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AKT/GSK-3β regulates stability and transcription of snail which is crucial for bFGF-induced epithelial–mesenchymal transition of prostate cancer cells

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ABSTRACT

Background: Epithelial–mesenchymal transition (EMT) plays a pivotal role in the development of metastatic cancers. Basic fibroblast growth factor (bFGF) is significantly elevated in metastatic prostate cancers, which has been mentioned mainly to induce EMT in normal cells. However, there is no description about bFGF induced EMT and its underlying mechanism in prostate cancer cells.
Methods: Western blotting, immunofluorescence and qRT-PCR assays were used to study protein or mRNA expression profiles of the EMT. Wound healing scratch, migration and invasion assays were used to test the motility of cells undergoing EMT. More methods were used to explore the underlying mechanisms.
Results: We demonstrated that bFGF promoted EMT and motility of human prostate cancer PC-3 cells. Both protein and mRNA expression of Snail were rapidly increased after bFGF treatment. Ectopic expression of Snail triggered EMT and enhanced cell motility in PC-3 cells, and knockdown of Snail almost abolished bFGF induced EMT, suggesting the critical role of Snail. Mechanistic study demonstrated that bFGF promoted the stability, nuclear localization and transcription of Snail by inhibiting the activity of glycogen synthase kinase 3 beta (GSK-3β) through phosphatidylinositide 3 kinases (PI3K)/protein kinase B (AKT) signaling pathway.
Conclusions: It is concluded that bFGF can promote EMT and motility of PC-3 cells, and AKT/GSK-3β signaling pathway controls the stability, localization and transcription of Snail which is crucial for this bFGF induced EMT.
General significance: To our knowledge, this is the first study to demonstrate that bFGF can induce EMT via AKT/GSK-3β/Snail signaling pathway in prostate cancer cells.

1. Introduction

Basic fibroblast growth factor (bFGF) is a growth factor that belongs to a large FGF family [16], and fulfills its functions mainly through activation of receptors [49]. bFGF activation of its receptors initiates FGF signaling cascades which are susceptible to hijack by cancer cells [32]. Recently, there is evidence from multiple cancer types to implicate FGF signaling in several oncogenic behaviors, including invasion and migration [31]. This promotes therapeutic targeting of FGFs and their receptors becoming a major area of drug development research [43].

Epithelial–mesenchymal transition (EMT) is one of multi-step events for cancer cell invasion and migration which occurs at the invasive front of many metastatic cancers [11,42]. Cells undergoing EMT exhibit a fibroblastic-like phenotype, acquire mesenchymal components and motile features, loss of epithelial components and cell adhesion [28]. Several transcription factors have been implicated in the control of EMT, and Snail, a zinc finger transcription factor has been proved as the key EMT regulator [30,35]. Snail binds to the promoter of E-cadherin gene and represses its transcription, which is one of the hallmark events of EMT and thought to suppress metastasis [17]. The important role of Snail in EMT regulation has been described in many cancer types [3,9,23,36]. Ectopic expression of Snail alone can trigger EMT and enhance cell motility in cancer cells [3,45]. Meanwhile, knockdown of Snail at least partially inhibits EMT and motility triggered by different stimuli [33,45,46].

Prostate cancer continues to be one of the most commonly diagnosed cancers in men in recent years [39]. The majority of deaths associated with prostate cancer are attributed to the failure to cure metastatic disease [41]. There are ample evidences that EMT plays a

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pivotal role in the development of metastatic prostate cancer [25,29]. Significantly increased plasma level of bFGF is found in metastatic prostate cancer patients [15]. In addition, bFGF is mentioned to induce EMT, but mainly in normal epithelial cells [8,24,37,40]. Thus, we wondered if bFGF is able to induce EMT in human prostate cancer cells and what are the underlying mechanisms. In this study, we revealed that bFGF can induce EMT in human prostate cancer PC-3 cells, and AKT/GSK-3β/Snail signaling pathway is crucial for this process.

2. Materials and methods

2.1. Chemicals and reagents

PI3K inhibitor LY294002, p38 MAPK inhibitor SB203580, TGF-β/Smad2 inhibitor SB431542 and JAK/Stat3 inhibitor AG490 and proteasome inhibitor MG132 were obtained from Sigma-Aldrich (St Louis, MO). Primary antibodies against E-cadherin, Snail, p-GSK-3β (Ser9), GSK-3β, p-Akt (Ser473), Akt, p-p38 (Thr180/Tyr182), p38, p-Smad2 (Ser465/467), Smad2, p-Stat3 (Tyr705), Stat3 and β-catenin were obtained from Cell Signaling Technology (MA, USA). Primary antibody against H2A.X was obtained from Bioworld (Bioworld Technology, Minneapolis, MN, USA). Protein A/G Sepharose and primary antibodies against N-cadherin, fibronectin, ubiquitin, β-actin and α-tubulin were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Horseradish peroxidase (HRP)-conjugated secondary antibody, Alexa Fluor 488/594 conjugated secondary antibody, DAPI and Lipofectamine 2000 were purchased from Invitrogen (Carlsbad, CA, USA). Recombinant human bFGF protein was obtained from PeproTech. PrimeScript® RT Reagent Kit and SYBR® Premix Ex Taq™ were products of TaKaRa. E.Z.N.A® HP Total RNA Kit was purchased from Omega Bio-Tek (Doraville, USA). Smart pool siRNA against human Snail (siSnail) and control (siNC) were purchased from RiboBio (Guangzhou, China). Vectors (pGL3-Basic and pRL-TK) and dual-luciferase assay kit were products of Promega (Madison, WI, USA). pGL3-Snail-luc reporter gene plasmid was previously constructed and examined in our group [18].

2.2. Cell culture

The PC-3 cell line was obtained from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). Cells were maintained in RPMI 1640 culture medium (Gibco BRL) supplemented with heat-inactivated endotoxin-free 10% fetal bovine serum, 100 μg/ml streptomycin and 100 units/ml penicillin under a humidified 5% CO_2 atmosphere at 37 °C in incubator.

2.3. Wound healing scratch assay

Cells were grown as monolayers in triplicates in 12-well plates (2×10^5/well) until confluent. Cells were then pre-treated with or without bFGF for 24 h and an artificial scratch wound was created. Cell debris was removed by washing with PBS. Cells were then cultured for additional 24 h. Cell migration was photographed and the width of the wound was measured.

2.4. Migration and invasion assay

Migration and invasion assays were performed in Boyden chambers [2]. The polycarbonate filters (8 μm pore size, Corning) pre-coated with Matrigel Matrix (BD Biosciences) were used for invasion assay, and uncoated filters were used for migration assay. Cells (1×10^5) in 300 ml medium (containing 0.1% FBS) pre-treated with or without 20 ng/ml bFGF for 24 h were seeded in the upper chamber. Then 600 ml medium with 10% FBS was added to the lower chamber and served as a chemotactic agent. After 24 h of incubation, for migration assay, the cells migrated and adhered onto the lower chamber were fixed in 4% paraformaldehyde for 20 min, stained with hematoxylin and counted under upright microscope (5 fields per chamber). For invasion assay, the cells in the upper chamber were fixed in 4% paraformaldehyde for 20 min. Then the Matrigel was mechanically removed from the filter with a cotton swab. The cells adhering to the under-side of the filter were stained with hematoxylin and counted under upright microscope (5 fields per chamber). Each migration and invasion assay was repeated in three independent experiments.

2.5. Preparation of nuclear extracts

Nuclear extract was prepared according to the method of Schreiber et al. [38]. Cells were washed with PBS, scraped and pelleted by centrifugation, resuspended in the cell lysis buffer [10 mM HEPES (pH 7.5), 10 mM KCl, 0.1 mM EDTA, 1 mM dithiothreitol (DTT), 0.5% Nonidet-40 and 0.5 mM PMSF along with the protease inhibitor cocktail] and allowed to swell on ice for 15-20 min with intermittent mixing. Tubes were vortexed to disrupt cell membranes and then centrifuged at 12,000 *g* at 4 °C for 10 min. The supernatant was stored at −70 °C till further use as cytoplasmic extract. The pelleted nuclei were washed thrice with the cell lysis buffer, resuspended in the nuclear extraction buffer [20 mM HEPES (pH 7.5), 400 mM NaCl, 1 mM EDTA, 1 mM DTT, 1 mM PMSF with protease inhibitor cocktail] and incubated in ice for 30 min. Nuclear extract was collected by centrifugation at 12,000 *g* for 15 min at 4 °C. The protein level in each fraction was estimated using Bradford's reagent (BioRad, USA). Each extract was either immediately used or stored at −70 °C till further use.

2.6. Western blotting analysis

Cells were washed three times with ice-cold phosphate buffer solution (PBS) and lysed in cell lysis buffer containing 1% NP-40, 20 mM Tris–HCl (pH 7.6), 0.15 M NaCl, 3 mM EDTA, 3 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 20 mg/ml aprotinin, and 5 mg/ml leupeptin. Lysates were cleared by centrifugation and denatured by boiling in loading buffer. Equal amounts of protein samples were separated on 10% sodium dodecyl sulfate (SDS)-polyacrylamide gels and electrophoretically transferred to PVDF membranes. Following blocking with 5% non-fat milk at room temperature for 2 h, membranes were incubated with the primary antibody at 1:1000 dilution overnight at 4 °C and then incubated with a horseradish peroxidase-conjugated secondary antibody at 1:5000 dilution for 2 h at room temperature. Specific immune complexes were detected using Western blotting Plus Chemiluminescence Reagent (Life Science, Inc., Boston, MA).

2.7. Immunoprecipitation

Cells were washed three times with ice-cold PBS and harvested at 4 °C in immunoprecipitation lysis buffer containing 1% NP-40, 20 mM Tris-HCl (pH 7.6), 0.15 M NaCl, 3 mM EDTA, 3 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 20 mg/ml aprotinin, and 5 mg/ml leupeptin. Equal amounts of protein were immunoprecipitated using anti Snail antibody, and the immune complexes were bound to protein A/G Sepharose. The beads were washed with lysis buffer for five times and subjected to Western blotting with anti-ubiquitin, anti-Snail or anti-β-catenin antibody.

2.8. Immunofluorescence

Cells were cultured on confocal dishes and then exposed to bFGF for the indicated time. Cells were washed three times with PBS, fixed with 4% paraformaldehyde for 20 min and permeabilized with 0.3% Triton X-100 for 10 min. After blocking with goat serum for 2 h at room temperature, cells were incubated with antibodies against E-cadherin, N-cadherin, fibronectin and Snail (1:100 dilution) at 4 °C overnight. Dishes were washed three times with PBS and incubated with Alexa Fluor 488 or Alexa Fluor 594-conjugated secondary antibodies