell line with the lowest SDF-1 expression for subsequent experiments.

MCF-7 cell mammospheres were cultured according to previously published methods by Wang *et al* (22). Briefly, MCF-7 cells were suspended in serum-free DMEM/F12 (1:1) medium containing 20 ng/ml epidermal growth factor (EGF, PeproTech, St. Louis, MO, USA), 10 ng/ml basic fibroblast growth factor (b-FGF, PeproTech), B27 (Gibco), and ITS (insulin, transferrin and selenium, Sigma-Aldrich, St. Louis, MO, USA), and seeded into ultralow attachment plates (Corning, NY, USA) at a density of $1x10^5$ cells/ml. Fresh mammosphere media (2 ml) was added into the well every 2-3 days without removing the old media. Mammospheres were collected every seven days for subsequent experiment.

Conduction of SDF-1 overexpressing vector and transfection. Full length SDF-1 coding sequences were amplified by PCR,

and cloned into the *Eco*RI-*Bam*HI fragment of pEGFP-N1 expression vector. The primers of SDF-1 were designed as follows: forward, 5'-TCA<u>GAATTC</u>ATGAACGCCAAGGT CGTGG-3' (the underline represents *Eco*RI site); reverse, 5'-CCTC<u>GGATCC</u>TCACATCTTGAACCTC-TTG-3' (the underline represents *Bam*HI site). Subsequently, the plasmid pEGFP-N1-SDF-1 was transfected into MCF-7 cells using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA) strictly following the manufacturer's instructions. The non-transfected control cells and the cells with the empty vector transfected (pEGFP-N1) were evaluated in parallel as control. The stably transfected cells were screened by G418 (400 µg/ml, Invitrogen) after 24 h of transfection. As pEGFP-N1 plasmid contains green fluorescent protein, we employed a fluorescence microscope to investigate and calculate the efficiency after 24 h of transfection which was 25-35%. Then the expression of SDF-1 was examined at 7-14 days of transfection.

Real-time (RT)-PCR. Total RNA from MCF-7 cells was extracted by High Purity Total RNA Fast Extraction kit (BioTeke, Beijing, China) following the manufacturer instructions and then reverse-transcribed into cDNA. RT-PCR was carried out by Exicycler™ 96 (Bioneer, Daejeon, Korea) using SYBR Green mastermix (Solarbio, Beijing, China) with the following protocol: initial denaturation at 95˚C for 10 min, 40 cycles consisting of 95˚C for 10 sec, 60˚C for 20 sec and 72˚C for 30 sec. Primer sequences were: OCT4, 5'-AGCGATCAAG CAGCGACTA-3' (forward) and 5'-GGAAAGGGACCGAGG AGTA-3' (reverse); Nanog, 5'-GCAGGCAACTCACTTTA TCC-3' (forward) and 5'-CCCACAAATCACAGGCATAG-3' (reverse); SDF-1, 5'-GTGCCCTTCAGATTGTAGCC-3' (forward) and 5'-CCTTCCCTAACACTGGTTTCA-3' (reverse); SOX2, 5'-CATCACCCACAGCAAATGAC-3' (forward) and 5'-CAAAGCTCCTACCGTACCACT-3' (reverser); β-actin, 5'-CTTAGTTGCGTTACACCCTTTC TTG-3' (forward) and 5'-CTGTCACCTTCACCGTTCCAG TTT-3' (reverse). Relative expression was obtained by $2^{-\Delta\Delta CT}$ method. β-actin served as an internal control.

Colony formation assay. Cells were resuspended in DMEM complete media and seeded in 35-mm plates at a density of 10^2 cells/ plate. All plates were incubated at 37˚C in an atmosphere of 5% CO_2 for ~14 days. The suspension was decanted and replaced with fresh medium every 3 days. Then cells were fixed in 4% formaldehyde for 20 min. After being washed twice with phosphate-buffered salines (PBS, pH 7.4), cells were stained with Wright-Giemsa dye composite for 5 min. The number of colonies was calculated with an inverted microscope. Cells containing >50 cells were counted as a colony.

MTT analysis. Cells were plated in 96-well plates at a density of $2x10^3$ per well with five replicates for each testing point and cultured in a 37˚C, 5% CO_2 incubator for 24, 48, 72 and 96 h, respectively. Thereafter, cells in each well were exposed to MTT (0.2 mg/ml, Sigma-Aldrich) for 4 h followed by incubation with 200 µl DMSO (Sigma-Aldrich) to dissolve the dark blue crystals before reading the optical density (OD) at 490 nm in a microplate reader (BioTek, VT, USA).

Wound healing assay. Cells were inoculated in 6-well plates until 80-90% confluence. A wound was gently created with a 200-μl pipette tip on each cell monolayer, and each well was rinsed with a serum-free culture medium to remove detached cells. The migrating cells were imaged under an inverted microscope at 0, 12 and 24 h of culturing. The result was the ratio of the migrated distance to the initial distance.

Matrigel-based invasion analysis. The Matrigel-based invasion analysis was performed in a 24-well Transwell system (Corning, Tewksbury, MA, USA) with a Matrigel (BD Biosciences, San Jose, CA, USA) pre-coated polycarbonate membrane in the top chamber. Cells were harvested and resuspended in FBS-free DMEM, then plated in the top chamber at a density of $2x10^4$ per well. DMEM (800 μl) supplemented with 30% FBS was added into the lower chamber as a chemoattractant. After 24 h of incubation, the non-invading cells on the upper-surface of membrane were removed with cotton swabs, and the invading cells on undersurface of the membrane were fixed with 4% paraformaldehyde for 20 min and stained with crystal violet for 5 min. Invasion was analyzed in five randomly selected areas under an inverted microscope in a blinded manner.

Flow cytometry assay. Cells in each group were trypsinized, fixed with 70% ethanol, and washed with fresh medium. Thereafter, the collected MCF-7 cells were stained with FITC-conjugated CD44 antibody and PE-conjugated CD24 antibody (BD Biosciences). Then the mixture was incubated for 30 min at room temperature in the dark. The labeled cells were washed and analyzed immediately on a FACS (fluorescence activated cell sorting) Vantage (BD Biosciences).

Aldehyde dehydrogenase (ALDH) activity assay. The Aldehyde Dehydrogenase Activity Colorimetric Assay kit (Sigma-Aldrich) was employed to analyze ALDH enzymatic activity. In brief, mammospheres were resuspended in ALDH binding buffer (200 μl per $1x10^6$ cells) then centrifuged at 13,000 g for 10 min. Fifty microliters supernatant from each sample was mixed with 43 μl ALDH binding buffer, 2 μl ALDH substrate, and 5 μl acetaldehyde in the dark. The absorbance values at OD 450 nm were detected at 2-3-min intervals from 5 min (T initial) up to the standard value, the last but one absorbance value was defined as T final, and ALDH activity was calculated based on T initial and T final.

Immunofluorescence staining. Cells grown on coverslips were fixed with 4% formaldehyde for 15 min and permeabilized with 0.1% Triton X-100 (Amresco, Cochran Road Solon, OH, USA) for 30 min, cells were washed with PBS for three times at the end of each step. Subsequently, the non-specific bindings were blocked by goat serum (Solarbio) at room temperate for 15 min. Thereafter, cells were incubated overnight at 4°C with primary antibody against E-cadherin (1:200 diluted, Boster, Wuhan, China) followed by incubated with Cy3-labeled goat anti-rabbit IgG secondary antibody (1:200, Beyotime, Haimen, China) for 1 h at room temperature. The unbound antibodies in each step were washed with PBS three times. After counterstaining with 4', 6-diamidino-2-phenylindole (DAPI) and finally rinsed with PBS, each coverslip was mounted inversely

onto a slide with anti-fluorescent mounting media (Solarbio) added. Images were captured by a laser scanning confocal microscope.

NF-κB p65-siRNA interference. siRNA for NF-κB p65 and the control siRNA were designed and synthesized by GenePharma Co., Ltd. (Shanghai, China). The sequences were: NF-κB p65-siRNA: 5'-AGGACAUAUGAGACCUUCA-3', control siRNA: 5'-UUCUCCGAACGUGUCACGU-3'. NF-κB p65-siRNA (75 pmol) and control siRNA were transfected into the indicated MCF-7 cells, respectively, using the Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's instructions. Cells were harvested at 24 h after transfection for western blot analysis.

Western blot analysis. Cells in each sample were lysed with RIPA lysate (Beyotime) including 1% phenylmethanesulfonyl fluoride (PMSF, Beyotime). For NF-κB p65 detection, nuclear and cytoplasmic fraction proteins were extracted using Nuclear and Cytoplasmic Protein Extraction kit (Beyotime) following the manufacturer's instructions. All protein concentrations were detected using a bicinchoninic acid (BCA) protein assay kit (Beyotime). Then equal amounts of different proteins were loaded and separated using SDS-polyacrylamide gel electrophoresis (PAGE) and electrotransferred onto polyvinylidene fluoride (PVDF) membranes (Millipore, Bedford, MA, USA). After being blocked with 5% non-fat milk for 1 h, the membranes were probed with primary antibodies against SDF-1, Nanog, SOX2 (all 1:200 diluted, Santa Cruz Biotechnology, Santa Cruz, CA, USA), E-cadherin, fibronectin, NF-κB p65 (all 1:400 diluted, Boster), CXCR4, CXCR7 (both 1:500 diluted, Biosharp), OCT4, Vimentin, Slug, p-IκB, IκB (all 1:500 diluted, Bioss, Beijing, China) at 4°C overnight and subsequently incubated with their corresponding secondary antibodies (1:5,000 dilution, Beyotime) for 45 min at 37°C. Unbound antibodies in each step were washed with TBST four times. The positive bands were visualized through enhanced chemiluminescence (ECL) solution (Qihai Biotec, Shanghai, China) and measured by Gel-Pro-analyzer software (Bethesda, MD, USA). Histone H3 and β-actin both served as internal controls.

Statistical analysis. All values are reported as mean ± standard deviation (SD). Kolmogorov-Smirnov (K-S) test and homogeneity of variance test were employed in each experiment and one-way analysis of variance (ANOVA) was used followed by the Bonferroni post hoc test to compare differences between groups. All statistical analysis was performed by GraphPad Prism 5.0 software. P<0.05 was considered statistically significant.

Results

Stable overexpression of SDF-1 in breast cancer cells. To explore the potential effects of SDF-1, we first detected the expressions of SDF-1, CXCR4 and CXCR7 in MDA-MB-231, MDA-MB-435, and MCF-7 cells, respectively. Western blot analysis showed that MCF-7 cell line had the lowest expression of SDF-1 and its receptors (Fig. 1A). Hence we selected MCF-7 cell line for detailed mechanistic studies. Next, the
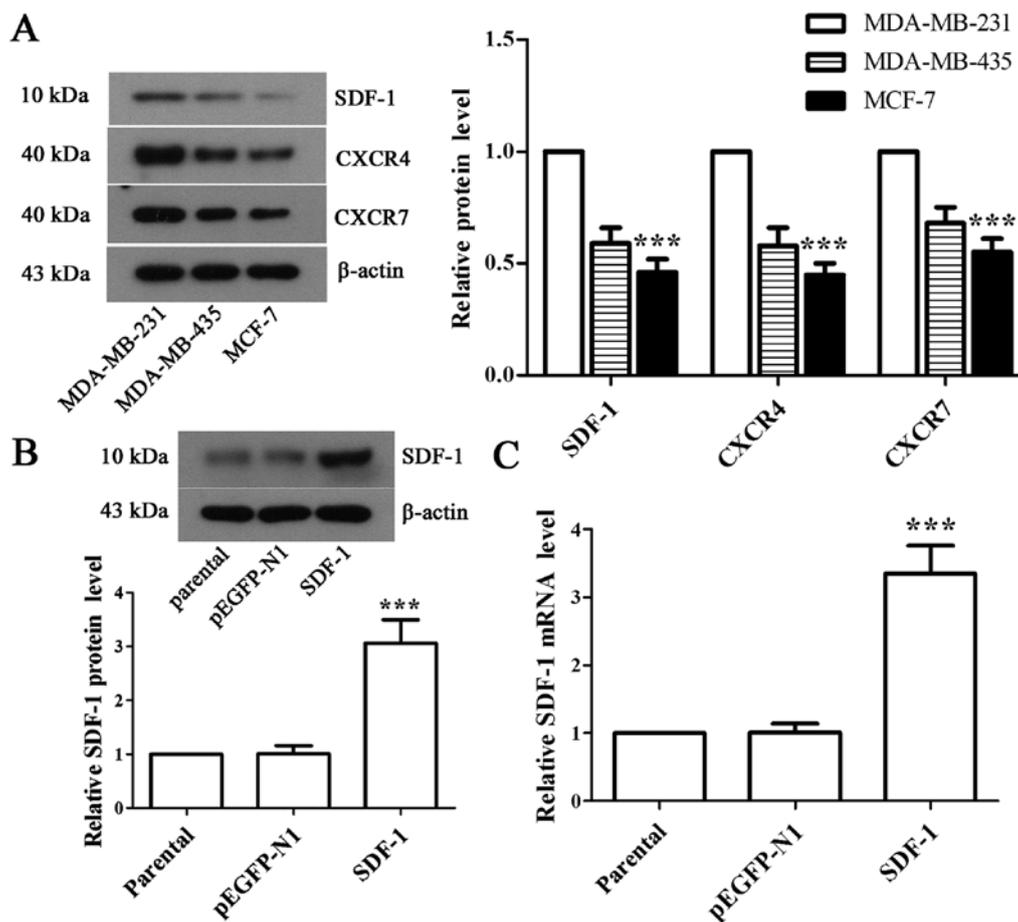
Figure 1. Stable overexpression of SDF-1 in breast cancer cells. (A) Representative photographs of western blot analysis on SDF-1, CXCR4 and CXCR7 protein expression levels in MDA-MB-231, MDA-MB-435 and MCF-7 cells. β-actin was used as an internal control. ***P<0.001 vs MDA-MB-231. (B) The pEGFP-N1-SDF-1 plasmid was transfected into MCF-7 cells, and the protein expression level of SDF-1 in MCF-7 cells was detected by western blot analysis and normalized to β-actin. The typical blots are shown, and the parental MCF-7 cells and pEGFP-N1 transfected MCF-7 cells served as controls. ***P<0.001 vs pEGFP-N1. (C) Real-time (RT) PCR analysis of SDF-1 mRNA expression levels. ***P<0.001 vs pEGFP-N1. The above results are given as mean ± SD, and the error bars represent the SD from three independent experiments.

recombinant pEGFP-N1-SDF-1 plasmid was transfected into MCF-7 cells, and western blot analysis and RT-PCR were performed to validate the expression of SDF-1 in positive monoclonal cells. The results showed that the expression of SDF-1 was increased by 3.03-fold (Fig. 1B, P<0.001) and 3.32-fold (Fig. 1B, P<0.001) at protein and mRNA levels, respectively, in SDF-1-transfected MCF-7 cells compared with pEGFP-N1-transfected MCF-7 cells, which suggested stable overexpression of SDF-1 in MCF-7 cell line.

Overexpression of SDF-1 promotes the proliferation, migration and invasion of MCF-7 cells. Colony formation and MTT assay were employed to visualize the effect of SDF-1 on the proliferation of MCF-7 cells. We found that the colony formation rate in overexpressing SDF-1 MCF-7 cells was enhanced by 1.54-fold compared with parental (Fig. 2A, P<0.01). At the same time, the proliferation capability of MCF-7 cells with SDF-1 transfection was increased significantly from 48 to 96 h as compared to parental (Fig. 2B, P<0.05). The above results indicated that SDF-1 was able to enhance the proliferation of MCF-7 cells.

We further evaluated the effect of SDF-1 on the migratory and invasive potential of MCF-7 cells through wound healing

and Transwell assay. The results at 12 and 24 h after wounding both showed that the wound healing rate in SDF-1- overexpressed MCF-7 cells was notably elevated compared with parental (Fig. 2C, P<0.01). Moreover, the number of invading cells was 54±5.7 in SDF-1 overexpressed-MCF-7 cells in the present of FBS, which was increased compared with parental (27.6±2.7) (Fig. 2D, P<0.001). Thus, the results strongly supported that overexpression of SDF-1 could promote migration and invasion of MCF-7 cells.

Overexpression of SDF-1 induces CSC-like phenotype formation from MCF-7 cells. To observe whether overexpression of SDF-1 contributes to the formation of CSC-like phenotypes in MCF-7 cells, we detected the proportion of CD44⁺/CD24⁻ cells through flow cytometry. Our analysis showed that SDF-1 caused an increased accumulation of cell population with CD44⁺/CD24⁻ phenotype (Fig. 3A, P<0.01). Similarly, ALDH activity was also dramatically elevated by overexpressing SDF-1 in MCF-1 cells (Fig. 3B, P<0.01). It was further observed that the expression of OCT-4, Nanog, and SOX2 in both mRNA and protein levels was elevated significantly in SDF-1-overexpressed MCF-7 cells compared with parental as observed by RT-PCR and western blot analysis (Fig. 3C and D,
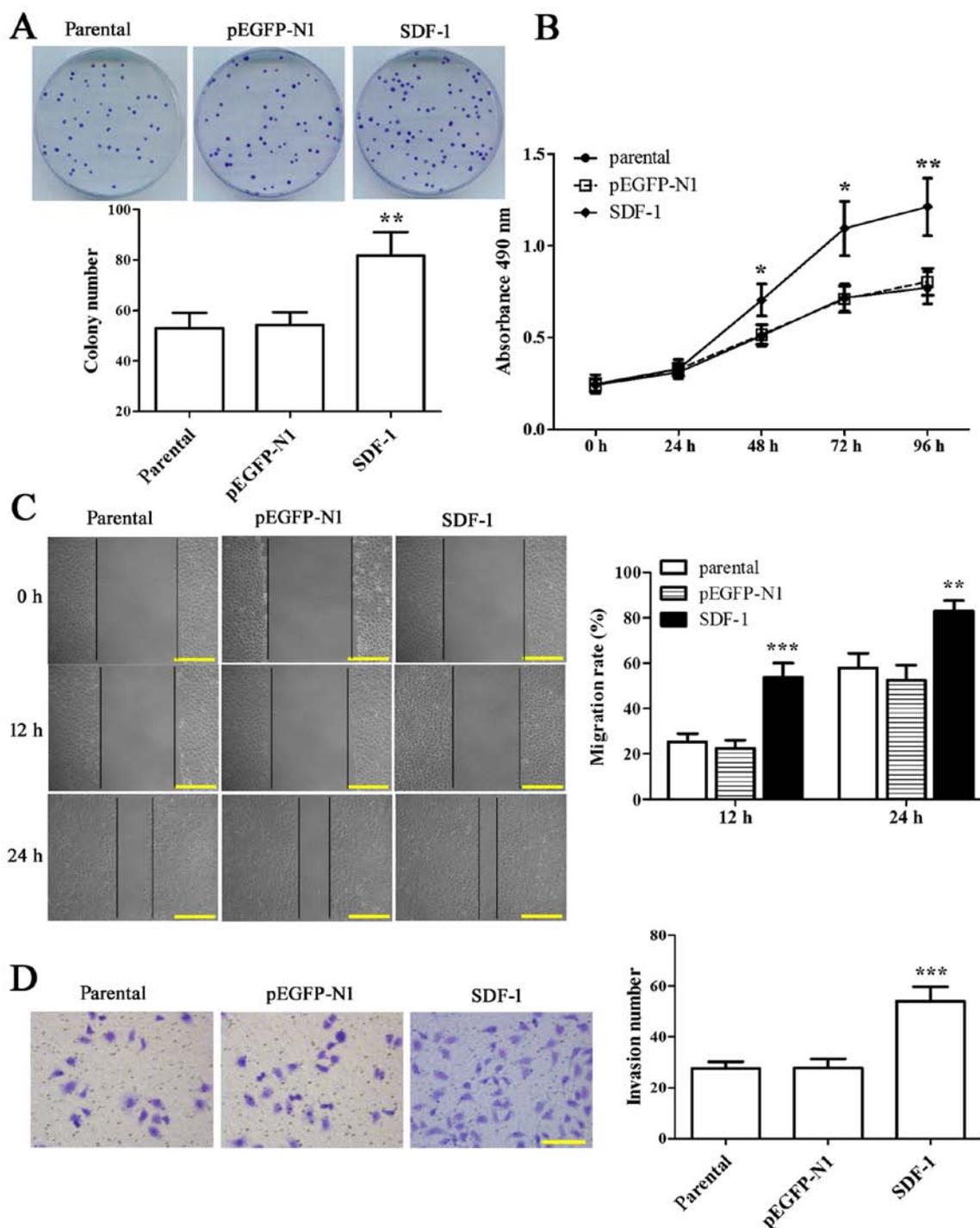
Figure 2. Overexpression of SDF-1 promotes the proliferation, migration and invasion of MCF-7 cells. (A) Cells were seeded in 35-mm plates and incubated for 14 days before being fixed with 4% formaldehyde. Then the cells were stained with Wright-Giemsa dye composite to calculate the number of colony formation, and the representative images are shown. (B) Cells were plated in 96-well plates with five replicates for each testing point and cultured for 24, 48, 72 and 96 h, respectively. MTT assay was used to evaluate cell viability at OD 490 nm. (C) A wound was created on cell monolayer in each well, cells were cultured for 12 or 24 h, and the distance migrated was measured. Representative photomicrographs are shown on the left. Scale bars indicate 200 μm at x200 magnification. (D) The Matrigel-based invasion assay was carried out in a Transwell system. After 24 h of incubation, the invading cells on undersurface of the membrane were fixed with 4% paraformaldehyde, stained with crystal violet and counted with five randomly selected microscopic fields. Representative examples of photographs are shown on the left. Scale bar indicates 200 μm at x200 magnification. Experiments were all done in triplicates for statistical significance, and the results are expressed as mean ± SD. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ vs parental.

P<0.01). These results suggested that MCF-7 cells obtained the CSC-like phenotype by overexpressing SDF-1.

Overexpression of SDF-1 boosts EMT of MCF-7 cells. To address the effect of SDF-1 on EMT of MCF-7 cells, we first observed the loose connections between cells with SDF-1 overexpression (Fig. 4A). Then we identified the significantly downregulated E-cadherin and notably upregulated slug, fibronectin and vimentin in SDF-1-overexpressed MCF-7 cells compared with parental (Fig. 4B, D and E, P<0.01).
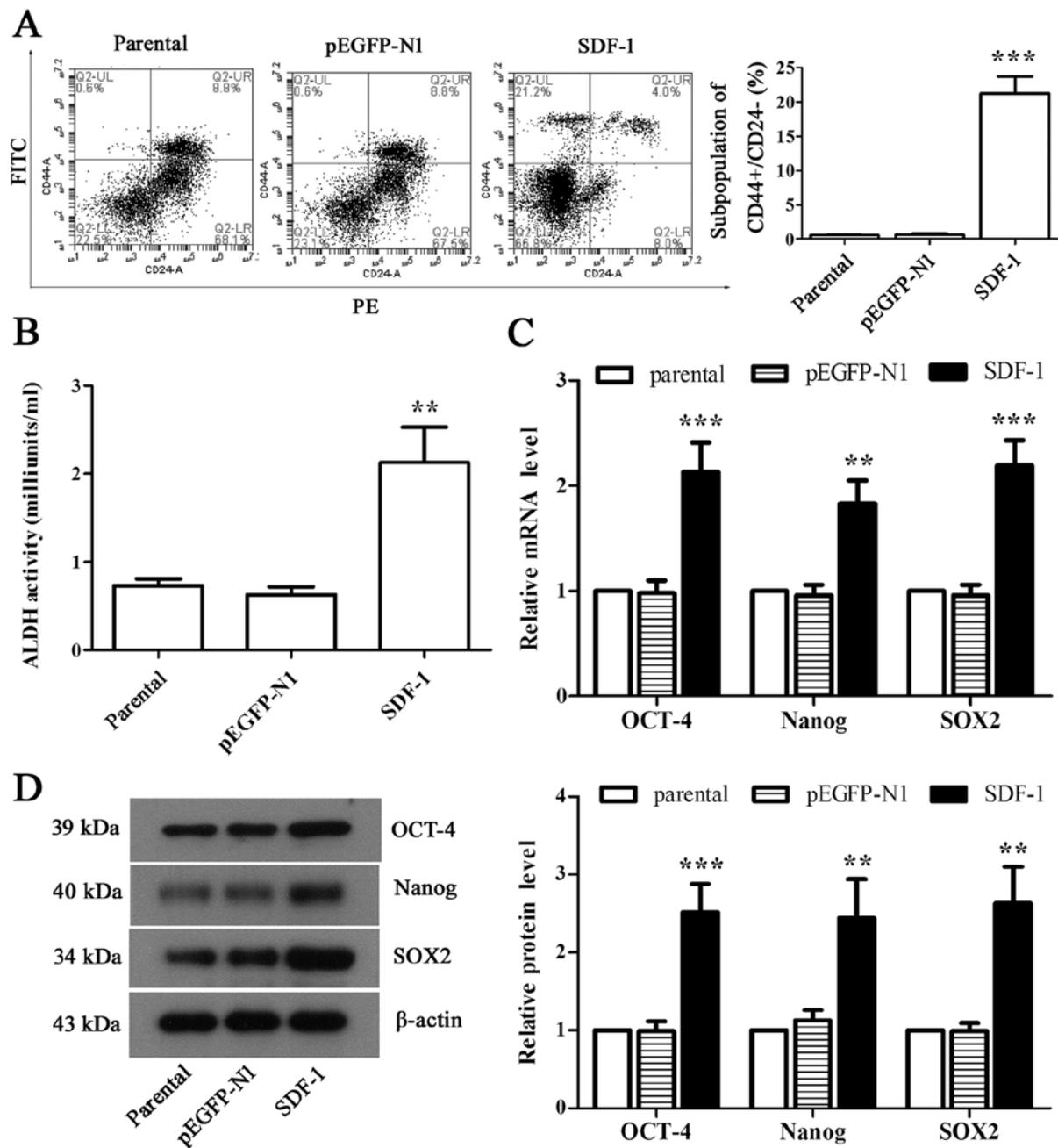
Figure 3. Overexpression of SDF-1 induces CSC-like phenotype formation from MCF-7 cells. (A) Transwell MCF-7 cells were stained with FITC-conjugated CD44 antibody and PE-conjugated CD24 antibody. The percentage of different CD44/CD24 subpopulations was determined by flow cytometry assay. A series of representative illustrations of cell subpopulation distributions are shown. (B) Colorimetry was carried out to assess ALDH activity in mammospheres at OD 450 nm. (C) Relative mRNA levels of OCT-4, Nanog, and SOX2 were evaluated by RT-PCR. (D) Relative protein levels of OCT-4, Nanog, and SOX2 were evaluated by western blot analysis, and the representative images are shown. β-actin served as internal control. The above two experiments were repeated three times. Data are presented as mean ± SD. $^{**}P<0.01$, $^{***}P<0.001$ vs parental.

Accordingly, the fluorescence intensity of E-cadherin was appeared to be reduced obviously in SDF-1 overexpressed-MCF-7 cells as compared with parental (Fig. 4C). Collectively, these data revealed that overexpression of SDF-1 could induce EMT in MCF-7 cells.

NF-κB pathway is involved in SDF-1-mediated EMT in MCF-7 cells. To investigate the mechanism by which SDF-1 induced EMT in MCF-7 cells, western blot analysis was applied to examine the expression levels of related proteins in NF-κB

pathway. We discovered a apparent decrease of cytoplasmic p65 level and a significant increase of nuclear p65 level in SDF-1 overexpressed MCF-7 cells compared with parental (Fig. 5A, P<0.01), along with the notably elevated phosphorylation level of cytoplasmic IκB (Fig. 5B, P<0.001), suggesting the activation of NF-κB pathway. We further employed BAY 11-7028, an antagonist target NF-κB pathway, to inhibit NF-κB pathway, and performed siRNA interference to silence NF-κB p65 gene in SDF-1 overexpressed-MCF-7 cells. Both results showed the significantly upregulated E-cadherin expression (Fig. 5C
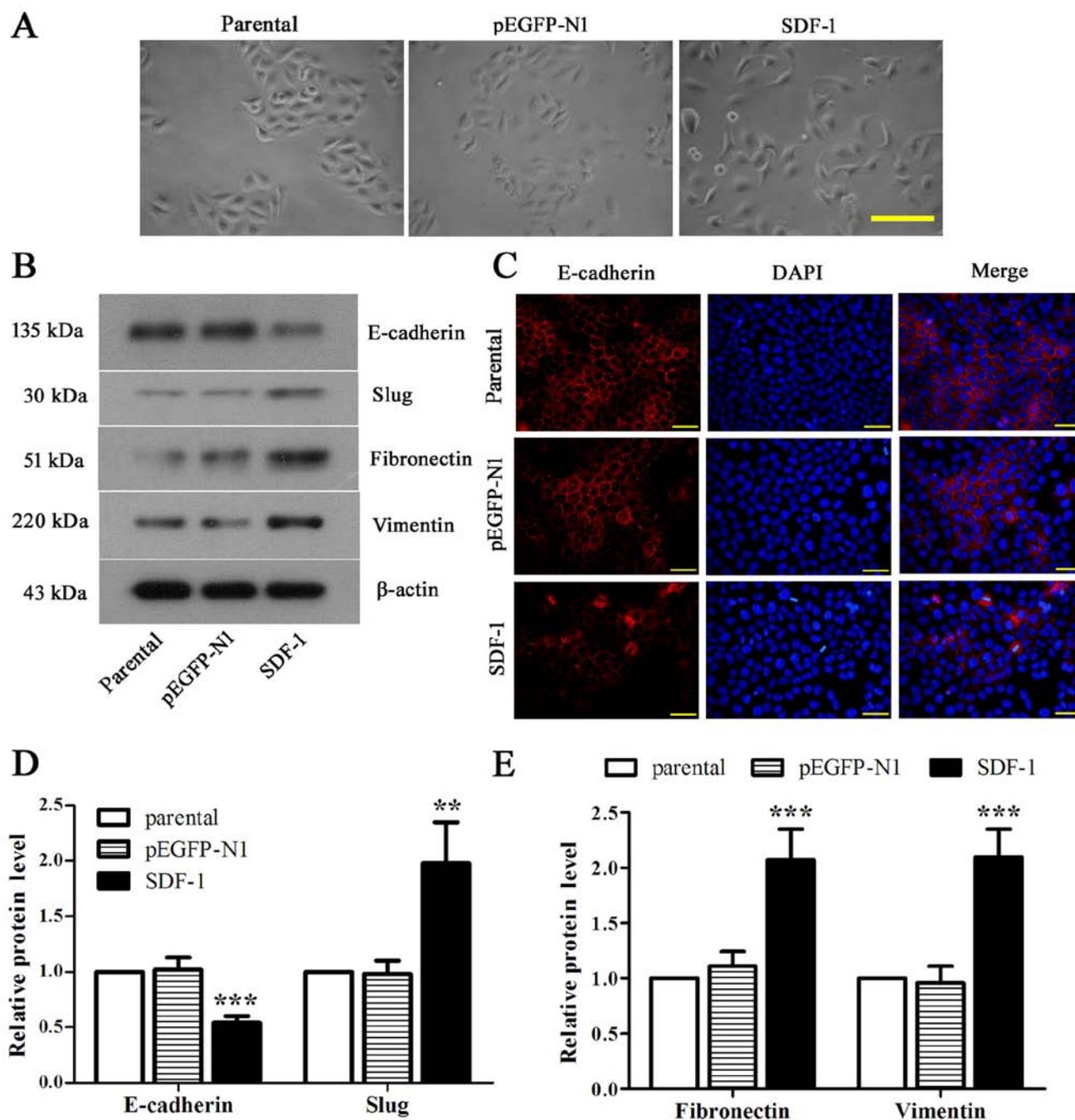
Figure 4. Overexpression of SDF-1 boosts EMT of MCF-7 cells. (A) Representative photomicrographs of MCF-7 cells in each group under phase contrast microscope. Scale bars, 200 μm. (B) Representative blots of E-cadherin, slug, fibronectin and vimentin, and β-actin was used as internal control. (C) Immunofluorescence staining was performed to investigate the expression of E-cadherin. Representative immunofluorescence images are shown. E-cadherin was visualized with Cy3-labeled red by goat anti-rabbit IgG, and cell nuclei were stained blue by DAPI. Scale bar indicates 100 μm at x400 magnification. (D) Relative protein levels of E-cadherin and slug were measured by gray scale analysis. (E) Relative protein levels of fibronectin and vimentin were measured by gray scale analysis. Data in each group are given as mean ± SD from three independent experiments. **P<0.01, ***P<0.001 vs parental.

and D, P<0.001), which indicated that NF-κB played a prominent role in the progression of EMT in SDF-1 overexpressed breast cancer cells.

Discussion

SDF-1 is involved in a broad range of biological procedures including cell adhesion, migration, invasion, chemotaxis, cell cycle, proliferation, apoptosis, angiogenesis, and cell commu-

nication. However, whether SDF-1 could induce CSC-like phenotypes and EMT of breast cancer cells and the detailed mechanism remain unclear. Here, we overexpressed SDF-1 in the poorly invasive MCF-7 cells (23). Then we found that overexpressing SDF-1 could trigger EMT in MCF-7 cells by activating NF-κB pathway and induce CSC-like phenotypes to increase the abilities of proliferation, migration, and invasion. Overall, these results further identified the roles of SDF-1 in the metastases of breast cancer cells.
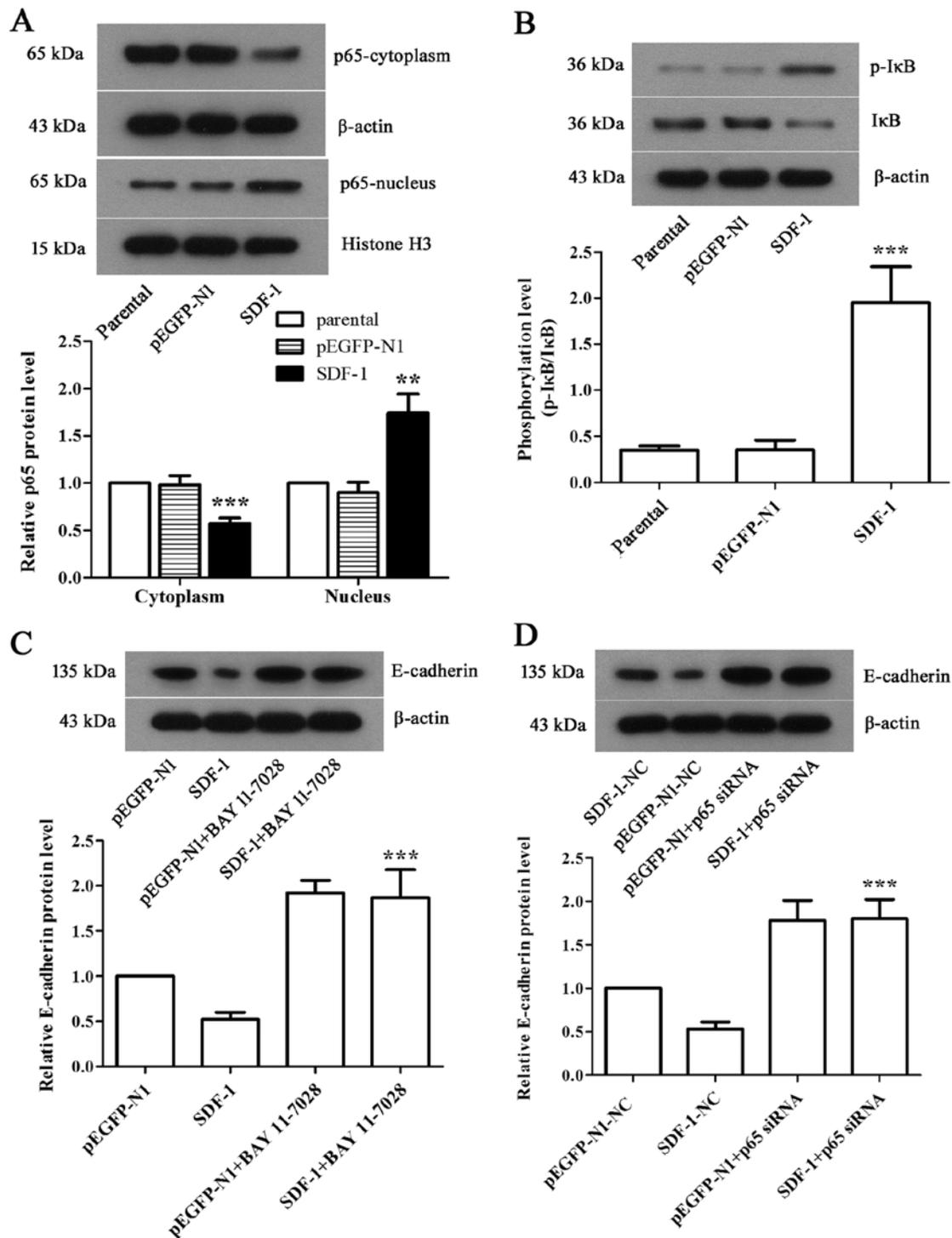
Figure 5. NF-κB pathway is involved in SDF-1-mediated EMT in MCF-7 cells. (A) Western blot analysis on the expression of p65 in cytoplasm and nucleus. (B) Western blot analysis on the phosphorylation level of IκB. (C) pEGFP-N1 transfected cells and SDF-1 transfected cells were both treated with BAY 11-7028 for 48 h, then western blot analysis was employed to detect the expression of E-cadherin in MCF-7 cells. (D) p65 gene in both pEGFP-N1 transfected cells and SDF-1 transfected cells was silenced by RNA interference, and western blot analysis was used to examine the expression of E-cadherin in MCF-7 cells. Representative blots are all presented and corresponding gray scale analysis is shown as mean ± SD of three independent experiments. β-actin and histone H3 served as internal controls. **P<0.01, ***P<0.001 vs parental.

The interactions between SDF-1 and receptors CXCR4 and CXCR7 control multiple steps of tumor growth and metastasis in >20 human malignancies, including breast cancer (24). In addition, overexpression of SDF-1 can recruit more macrophages. The increased recruitment of cancer-associated macrophages (CAMs) is not contributed only to tumor angiogenesis by releasing vascular endothelial growth factor (VEGF) but also capable of inducing tumor cell motility and invasion through paracrine loop signaling (25-28). On the contrary, hypoxia has been reported as an important driving force for the multistep process of metastasis, and the accumulated CAMs could exacerbate the oxidant microenvironment

of the tumor. Hypoxia improves metastatic seeding of cancer cells by enhancing CXCR4 expression to enable tumor cells to home to SDF1 highly expressed secondary organs (29). In our study, we created an autocrine loop of SDF-1 and its receptors and found that overexpression of SDF-1 could increase the growth, migration, and invasion in MCF-7 cells without recruiting CAMs, consistent with Kang and colleagues (30).

CSCs are unresponsive to chemotherapeutic and apoptotic drugs since they can resist DNA damage (30). Breast cancer stem cells (BCSCs) have been identified as CD44$^+$/CD24$^-$ cells. Huang *et al* reported that SDF-1 boosted the proliferation of CD44$^+$/CD24$^-$ cells through SDF-1/CXCR4 signaling (1). We found that the subpopulation of MCF-7 cells with CD44$^+$/CD24$^-$ phenotypes was elevated after overexpressing SDF-1, suggesting the acquirement of CSC phenotypes in overexpressing SDF-1 MCF-7 cells. ALDH1 exhibits low or absent expression in normal breast tissue, breast cells with increased ALDH1 expression indicate stem or progenitor properties with broadest differentiation potential and greatest growth capacity (31). The transcription factors OCT4, Nanog and SOX2 are all embryonic stem cell (ESC) markers and play an important role in maintaining the pluripotent self-renewal of ESCs, which are downregulated in the differentiated somatic cells (32). In response to hypoxic conditions, hypoxia-inducible factors (HIFs) reprogram non-stem cancer cells to a stem-like phenotype by increasing the transcription of ALDH and inducing the expression of OCT4 and Nanog (33,34). Here, *in vitro*, the mammosphere cells with increased ALDH activity was observed, along with the upregulation of OCT4, Nanog and SOX2 in SDF-1 overespressed MCF-7 cells, suggesting that SDF-1 could induce CSC-like phenotypes of breast cancer cells. It has been indicated that the emergence of CSCs occurs in part because of EMT (5). Hence, further experiments were required to identify the effect of SDF-1 on EMT in MCF-7 cells.

E-cadherin is an epithelial cell junction marker; vimentin and fibronectin are mesenchymal markers. During the progression of EMT, slug could repress E-cadherin transcription at promoter level to break down the adherence junction (7,35). In this study, we found that overexpression of SDF-1 increased the expression of slug leading to downregulation of E-cadherin, along with the elevated expression of vimentin and fibronectin, suggesting that EMT was triggered by overexpressing SDF-1 which may subsequently induced CSC phenotypes in MCF-7 cells to facilitated breast cancer cells metastasis.

NF-κB pathway is associated with cell proliferation, apoptosis, and inflammation, and involved in diverse progressions of cancer development (36,37). Recent studies have identified that NF-κB was an important regulator of EMT in several cell types (38-40) governing the induction, metastasis and maintenance of EMT (41). Jiang *et al* reported that longer times for EMT would increase activation of IκB and NF-κB, then the expression of stem cell markers were enhanced to promote neoplastic transformation of human keratinocytes (42). It is a well established fact that the promoter of CXCR4 contains several binding sites for NF-κB, we therefore speculated that NF-κB pathway may be involved in SDF-1-induced EMT of MCF-7 cells. Our results showed the activation of NF-κB pathway in SDF-1 overexpressing MCF-7 cells, and the expression of E-cadherin was increased remarkably after incubating

with NF-κB inhibitor drug or silencing NF-κB p65 gene in SDF-1-overexpressed MCF-7 cells, indicating that NF-κB pathway regulates SDF-1-induced EMT of breast cancer cells. For the downstream pathways of SDF-1/CXCR4 in breast CSCs, Yi *et al* identified SDF-1/CXCR4-PKA-MAP2K2-ERK signaling pathway and demonstrated the feedback regulation on MEK, ERK1/2, δ-catenin, and PPP1Cα in breast CSCs treated with 100 ng/ml SDF-1 (43). As EMT is an important inducer of CSCs, there must be connections between NF-κB pathway and PKA-MAP2K2-ERK signaling pathway which is one of the strategies of our subsequent study.

In conclusion, our data indicated that overexpression of SDF-1 could trigger EMT of MCF-7 cells through NF-κB pathway to further gain the CSC-like phenotypes, subsequently promoting metastasis of MCF-7 cells. Our findings preliminarily identified the significant roles of SDF-1 in MCF-1 cells, and suggest that SDF-1 may become a promising candidate for breast cancer therapy.

Acknowledgements

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