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 cancers 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 overexpressed 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). 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% CO2 incubator. When cells reached 80-90% confluence, cells were resuspended in DMEM containing 10% FBS and 1% Pen/Strep (v/v) and decanted and replaced with fresh medium every 3 days. Cells were cultured in a 37˚C, 5% CO2 incubator. Dishes were changed twice a week.

**Colony formation assay.** Cells were resuspended in DMEM complete media and seeded in 35-mm plates at a density of 102 cells/plate. All plates were incubated at 37°C in an atmosphere of 5% CO2 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 2×103 per well with five replicates for each testing point and cultured in a 37°C, 5% CO2 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).

**Real-time (RT)-PCR.** Total RNA from MCF-7 cells was extracted by High Purity Total RNA Fast Extraction kit (Biofeke, 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'-AGGGATCCAGAGCGACTA-3' (forward) and 5'-GGAAAGGGACCGAGGAGTA-3' (reverse); Nanog, 5'-GACGCACTACTTATTTCC-3' (forward) and 5'-CCCCAAAATCACAGGATCAGAGTAG-3' (reverse); SDF-1, 5'-GTGCCCTTCAGATTGTAGCC-3' (forward) and 5'-CCCACAAATCACAGGCATAG-3' (reverse); SOX2, 5'-CATCACCACACAGCAAATGC-3' (forward) and 5'-CAAAGCTCCTACGTTGTTTA-3' (reverse); β-actin, 5'-CTTCTGATCTACTGCTTTGA-3' (forward) and 5'-CTGTCACCTACGTTGTTTA-3' (reverse).

Relative expression was obtained by 2^-ΔΔCT method. β-actin served as an internal control.
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. 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 temperature 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'-AGGACAUAGAGACCCUUCA-3', control siRNA: 5'-UUUCUCGAGGUUCACGU-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 lysis (Beyotime) including 1% phenylmethylsulfonyl 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 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-1αB, ICβ (all 1:500 diluted, Bios, 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
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 OCT4, 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, respectively).
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).
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.

\textit{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).
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 communication. 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.
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 overexpressed 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 PP12. 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.

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References


