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The effect of TGF-beta-induced epithelial–mesenchymal transition on the expression of intracellular calcium-handling proteins in T47D and MCF-7 human breast cancer cells

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The contribution of Ca^{2+} in TGF-β-induced EMT is poorly understood. We aimed to confirm the effect of TGF-β on the gene expression of intracellular calcium-handling proteins and to investigate the potential underlying mechanisms in TGF-β-induced EMT. T47D and MCF-7 cells were cultured *in vitro* and treated with TGF-β. The mRNA expression of EMT marker genes and intracellular calcium-handling proteins were quantified by qRT-PCR. qRT-PCR and Western blot analysis results verified the changes of EMT marker gene expression. Furthermore, we found that TGF-β induced cell morphological changes significantly with an increase of cell surface area and cell length. These results indicated that TGF-β induced EMT. The mRNA expression levels of SPCA1, SPCA2 and MCU were not influenced by TGF-β treatment, while NCX1 expression was decreased in T47D cells. In addition, the mRNA levels of SERCAs and IP_3Rs were significantly changed due to TGF-β-induced EMT. The TGF-β-treated T47D cells exhibited markedly greater response to ATP than the control cells, and the descent velocity of cytosolic calcium concentration was faster in TGF-β-treated cells than in control cells. This is the first report to demonstrate that TGF-β-induced EMT in human breast cancer cells is associated with alterations in endoplasmic reticulum calcium homeostasis.

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1. Introduction

Breast cancer is the most commonly diagnosed cancer in women worldwide. Metastasis is the leading cause of cancer-related death [1]. One of the critical steps during tumor metastasis is tumor cell migration and invasion. Epithelial-mesenchymal transition (EMT) is characterized by the loss of epithelial junctions and cell polarity and the gain of mesenchymal characteristics, such as fibroblastic-like morphology, migration and invasion ability [2]. EMT is involved in developmental processes and plays a critical role in cancer progression [3]. Most of the cell biomarkers change during EMT in different breast cancer cell lines; epithelial markers (E-cadherin, cytokeratin, ZO-1) are down-regulated, mesenchymal markers (N-cadherin, vimentin, fibronectin) and EMT-inducing transcription factors (Snail, Slug, and Twist) are up-regulated [4]. Downregulation of E-cadherin expression and loss of E-cadherin-related adhesions in epithelial tumor cells are associated with TGF-β stimulated EMT [5,6]. In contrast, over-expression of vimentin alters epithelial cell shape and increases epithelial cell motility [7]. Transforming growth factor beta (TGF-β) is correlated with a high incidence of distant metastasis as TGF-β acts on the tumor cells and the surrounding stroma to promote EMT, extracellular matrix degradation, cell migration and invasion [8]. TGF-β activates the phosphatidylinositol 3-kinase (PI3K) signaling pathway and its downstream effector AKT/PKB to cooperatively induce EMT in breast cancer cells [9].

Abbreviations used: TGF-β, transforming growth factor beta; EMT, epithelial–mesenchymal transition; qRT-PCR, real-time fluorescent quantitative reverse transcription polymerase chain reaction; SPCAs, secretory pathway calcium ATPases; MCU, mitochondrial calcium uniporter; NCX1, sodium/calcium exchanger 1; SERCAs, sarco/endoplasmic reticulum calcium ATPases; IP_3Rs, inositol 1, 4, 5-trisphosphate receptors; PSS, physiological salt solution; SOCE, store-operated Ca^{2+} entry.

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The ubiquitous second messenger Ca^{2+} regulates various cellular processes such as proliferation and migration [10–12]. Intracellular Ca^{2+} signaling has a crucial role in cancer cell migration and invasion [12]. The predominant Ca^{2+} entry mechanism in non-excitable cells is the store-operated Ca^{2+} entry (SOCE). Orai and STIM proteins are responsible for SOCE activation [13]. Orai proteins (Orai1–3) are pore-forming subunits in the plasma membrane and STIM1 is a Ca^{2+} sensor in the endoplasmic reticulum [14,15]. Secretory pathway calcium ATPases (SPCAs) are key regulators of Golgi apparatus luminal calcium pumps. SPCAs are partially responsible for Ca^{2+} transport to the secretory pathway and play an important role in the secretion of calcium into milk [16,17]. The mitochondrial calcium uniporter (MCU) is a distinctive Ca^{2+} selective channel of the inner mitochondrial membrane and a necessary element of the mitochondrial Ca^{2+} uniporter [18]. The sodium/calcium exchanger 1 (NCX1) is a reversible transporter that can move Na^{+} across the membrane in exchange for Ca^{2+} and plays a key role in maintaining Ca^{2+} homeostasis of mitochondria in cardiomyocytes [19].

The endoplasmic reticulum is a chief store of intracellular calcium in epithelial cells and engages in metabolic processes, and calcium is accumulated in the endoplasmic reticulum by sarco/endoplasmic reticulum calcium ATPases (SERCAs) calcium pumps [20]. SERCAs are encodes by three genes (ATP2A1, 2 and 3) in which SERCA1 is expressed in skeletal muscle, SERCA2 is mainly found in cardiac muscle and smooth muscle, and SERCA3 is detected in various cells and tissues [20]. Different endoplasmic reticulum calcium channels in endoplasmic reticulum membrane control the release of Ca^{2+} by multiple Ca^{2+} effectors such as inositol 1, 4, 5-trisphosphate receptors (IP_3Rs) and ryanodine receptors (RyRs) [21]. The IP_3Rs are intracellular Ca^{2+}-release channels located on the endoplasmic reticulum. IP_3 binds to and activates the IP_3R, leading to Ca^{2+} release from the endoplasmic reticulum [22]. ATP-specific P2-purinoceptors (P2Y2) are G protein-coupled receptors that are implicated in the release of intracellular Ca^{2+} from the endoplasmic reticulum by activation of phospholipase C (PLC), IP_3 synthesis and, ultimately, IP_3R binding [23].

Calcium plays an important role in cell migration. One of EMT characteristics is the increase of cell migration and invasion ability. So our hypothesis is that calcium may play a role in TGF-β-induced EMT. However, little is known about the pathological function of Ca^{2+} dysregulation in TGF-β-induced EMT. The intracellular Ca^{2+}concentration is tightly controlled by Golgi apparatus, mitochondrial and endoplasmic reticulum calcium channels, pumps and exchanger. In this study, we demonstrated TGF-β-induced EMT by qRT-PCR, Western blot analysis and morphological analysis. Subsequently, we determined the mRNA levels of Golgi apparatus, mitochondrial and endoplasmic reticulum calcium channels, pumps and exchanger with TGF-β-induced EMT in T47D and MCF-7 human breast cancer cells. We then evaluated the response to ATP in TGF-β-treated T47D cells.

Our data show that variations in mRNA levels of Golgi apparatus, mitochondrial and endoplasmic reticulum calcium channels, pumps and exchanger are associated with TGF-β stimulated EMT in T47D and MCF-7 cells. Furthermore, we demonstrate that TGF-β-treated cells exhibited greater response to ATP than the control cells, and the descent velocity of cytosolic calcium concentration was faster in TGF-β-treated cells than in control cells.

2. Materials and methods

2.1. Cell culture

The human breast cancer cell lines T47D and MCF-7 were obtained from American Type Culture Collection (Manassas, USA). Cells were cultured in 6-well plate in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, USA) supplemented with 10% fetal bovine serum (FBS, Hyclone, USA), 3.75 g/L sodium bicarbonate, 100 U/ml penicillin and 100 μg/ml streptomycin. Cells were cultured at 37 °C in a humidified incubator containing 5% CO_2. Before stimulation with TGF-β (PeproTech, USA), cells were serum-starved (1% fetal bovine serum) for 12 h and stimulated with 5 ng/ml TGF-β for 6 h, 12 h, 24 h (calcium assays: only 24 h) and 48 h (Western blot analysis and cell morphology analysis: only 48 h). For morphological images and measurement of Ca^{2+} responses to ATP, cells were cultured on 10 mm diameter sterile glass coverslips in 6-well plate using the above condition.

2.2. RNA isolation and real-time fluorescent quantitative reverse transcription polymerase chain reaction (qRT-PCR)

Total RNA was extracted by TRIzol reagent (Invitrogen, USA) according to the suggested protocol. RNA (2 μg) was used to synthesize the first-strand cDNA with the Superscript first-strand synthesis system (Invitrogen, USA) for qRT-PCR according to the manufacturer's recommendation. Gene-specific primers for qRT-PCR analysis were synthesized commercially by Beijing Ruibo Biotechnology Company, China (Table 1). The qRT-PCR was performed using the SYBR Green qPCR Kits (Invitrogen, USA) and Mx3000P continuous fluorescence detection system (Agilent Stratagene, USA) according to the manufacturer's instruction. All results were obtained from at least three independent experiments using different RNA samples. Relative quantification was calculated with reference to GAPDH and analyzed using the comparative C_T method.

2.3. Western blot analysis

After washing with ice-cold PBS, T47D and MCF-7 cells were lysed in RIPA lysis buffer. Protein concentrations were determined by Biodropsis BD-2000 Ultra Micro Ultraviolet Spectrophotometer (Biodropsis Technologies, Merinton Ltd., China). Total cellular protein (30 μg/lane) was separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were transferred to polyvinylidene difluoride membrane (Millipore, Billerica, USA) and the membranes were blocked in 5% skim milk dissolved in TBST (10 mM Tris-Base, 0.15 M NaCl, 0.05% Tween-20, pH 7.6). The membranes were then incubated with the primary antibody (1:1000 dilution of anti-E-cadherin antibody or anti-vimentin antibody) at 4 °C overnight, washed three times with TBST and followed by incubation with secondary antibody (HRP-conjugated rabbit anti-goat IgG, Thermo Scientific) at a dilution 1:1000 and HRP-conjugated anti-GAPDH antibody (1:10,000) at 37 °C for 1 h. After washing three times with TBST, the immunoblot was visualized with SuperSignal® West Dura Extended Duration Substrate (Thermo Scientific) and detected by MiniChemi II Chemiluminescence Imaging System (SageCreation, Beijing, China).

Polyclonal antibodies against E-cadherin and vimentin were obtained from Bioworld Technology (MN, USA). Monoclonal antibody against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was obtained from KangChen Bio-tech (Shanghai, China).

2.4. Cell morphology and measurement

After treatment, cells were washed with fresh medium to remove dead cells. Images were acquired by phase contrast microscope (IX71, Olympus, Japan) using a 20 × objective. The area, major axis and minor axis were measured using ImageJ software (NIH, USA). The aspect ratio of major axis/minor axis was calculated by Microsoft Office Excel 2010.

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