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Original Article

Transforming growth factor-β1 regulates epithelial-mesenchymal transition in association with cancer stem-like cells in a breast cancer cell line

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Abstract: Epithelial-mesenchymal transition (EMT) is associated with altered connection and junctions between cells and changes in abilities of invasion and migration. In this study, we investigated whether SK-BR-3 breast cancer cells induced to undergo EMT exhibit changes in morphological and invasion abilities after Transforming growth factor β1 (TGF-β1) treatment. Serum-deprived SK-BR-3 cells were treated with TGF-β1 (0, 10 ng/mL) for 24 h. The cells morphological changes were observed and imaged using inverted phase contrast microscope. Scratch experiment and invasion experiment were employed to detect changes of invasion ability, cell-flow experiment was used to assess cell cycle, immunohistochemistry technique was used to detect epithelial and mesenchymal markers after the crawling cells were fixed. Our research reveal that SK-BR-3 cells become larger and more messy, the elongated cells extend pseudopodia, the link of the cells became more loosely and cell gap widened after TGF-β1 treatment. SK-BR-3 cells showed faster growing and improved invasion abilities after TGF-β1 treatment, and reduced G_1 phase cells proportion in the total number of cells after the conversion, in contrast the S phase cells accounted for the proportion of the total number of cells increased. These findings indicate that TGF-β1-induced EMT in breast cancer cells may be associated with major alterations in morphological and invasion abilities.

Keywords: Breast cancer, epithelial-mesenchymal transition (EMT), Transforming growth factor β1 (TGF-β1), invasion ability

Introduction

Epithelial-mesenchymal transition (EMT) facilitates cancer cell invasion and metastasis formation [1, 2], and has also been linked to the acquisition of a stem cell-like phenotype [3], anchorage-independent growth and chemoresistance in cancer cell lines and clinical samples [4-11]. The phenotypic changes associated with Transforming growth factor β1 (TGF-β1)-induced EMT are well characterized in the multiple human cancer cell lines, and include changes in cell morphology; increased expression of the transcription factor Twist and the intermediate filament protein vimentin, and reduced E-cadherin expression following TGF-β1 treatment [12-14].

In this study we assessed whether alterations in morphological changes, invasion abilities were associated with TGF-β1-induced EMT in SK-BR-3 breast cancer cells.

Materials and methods

Cell culture

SK-BR-3 cells were maintained in Dulbecco's Modified Eagle's Medium (D6546, Sigma Aldrich) supplemented with penicillin (100 U/mL, Invitrogen), streptomycin (100 μg/mL, Invi-

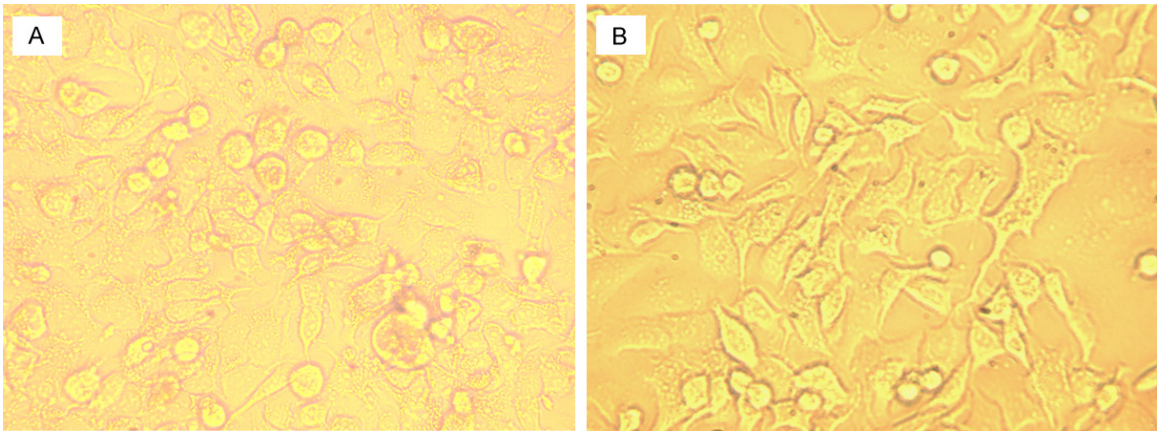

Figure 1. Morphological changes in SK-BR-3 cells. A. SK-BR-3 cells treated with TGF-β1 (0 ng/ml) for 24 h. B. SK-BR-3 cells treated with TGF-β1 (10 ng/ml) for 24 h.

Table 1. Scratch wound healing assay of SK-BR-3 cells

TGF-β1 (ng/ml)	Width of wound (μm)		
	0 h	24 h	Rate of healing (%)
0	19.5±2.3	12.4±1.6	36.41±5.21
10	19.7±3.1	9.2±1.3**	42.22±4.63**

SK-BR-3 cells were treated with TGF-β1 for 24 h. **; $p<0.01$.

trogen), fetal bovine serum (10%, Sigma Aldrich) and L-glutamine (4 mM, Invitrogen). To induce EMT, SK-BR-3 cells were serum-starved (0.5% fetal bovine serum) for 24 h and stimulated with TGF-β1 (0, 10 ng/mL, Sigma Aldrich) for 24 h. These time points were chosen because changes in mRNA levels are expected to precede changes in functional responses. SK-BR-3 cells were maintained in a humidified incubator at 37°C with 5% CO_2 and routinely tested negative for mycoplasma infection (MycoAlert, Lonza).

Migration assay

We scribed five paralleled lines on the bottom of six-well plates using a marker pen and seeded cells at a density of 4.0×10^5 cells per well in triplicate for 48 h. A perpendicular scratch wound was generated by scratching with a pipette tip. After rinsing with PBS to remove the detached cells, medium containing different concentrations of TGF-β1 (0, 10 ng/mL) was added. Photographic images were taken from each well at 0 h and 24 h. The distance that cells migrated through the area created by scratching was determined by measuring the wound width at the above times and subtracting it from the wound width at the start. The values obtained were then expressed as the rate of wound healing. The experiment was repeated three times.

Invasion assay

Transwell chambers (Corning-Costar) were used to examine the ability of cells to invade through a Matrigel-coated filter following the manufacturer's instructions. DMEM medium was added to the upper chambers and allowed to hydrate for 2 h at 37°C with 5% CO_2. Next, 5×10^4 SK-BR-3 cells treated with various concentrations of TGF-β1 (0, 10 ng/mL) were added to the upper chamber and grown in medium containing 2% fetal bovine serum on 8.0 μm porous polycarbonate membranes, which were coated with diluted Matrigel basement membrane matrix. The lower chambers were filled with DMEM medium containing 10% fetal bovine serum. After 24 h incubation, the cells remaining on the upper surface of the filter were removed using cotton tips, and the cells that invaded to the underside of the membrane were fixed with 4% paraform and stained with crystal violet. Cells in 10 random fields of view at 400 × magnification were counted and expressed as the average number of cells/field of view.

Flow cytometry

SK-BR-3 seeded in six-well plates were treated with different concentration (0, 10 ng/mL) TGF-

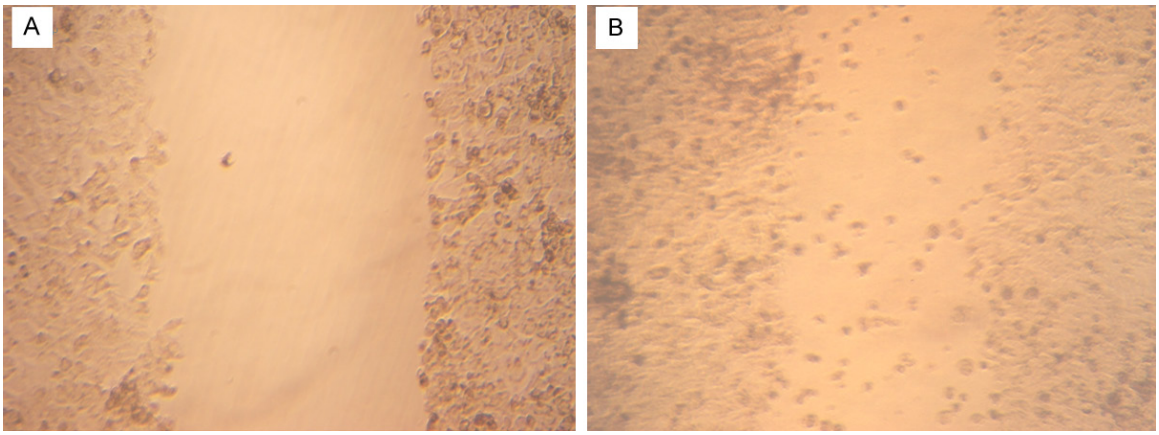

Figure 2. Wound healing test of SK-BR-3 cells. A. Wound healing test of SK-BR-3 cells treated with TGF-β1 (0 ng/ml) for 24 h. B. Wound healing test of SK-BR-3 cells treated with TGF-β1 (10 ng/ml) for 24 h.

Table 2. Invasion assay of SK-BR-3 cells

TGF-β1 (ng/ml)	Number of invading cells (Mean ± SD)
0	57.12±3.14
10	129.47±5.83**

SK-BR-3 cells were treated with TGF-β1 for 24 h. **; $p<0.01$.

β1 for 24 h at a cell density of 1.5×10^5 cells/mL. Cells were processed using the following assay. (1) Cells were resuspended by adding 500 μL binding buffer, followed by adding 5 μL annexin V-FITC and 5 μL propidium iodide (PI) dye. After mixing at room temperature in the dark for 5-15 min, flow cytometry analysis was performed. Annexin V-FITC-positive and PI-negative cells were considered as apoptotic cells; (2) Cells were resuspended by adding 100 μL RNase A H_2O and incubated in water at 37°C for 30 min. After adding 400 μL PI and mixing at 4°C for 30 min in the dark, flow cytometry analysis was performed. The G_0/G_1, S and G_2/M stages were compared. The experiment was repeated three times.

IHC studies

SK-BR-3 cells seeded in 6-well plates in the coverslip, fixed with cold acetone for 10 min, put it aside at room temperature 1 h, closed non-specific binding, no cleaning. Immunohistochemistry (IHC) was performed with monoclonal mouse antihuman E-cadherin antibody (ZM0092, Beijing ZSGB Company, China) and

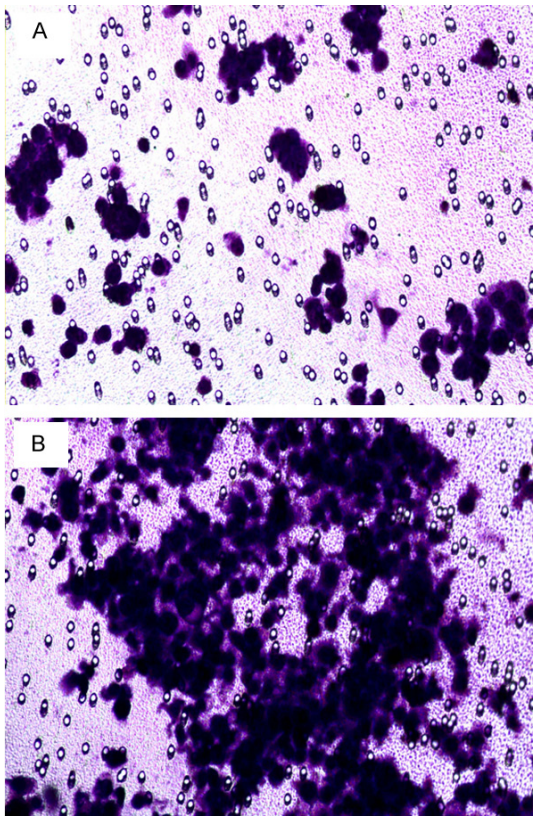

Figure 3. Invasion ability of SK-BR-3 cells. A. Invasion ability of SK-BR-3 cells treated with TGF-β1 (0 ng/ml) for 24 h. B. Invasion ability of SK-BR-3 cells treated with TGF-β1 (10 ng/ml) for 24 h.

monoclonal mouse antihuman N-cadherin antibody (ZM0094, Beijing ZSGB Company, China), dropwise primary antibody (1:100), 37 incubated for 2 h; PBS wash 5 min, 3 times; followed by

Table 3. Flow cytometry detection of SK-BR-3 cells

TGF-β1 (ng/ml)	G_1 (%)	S (%)	Apoptosis
0	80.65±7.86	15.48±2.54	26.62±3.81
10	50.38±6.38**	41.88±5.64**	24.67±2.95

SK-BR-3 cells were treated with TGF-β1 for 24 h. **; p<0.01.

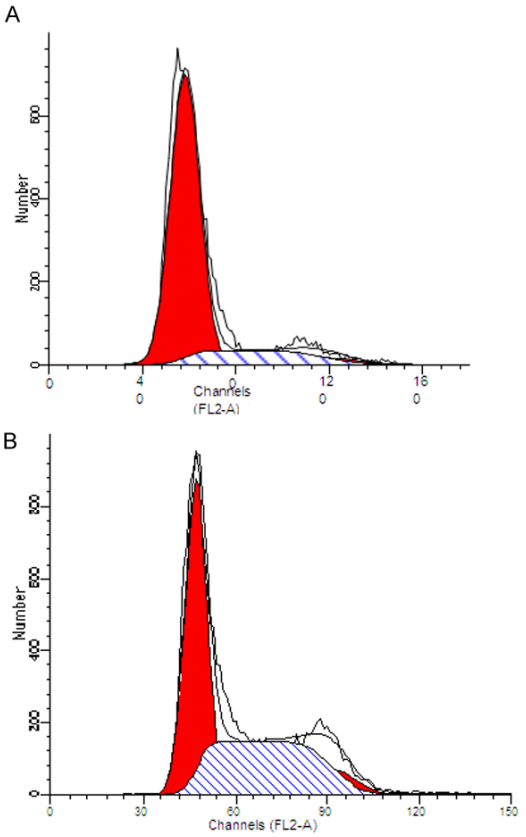

Figure 4. Flow cytometry detection of SK-BR-3 cells. A. SK-BR-3 cells treated with TGF-β1 (0 ng/ml) for 24 h. B. SK-BR-3 cells treated with TGF-β1 (10 ng/ml) for 24 h.

incubation with the anti-mouse IgG at 37°C for 30 min, washed again with PBS, followed by incubation with streptavidin-peroxidase complex for 30 min at 37°C, stained with DAB for 10 min and counterstained with hematoxylin solution for 5 min.

Statistical analysis

The data were analyzed with single factor analysis of variance and a Student's t test using SPSS 13.0 software. Data were represented as mean ± SD. P<0.05 was considered statistically significant.

Results

Morphological changes in SK-BR-3 cells

SK-BR-3 breast cancer cells treated with TGF-β1 (10 ng/mL) for 24 h become larger and more messy, the elongated cells extend pseudopodia, the link of the cells became more loosely and cell gap widened after TGF-β1 treatment (**Figure 1**).

Growing and invasion abilities of SK-BR-3 cells

SK-BR-3 cells showed faster growing and improved invasion abilities after TGF-β1 treatment. In wounding healing test, the width of wound decreased significantly, while the rate of wound healing improved after TGF-β1 treatment (**Table 1**, **Figure 2**). The migration ability of TGF-β1 treated SK-BR-3 cells also increased in the invasion test (**Table 2**, **Figure 3**).

Flow cytometry detection

SK-BR-3 cells treated with TGF-β1 showed reduced G_1 phase cells proportion in the total number of cells after the conversion, in contrast the S phase cells accounted for the proportion of the total number of cells increased, there was no difference in the number of apoptotic cells (**Table 3**, **Figure 4**).

IHC studies

TGF-β1 treated SK-BR-3 cells exhibited increased N-cadherin expressing, while the expression of E-cadherin decreased coordinately (**Figures 5**, **6**).

Discussion

Breast cancer is a highly malignant carcinoma, and most deaths of breast cancer are caused by metastasis [15]. Nevertheless, the mechanism of breast cancer metastasis remains unclear [16-19]. The alterations associated with EMT may be related to the cancer cell metastasis [20, 21]. EMT is a process by which an epithelial cell alters its phenotype to that of a mesenchymal cell and plays a critical role in embryonic development [22, 23], tumor invasion and metastasis and tissue fibrosis [24, 25]. EMT occurs in organogenesis throughout embryonic development and is recapitulated

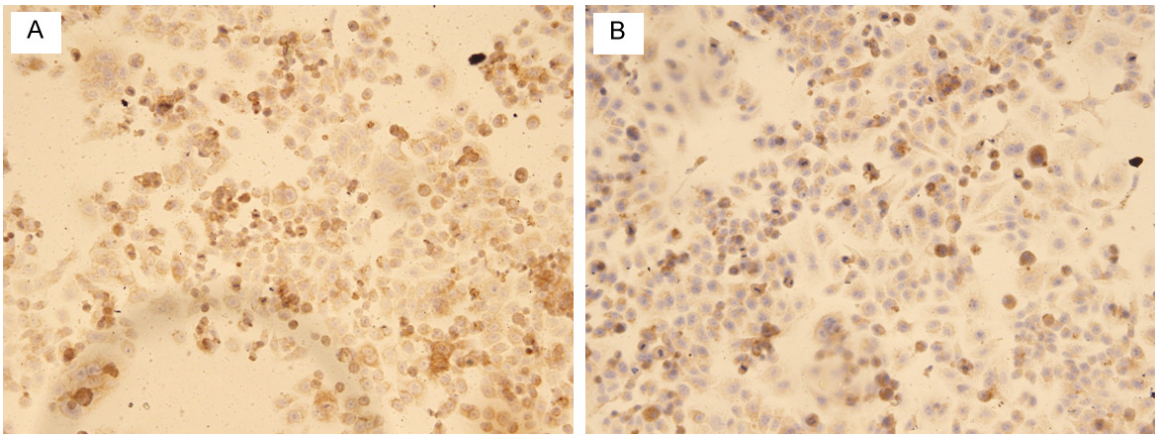

Figure 5. Immunohistochemical analysis of E-cadherin. A. SK-BR-3 cells treated with TGF-β1 (0 ng/ml) for 24 h, strong cytoplasmic and cytomembrane reactivity to E-cadherin. B. SK-BR-3 cells treated with TGF-β1 (10 ng/ml) for 24 h, weak cytoplasmic and cytomembrane reactivity to E-cadherin.

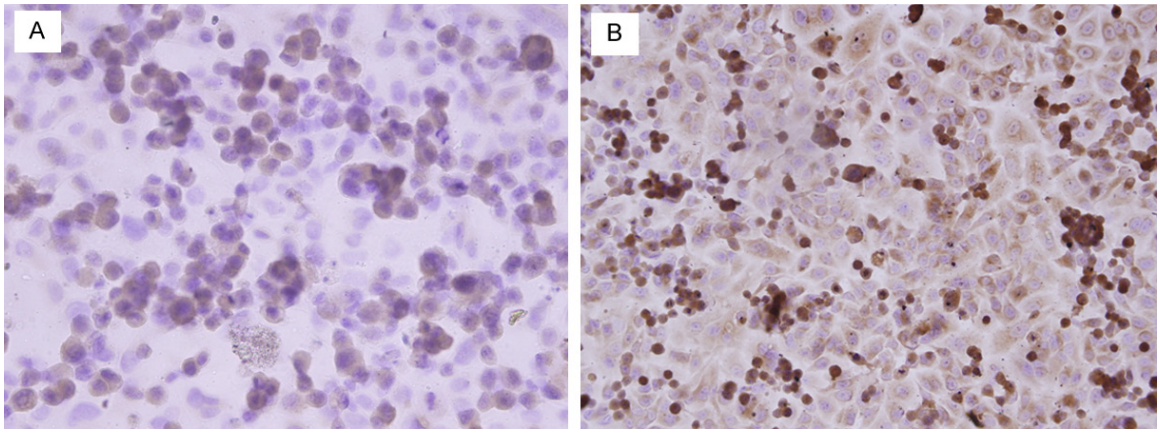

Figure 6. Immunohistochemical analysis of N-cadherin. A. SK-BR-3 cells treated with TGF-β1 (0 ng/ml) for 24 h, weak cytoplasmic reactivity to N-cadherin. B. SK-BR-3 cells treated with TGF-β1 (10 ng/ml) for 24 h, strong cytoplasmic reactivity to N-cadherin.

during epithelial tissue injury and in carcinoma progression [26], and is regulated by complex, precisely orchestrated cell signaling and gene expression networks [27], with the participation of key developmental pathways [28, 29]. Metastasis of tumor cells is associated with EMT, which is a process whereby epithelial cells lose their polarity and acquire new features of mesenchyme [30-32].

TGF-β1 is established as a central mediator involved in tissue repair and the progression of fibrosis as well as inducing EMT in multiple organs [33, 34]. Series of studies were conducted to investigate whether TGF-β1 could induce changes of, such as cell morphology, expression of relative protein markers, and cellular motile and invasive activities [35]. Several elegant studies have provided evidence of TGF-β1-induced EMT in experimental animal fibrosis models and human alveolar epithelium [36-39]. However, its mechanism remains elusive.

The aim of the present study was to determine whether EMT occurs in cultures of human breast cancer cells after TGF-β1 exposure. In this study, we demonstrate that TGF-β1-induced EMT characterized by the transition from a typically epithelial morphology to a spindle-shaped cell, TGF-β1 treated SK-BR-3 cells become larger and more messy, the elongated cells extend pseudopodia, the link of the cells became more loosely and cell gap widened after TGF-β1 treatment. SK-BR-3 cells showed faster growing and

improved invasion abilities after TGF-β1 treatment, G_1 phase cells proportion in the total number of cells were reduced after the conversion, with S phase cells accounted for the proportion of the total number of cells increased. This was confirmed at immunohistochemical level, we further presented the increased expressing mesenchymal markers N-cadherin with a coordinate loss of epithelial proteins E-cadherin.

In conclusion, we provide the first evidence that Human breast cancer SK-BR-3 cells in culture undergo EMT in response to TGF-β1, these findings indicate that TGF-β1-induced EMT in breast cancer cells may be associated with major alterations in morphological and invasion abilities. Further studies are needed to investigate its signal pathways and mechanisms, besides, how these changes in epithelial phenotype affect the progression of the breast cancer metastasis.

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Disclosure of conflict of interest

None.

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