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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

(1:1000 dilution) for 1 h at room temperature. Nuclei were stained with DAPI (10 mg/ml) for 10 min. Samples were examined with Confocal Laser Scanning Microscopy (Zeiss) to analyze expression of E-cadherin, N-cadherin, fibronectin, and nuclear translocation of Snail.

2.9. Gene over-expression and RNA interference

Cells were seeded on a 6-well plate (2×10^5 cells/well) and cultured for 24 h. They were then transfected with 2 μg plasmid vector or 100 pmol siRNA mixed with Lipofectamine 2000 reagent in serum reduced medium according to the manufacturer's instructions. Medium was changed to complete culture medium 4 h later, and the cells were incubated at 37 °C in a CO_2 incubator for another 24 h before harvest.

2.10. Quantitative real-time PCR

Total mRNA of the cells was extracted after treatment for the indicated time. First strand cDNA synthesis was generated from 500 ng of total RNA. Quantification of target and reference (GAPDH) genes was performed in triplicate on LightCyclerH 480 II (Roche, Applied Science). The primers used in each reaction were as follows: E-cadherin forward 5′-TAC ACT GCC CAG GAG CCA GA-3′ and reverse 5′-TGG CAC CAG TGT CCG GAT TA-3′; N-cadherin, forward 5′-CGA ATG GAT GAA AGA CCC ATC C-3′ and reverse 5′-GGA GCC ACT GCC TTC ATA GTC AA-3′; fibronectin, forward 5′-CCC AGA CTT ATG GTG GCA ATT C-3′ and reverse 5′-AAT TTC CGC CTC GAG TCT GA-3′; Snail, forward 5′-GAC CAC TAT GCC GCG CTC TT-3′ and reverse 5′-TCG CTG TAG TTA GGC TTC CGA TT-3′; GAPDH, forward 5′-GCA CCG TCA AGG CTG AGA AC-3′ and reverse 5′-TGG TGA AGA CGC CAG TGG A-3′. After normalized to GAPDH gene, expression levels for each target gene were calculated using the comparative threshold cycle (CT) method. The Dct values were calculated according to the formula $\Delta ct = ct$ (gene of interest) $-$ ct (GAPDH) in correlation analysis, and the $2\text{-}\Delta\Delta ct$ was calculated according to the formula $\Delta\Delta ct = \Delta ct$ (control group) $- \Delta ct$ (experimental group) for determination of relative.

2.11. Dual-luciferase reporter gene assay

Cells cultured on 6-well plates were transiently co-transfected with pGL3-Snail-luc (2 μg) and pRL-TK (0.5 μg). After 24 h, these cells were treated with bFGF for the indicated time. Transcriptional activity was determined by the dual-luciferase reporter assay system. Results were calculated as the ratios between the activity of pGL3-Snail-luc and pRL-TK.

2.12. Statistical analysis

Results were expressed as mean ± standard deviation (SD) of three independent experiments unless otherwise specified. Data were analyzed by two-tailed unpaired Student's *t*-test between any two groups. These analyses were performed using GraphPad Prism Software Version 5.0 (GraphPad Software Inc., La Jolla, CA). A P-value of <0.05 was considered as statistically significant.

3. Results

3.1. bFGF induces EMT and promotes motility of PC-3 cells

Prostate cancer cells preferentially metastasize to bone to form bone metastases, which are a major cause of morbidity for men with prostate cancer [21]. As one of the three most used cell lines (PC-3, DU145 and LNCap) in prostate cancer research, PC-3 cells were initially isolated from the bone metastases of prostate cancer while the others were not [26]. Thus, PC-3 cells were used in the following studies. After treatment with bFGF (20 ng/ml) for 24 h, PC-3 cells became scattered and showed a typical fibroblast-like morphology of mesenchymal cells compared to controls (Fig. 1A). Furthermore, significant up-regulation of N-cadherin, fibronectin and down-regulation of E-cadherin protein and mRNA levels were observed compared to controls (Fig. 1B-D). We further studied the motility of PC-3 cells that underwent EMT induced by bFGF. Data in Fig. 2A and B showed that the wound healing ability, and the number of migrated and invaded cells were increased more than two folds in PC-3 cells after bFGF treatment for 24 h than controls (Fig. 2C and D).

3.2. Snail is crucial for bFGF induced EMT in PC-3 cells

Snail acts as the most important organizer regulating EMT by down-regulating the epithelial genes and up-regulating the mesenchymal genes [30]. Therefore, we first studied whether Snail was activated by bFGF. Results in Fig. 3A and B revealed that both protein and mRNA levels of Snail were elevated after 24 h bFGF exposure compared to controls. Then we overexpressed Snail to further investigate its roles in bFGF induced EMT of PC-3 cells. In accordance with the previous findings [44], our results confirmed that aberrant Snail expression can induce EMT-like morphological changes and E-cadherin down-expression in PC-3 cells. Except this, we else found that EMT marker N-cadherin and fibronectin were up-regulated and cell motility was enhanced (Fig. 3C and D). Based on these observations, we speculated that Snail may be crucial for bFGF induced EMT in PC-3 cells.

To verify the key role of Snail in the EMT process triggered by bFGF, Snail was almost knocked down by RNA interfering technology. It was found that bFGF induced morphological changes were almost abolished when Snail was knocked down (Fig. 4A). And the expression of EMT markers was almost reversed compared to siNC groups (Fig. 4B). It indicated that Snail mediated the bFGF-induced EMT in PC-3 cells. In addition, it was observed that the percentage of wound healing, and the number of migrated and invaded cells were dramatically decreased almost to normal level compared to siNC groups after the knockdown of Snail with bFGF treatment (Fig. 4B-D).

3.3. bFGF regulates stabilization and nuclear localization of Snail in PC-3 cells

It was observed that Snail expression was significantly increased within as short as 1 h (Fig. 5A). Therefore, we wondered if the stabilization of Snail was responsible for the rapid Snail accumulation. It is known that GSK-3β has been characterized as a main kinase to phosphorylate Snail, thereby promotes its degradation via ubiquitin–proteasome pathway and modulates its localization by inducing its export to the cytoplasm [4,50]. Therefore, we first detected if the activity of GSK-3β was influenced by bFGF. Results showed that phosphorylated GSK-3β was rapidly increased in PC-3 cells after bFGF treatment within 2 h (Fig. 5B). It indicated that bFGF inhibited the activity of GSK-3β, which might be responsible for rapid Snail accumulation. Thus, we hypothesized that the inhibition of GSK-3β activity by bFGF might repress Snail ubiquitination and export from unclear. Data in Fig. 6A showed that proteasome inhibitor MG132 increased the ubiquitination of Snail compared to control, and bFGF treatment suppressed the ubiquitination of Snail compared to MG132 treatment alone group. It indicated that ubiquitin mediated degradation regulates the stability of Snail and bFGF treatment suppresses the ubiquitination of Snail, thereby increases the stabilization of Snail in PC-3 cells. Furthermore, it was found that rapidly accumulated Snail was almost located in nucleus rather than cytoplasm in PC-3 cells treated with bFGF (Fig. 6B). Immunofluorescence results confirmed above observations (Fig. 6C). Similar phenomenon was observed in β-catenin which also can be regulated by GSK-3β (Fig. 6B). It further suggested that GSK-3β was the key molecular for the stabilization and localization of Snail. GSK-3β binds to and phosphorylates Snail to induce its cytoplasmic translocation and

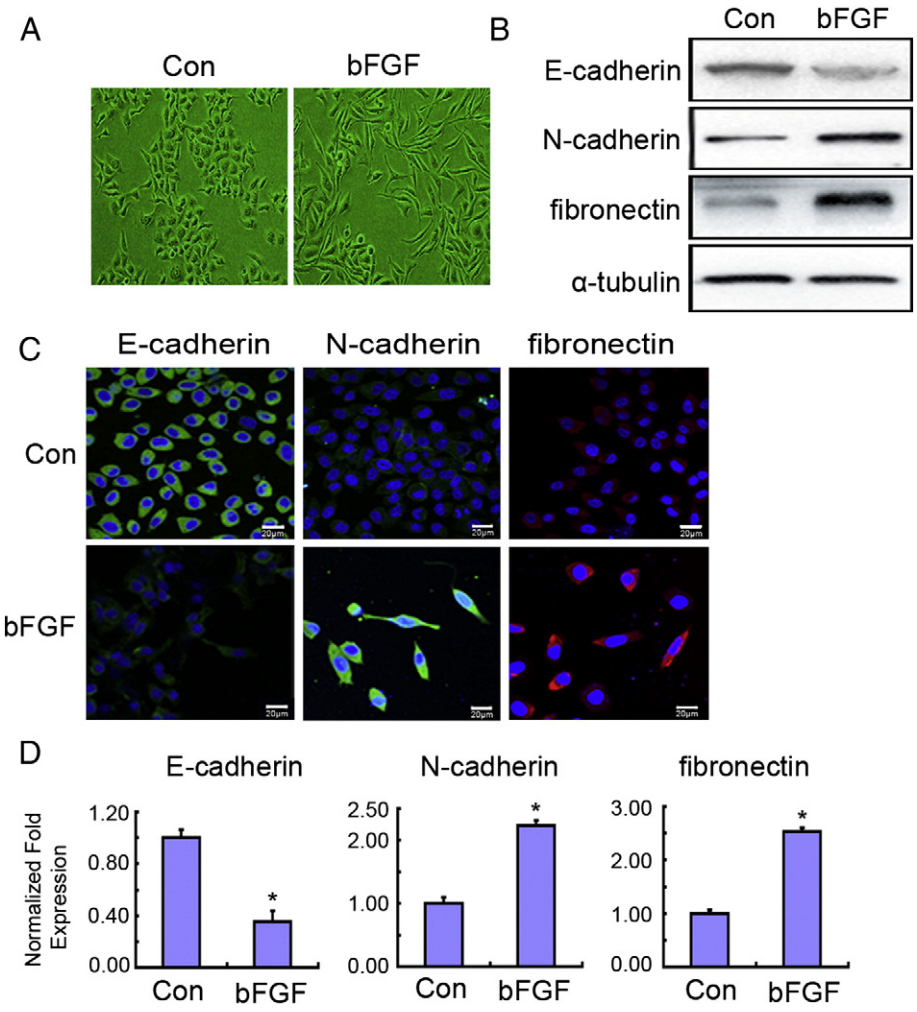

Fig. 1. bFGF induces EMT in PC-3 cells. (A) Cells were treated with or without bFGF (20 ng/ml) for 24 h, and then the morphological changes of EMT in PC-3 cells were detected by a phase contrast microscope. (B) Cells were treated with or without bFGF (20 ng/ml) for 24 h, and the expression of E-cadherin, N-cadherin, and fibronectin was analyzed by Western blotting. α-tubulin servers as the loading control. (C) PC-3 cells were treated with or without bFGF (20 ng/ml) for 24 h. Expression of E-cadherin, N-cadherin and fibronectin was analyzed by immunofluorescence staining. Nuclei were visualized with DAPI staining. Scale bars: 20 μm. (D) Cells were treated with or without bFGF (20 ng/ml) for 24 h. The mRNA levels of E-cadherin, N-cadherin and fibronectin were analyzed by qRT-PCR. Data represent the average of three independent experiments. *$p < 0.05$ compared with control.

degradation. Results in Fig. 6D confirmed that the protein level of GSK-3β binded with Snail was decreased after bFGF treatment for 1 h.

3.4. AKT/GSK-3β signaling pathway regulates stability of Snail in PC-3 cells

PI3K/AKT signaling pathway is known to inhibit GSK-3β activity and promote Snail protein stability. To confirm this notion, we detected the protein levels of p-AKT and AKT in PC-3 cells after bFGF treatment. It was observed that phosphorylated AKT was gradually increased in PC-3 cells after bFGF treatment within 2 h (Fig. 7A). Furthermore, inhibitors were used to test the potential roles of PI3K/AKT signaling in the regulation of phosphorylated GSK-3β induced by bFGF. It was showed that PI3K/AKT signaling inhibitor LY294002 abrogated the protein levels of phosphorylated GSK-3β and Snail after bFGF treatment (Fig. 7B). It suggested that PI3K/AKT signaling pathway was responsible for the inhibition of GSK-3β activity induced by bFGF treatment. bFGF is mentioned to activate several signaling pathways like p38 MAPK, TGF-β/Smad2 and JAK/Stat3 signaling pathways which are also involved in Snail regulation [6,10,14,27,34,47]. It was confirmed that bFGF could promote the phosphorylation of p38, Smad2 and Stat3 in PC-3 cells (Fig. 7C). We wondered if the activation of p38, TGF-β/Smad2 and JAK/Stat3 signaling pathways was also involed in the regulation of Snail. Inhibitors were used to study the roles of activated p38, TGF-β/Smad2 and JAK/Stat3 signaling pathways in Snail regulation. Results in Fig. 7D demonstrated that p38 inhibitor SB203580, Smads inhibitor SB431542 and Stats inhibitor AG490 all failed to inhibit the upregulation of Snail induced by bFGF in PC-3 cells. It indicated that p38, TGF-β/Smad2 and JAK/Stat3 signaling pathways were not involved in the up-regulation of Snail induced by bFGF in PC-3 cells.

3.5. AKT/GSK-3β signaling pathway regulates transcription of Snail in PC-3 cells

To study the effects of bFGF on transcription of Snail, we further detected the mRNA expression of Snail in PC-3 cells after bFGF treatment. Results showed that Snail mRNA expression was gradually increased in PC-3 cells after bFGF treatment within 2 h (Fig. 8A). It indicated that bFGF can also rapidly modulate the transcription of Snail in PC-3 cells. We wondered if the inhibition of GSK-3β activity was also responsible for the increased transcription of Snail. Interestingly, after inhibition of GSK-3β activity using PI3K/AKT signaling inhibitor LY294002, the up-regulation of Snail mRNA was abrogated compared to group with bFGF alone (Fig. 8B). Furthermore, results from dual-luciferase reporter assay revealed that pGL3-Snail-luc activity was significantly activated after bFGF treatment for 1 h in PC-3 cells, and pre-treatment with PI3K/AKT signaling inhibitor LY294002 abolished this activation (Fig. 8C).

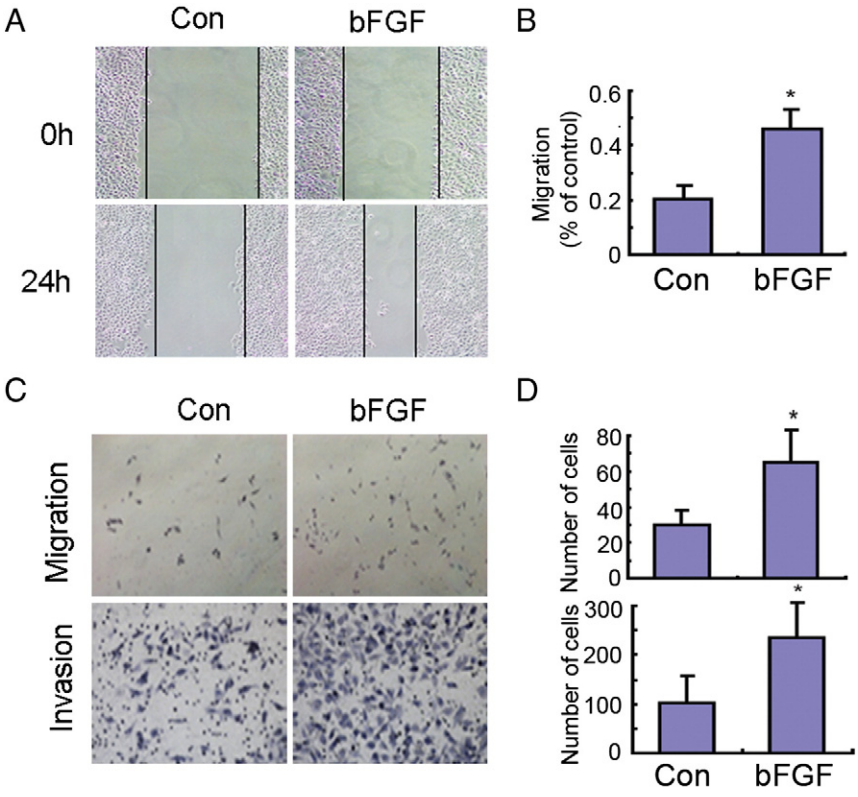

Fig. 2. bFGF promotes migration and invasion of PC-3 cells that underwent EMT. (A) Cells were pre-treated with or without bFGF (20 ng/ml) for 24 h, then cells were used to perform wound healing scratch assay for additional 24 h, representative phase-contrast images of cells migrating into the wounded area at time indicated were recorded. Magnification, 100×. (B) The percentage of wound healing was measured at 24 h. (C) Cells were pre-treated with or without bFGF (20 ng/ml) for 24 h, then cells were harvested to perform invasion and migration assays for additional 24 h. Cells that had migrated into the lower chamber or invaded through the matrix gel into the under-side of the filter were fixed, stained, and photographed. Magnification, 200×. (D) The number of migrated and invaded cells was recorded. Data represent the average of three independent experiments. *$p < 0.05$ compared with control.

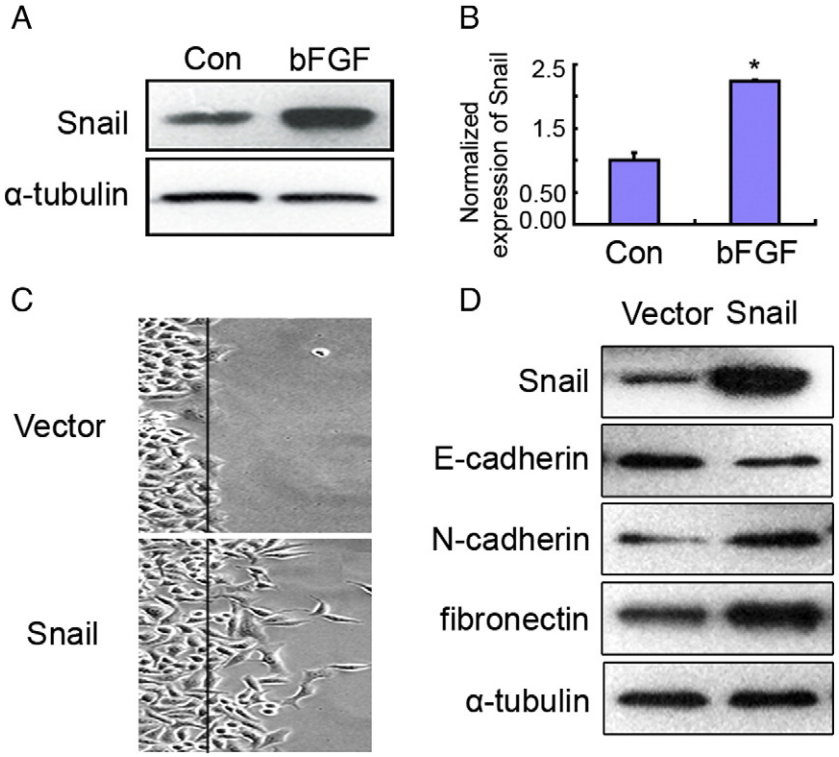

Fig. 3. Snail is crucial for bFGF induced EMT in PC-3 cells. (A) Cells were treated with or without bFGF (20 ng/ml) for 24 h, then the expression of Snail was analyzed by Western blotting. (B) Cells were treated with or without bFGF (20 ng/ml) for 24 h. The mRNA level of Snail was analyzed by qRT-PCR. (C) Cells were transfected with pcDNA-Snail (Snail) or control vector pcDNA-3.1 (vector) for 24 h. Cell morphological changes and wound healing ability were recorded by a phase contrast microscope, magnification, 200×. Then cells were harvested for the Snail, E-cadherin, N-cadherin and fibronectin detection by Western blotting (D). Data represent the average of three independent experiments. *$p < 0.05$ compared with control.

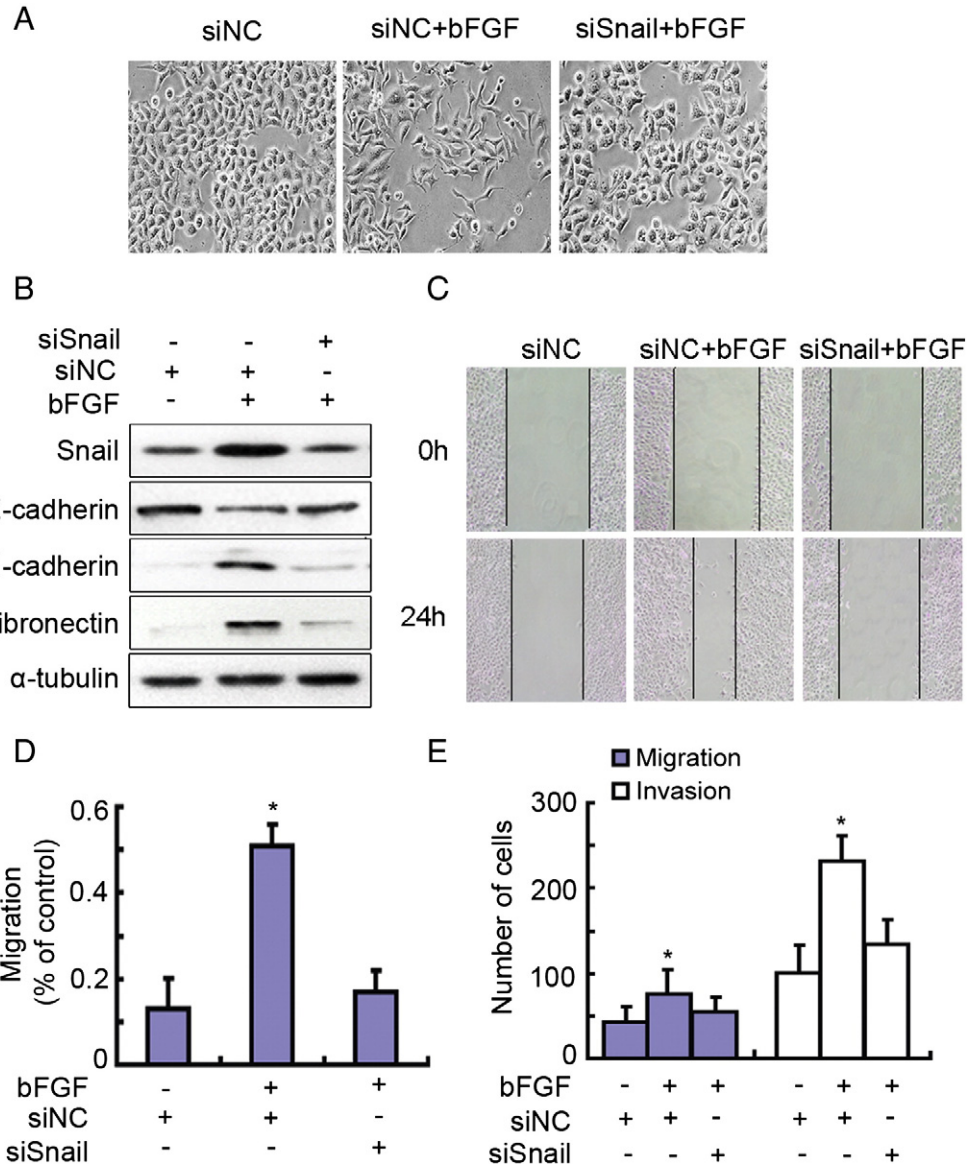

Fig. 4. Snail is crucial for the enhanced motility of PC-3 cells induced by bFGF. (A) After 24 h pre-transfection with si-Snail or si-NC siRNAs, cells were further treated with or without bFGF (20 ng/ml) for 24 h, and then the morphological changes of EMT in PC-3 cells were detected by a phase contrast microscope. And the expression of Snail, E-cadherin, N-cadherin and fibronectin was detected by Western blotting (B). (C) After 24 h pre-transfection with si-Snail or si-NC siRNAs, cells were further pre-treated with or without bFGF (20 ng/ml) for 24 h, then cells were used to perform wound healing scratch assay for additional 24 h, representative phase contrast images of cells migrating into the wounded area at time indicated were recorded. Magnification, 100×. (D) The percentage of wound healing was measured at 24 h. (E) After 24 h pre-transfection with si-Snail or si-NC siRNAs, cells were further pre-treated with or without bFGF (20 ng/ml) for additional 24 h. Then, cells were harvested to perform migration and invasion assays for 24 h. Cells that had migrated into the lower chamber or invaded through the matrix gel into the under-side of the filter were fixed, stained, and calculated. Data represent the average of three independent experiments. $^{*}p < 0.05$ compared with control.

4. Discusion

Prostate cancer is the most common malignancy and the second leading cause of cancer deaths in men in the USA [22]. It underscores the need for a more thorough molecular understanding of this resilient disease [1]. The lethal consequences of prostate cancer are related to its metastasis to other organ sites. It has been suggested that EMT is co-opted by prostate cancer cells during their metastatic dissemination from a primary organ to secondary sites [25,29]. bFGF is recorded to induce EMT mainly in normal tissue cells [24,40]. While there is still no report about bFGF induced EMT in prostate cancer cells. In metastatic prostate cancer patients, the plasma levels of bFGF are significantly elevated, which inspires us that bFGF may induce EMT in prostate cancer cells. In this study, our results indicated that bFGF induces EMT in prostate cancer PC-3 cells with downregulated expression of epithelial marker E-cadherin, upregulated mesenchymal marker N-cadherin and fibronectin. bFGF induced EMT in normal epithelial cells contributes to the cell differentiation or fibrosis. In this study, bFGF induced EMT conferred PC-3 cancer cells with mesenchymal phenotype and enhanced motility. To our knowledge, this is the first evidence to indicate that bFGF is able to promote the motility of prostate cancer cells through EMT.

Several transcription factors have been implicated in the control of EMT, Snail is a zinc-finger transcription factor which has been known as an essential player in EMT of prostate cancer cells including PC-3 cells [23,46]. Snail is aberrantly expressed in cytokines such as transforming growth factor beta (TGF-β) and tumor necrosis factor alpha (TNF-α) induced EMT, and function as the key organizer [48]. Previous studies found that Snail was aberrantly expressed and critical in TNF-α induced EMT in prostate cancer PC-3 and colon cancer cells [45,46]. Snail is upregulated in bFGF induced EMT in normal tissue cells or colon cancer HT29 and DLD-1 cells [37,40]. However, no study

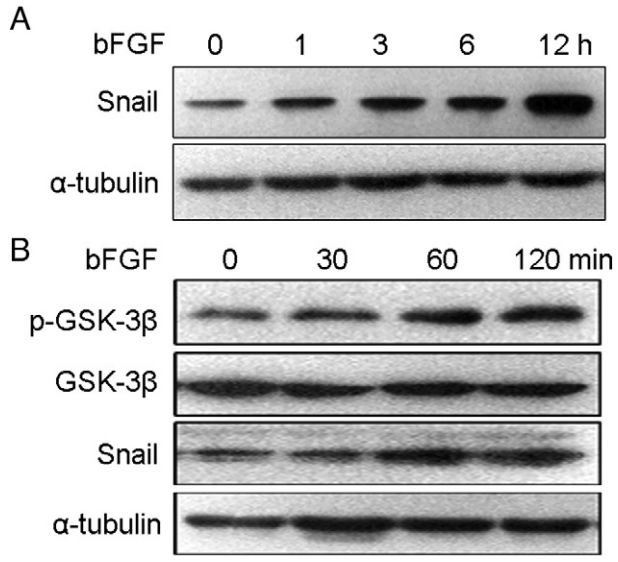

Fig. 5. bFGF induces rapid Snail accumulation and GSK-3β inhibition in PC-3 cells. (A) Cells were treated with bFGF (20 ng/ml) for the times indicated, then the expression of Snail was analyzed by Western blotting. (B) Cells were treated with bFGF (20 ng/ml) for the times indicated, then the expression of Snail, p-GSK-3β and GSK-3β was analyzed by Western blotting. α-tubulin servers as the loading control. Data represent the average of three independent experiments.

has illustrated the role of Snail in bFGF induced EMT in prostate cancer cells. In this study, our results revealed that Snail was up-regulated and played a critical role in bFGF induced EMT in PC-3 cells. It was found that the protein and mRNA level of Snail exhibited a rapid increase after bFGF stimulation. Similar with our previous findings, ectopic expression of Snail by transfection with pcDNA-Snail plasmid induced the expression of EMT markers, mesenchymal morphology and enhanced motility in PC-3 cells. Furthermore, EMT markers, mesenchymal morphology and enhanced motility induced by bFGF were almost abolished after Snail knock-down by siRNA.

Either enhanced stability or transcription of Snail can lead to Snail accumulation [48]. Snail can be upregulated by different stimuli, however, the underlying mechanisms are different. Previous studies have showed that TNF-α enhances the stability, while Nodal enhances both the stability and transcription of Snail to induce EMT in cancer cells respectively [13,46]. Snail up-regulation induced by FGFs always occur in the transcriptional level through different signaling pathways [12, 37,40]. However, it was indicated that bFGF not only promoted stability but also transcription of Snail to induce EMT in prostate cancer PC-3 cells in this study. Mechanically, bFGF treatment increased phosphorylation of GSK-3β at Ser9 residues, leading to the inhibition of GSK-3β activity and increase in stabilization of Snail. We further confirmed this notion by observing that ubiquitination of Snail was decreased after bFGF treatment in PC-3 cells. In addition, the protein level of GSK-3β binded with Snail was decreased, which may explain the less ubiquitination of Snail. Western blotting and immunofluorescence

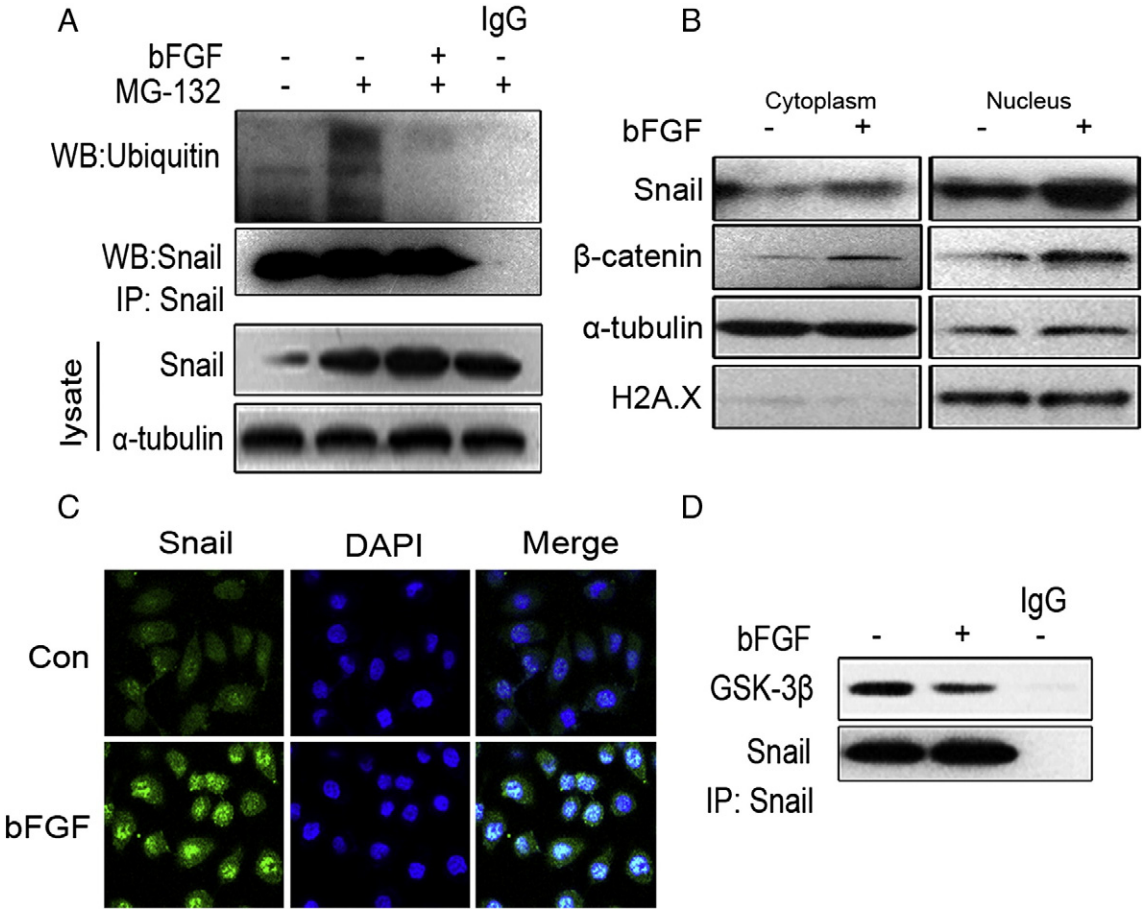

Fig. 6. bFGF regulates stabilization and nuclear localization of Snail in PC-3 cells. (A) Cells were treated with or without bFGF (20 ng/ml) or/and MG132 (10 μM) for 1 h as indicated. Then equal amount of Snail in lysates was immunoprecipitated, the ubiquitination of Snail was examined by western blotting. (B) Cells were treated with or without bFGF (20 ng/ml) for 1 h, Snail and β-catenin located at cytoplasm and nucleus were isolated respectively and then analyzed by western blotting. α-tubulin and H2A.X server as the loading controls for cytoplasm and nucleus proteins respectively. (C) Cells grown on con-focal dishes were treated with or without bFGF (20 ng/ml) for 1 h. Immunofluorescence and con-focal microscopy were performed as described. (D) Cells were treated with bFGF (20 ng/ml) for 1 h. Then equal amount of Snail in lysates was immunoprecipitated, the GSK-3β binded to Snail was examined by western blotting. Data represent the average of three independent experiments.

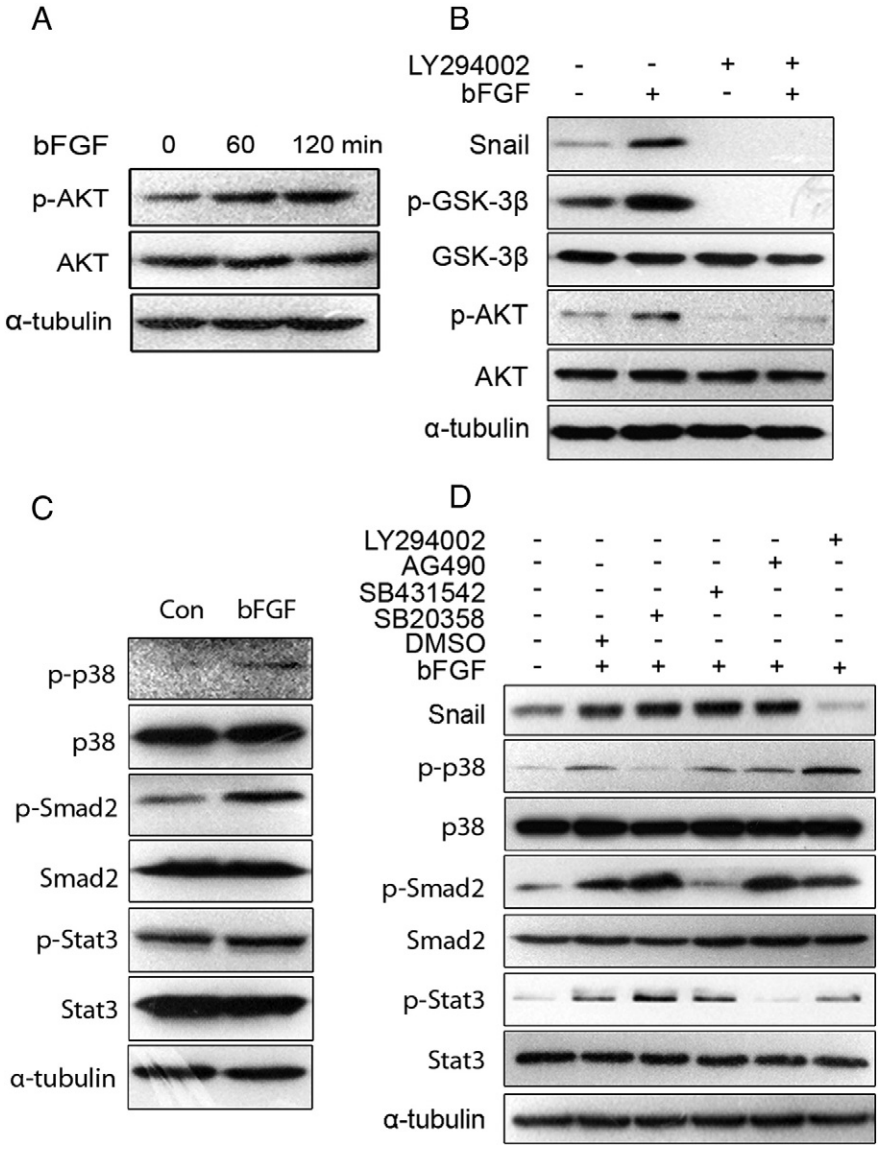

Fig. 7. AKT/GSK-3β signaling regulates stability of Snail in PC-3 cells. (A) Cells were treated with or without bFGF (20 ng/ml) for the times indicated, and the expression of p-AKT and AKT was analyzed by Western blotting. (B) Cells were pre-treated with or without LY294002 (20 μM) for 1 h, following with or without bFGF (20 ng/ml) treatment for additional 1 h. The expression of Snail, p-GSK-3β, GSK-3β, p-AKT and AKT was examined by Western blotting. (C) Cells were treated with or without bFGF (20 ng/ml) for 12 h, several signaling pathway key proteins were analyzed by Western blotting. (D) Cells were pre-treated with or without SB203580 (20 μM), SB431542 (20 μM), AG490 (20 μM) or LY 294002 (20 μM) for 1 h respectively, following with or without bFGF (20 ng/ml) treatment for additional 1 h. The expression of Snail and several signaling pathway key proteins were examined by Western blotting. α-tubulin servers as the loading control. Data represent the average of three independent experiments.

results showed that increased Snail was almost located in nucleus rather than cytoplasm, which facilitates Snail to fulfill its function as a transcription factor. At the transcriptional level, Snail is regulated by many cytokines such as TGF-β and EGF [5]. In this study, it was found that bFGF stimulation also rapidly triggered transcription of Snail. And results from dual-luciferase reporter assay suggested that this increased transcription of Snail might due to the enhanced Snail gene promoter activity induced by bFGF.

AKT/GSK-3β signaling pathway is frequently mentioned to modulate the stability of Snail [44,46]. FGF signaling is mentioned to inhibit GSK-3β activity via PI3K/AKT signaling network [19,20]. Factors like TNF-α and TGF-β are demonstrated to regulate Snail expression via inhibition of GSK-3β activity by PI3K/AKT signaling [7,45]. In this study, it was demonstrated that PI3K/AKT signaling is responsible for bFGF inhibited activity of GSK-3β then Snail up-regulation in PC-3 cells. Signaling pathways like p38 MAPK, TGF-β/Smads and JAK/Stat3 are mentioned to regulate expression of Snail [6,10,14,27,34,47]. However, those signalings do not involve in the up-regulation of Snail, although it was found that bFGF can slightly activate those signalings in PC-3 cells, Since Snail level remains unaffected in the presence of specific inhibitors. Interestingly, it was indicated that PI3K/AKT/GSK-3β signaling pathway not only controls the stability but also transcription of Snail induced by bFGF in PC-3 cells, although GSK-3β is mentioned as an endogenous inhibitor of Snail transcription in some other cellular contexts [4]. We speculated that GSK-3β activity inhibition may change the levels of transcription factors which promote the transcription of Snail induced by bFGF treatment. However, it needs more studies to fully understand this process.

In summary, we demonstrated that bFGF is able to induce EMT in PC-3 cells and Snail plays a critical role. We proposed a model in which bFGF promotes stabilization and transcription of Snail via activation of AKT and subsequent inhibition of GSK-3β activity. GSK-3β activity inhibition decreases the ubiquitination of Snail and increases Snail transcription. Then, up-regulated Snail is mainly stayed in the nucleus and promotes EMT, migration and invasion of PC-3 cells. These discoveries provide a deeper understanding of bFGF induced EMT and

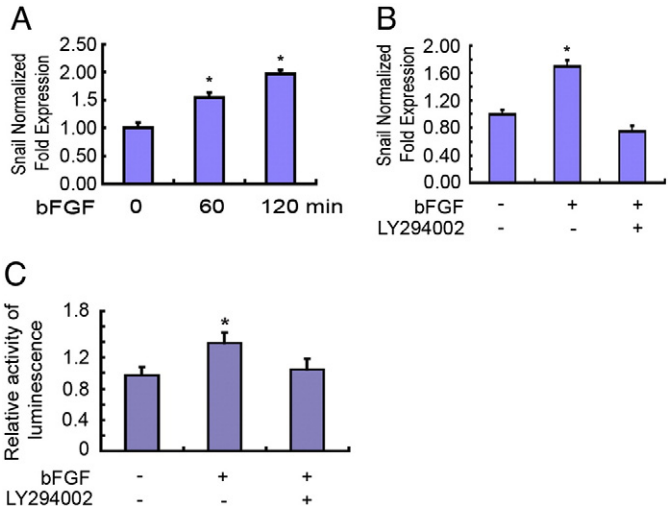

Fig. 8. AKT/GSK-3β signaling regulates transcription of Snail in PC-3 cells. (A) Cells were treated with or without bFGF (20 ng/ml) for the times indicated. The mRNA level of Snail was analyzed by qRT-PCR. (B) Cells were pre-treated with or without PI3K/AKT signaling inhibitor LY 294002 (20 μM) for 1 h, following with or without bFGF (20 ng/ml) treatment for additional 1 h. The mRNA levels of Snail were analyzed by qRT-PCR. (C) After 24 h pre-transfection with pGL3-Snail-luc or pRL-TK reporter gene plasmid, cells then were pre-treated with or without LY 294002 (20 μM) for 1 h, following with or without bFGF (20 ng/ml) treatment for additional 1 h. The relative activity of luminescence was analyzed by dual-luciferase reporter gene assay. Data represent the average of three independent experiments. *$p < 0.05$ compared with control.

introduce potential therapeutic targets for prostate cancer with over-activated FGF signaling.

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