

## Original Article

# Response gene to complement-32 mediates TGF- $\beta$ -induced epithelial-mesenchymal transition via Erk-/p38-MAPK signaling pathways in human pancreatic cancer cell line BxPC-3

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Received March 8, 2017; Accepted May 3, 2017; Epub June 1, 2017; Published June 15, 2017

**Abstract:** Pancreatic cancer (PC) is a highly life-destructive human digestive system carcinoma, the five-year survival of which is less than 6%. The PC progression was believed to be associated with epithelial-mesenchymal transition (EMT) which could be stimulated by TGF- $\beta$ . We previously found that response gene to complement-32 (RGC-32) mediated TGF- $\beta$ -induced EMT. However, the underlying mechanism remained unclear. In the present study, BxPC-3 cells were used and treated with 10 ng/mL TGF- $\beta$ 1 alone or in combination with 10  $\mu$ M U0126/SB203580/LY294002. The mRNA and protein expressions of RGC-32, E-cadherin and vimentin were analyzed by qRT-PCR and Western blot. The siRGC-32 was introduced to measure the expression of Snail and Zeb1 in BxPC-3 cells treated with or without TGF- $\beta$ 1. The results showed that U0126/SB203580 combined with TGF- $\beta$ 1 inhibited EMT development, decreased the mRNA and protein expressions of RGC-32 and vimentin, and increased those of E-cadherin compared with the cells treated with TGF- $\beta$ 1 alone. After combined use of siRGC-32 and TGF- $\beta$ 1, the EMT occurrence and the levels of RGC-32, snail and Zeb1 were all inhibited compared with the cells treated with siCtrl alone or siCtrl + TGF- $\beta$ 1. The present study demonstrated that the regulation of RGC-32 on TGF- $\beta$ -induced EMT might be associated with non-Smad pathways, and Erk-MAPK and p38-MAPK pathways seemed to play more important roles than PI3K-Akt pathway in PC progression.

**Keywords:** RGC-32, EMT, TGF- $\beta$ , pancreatic cancer, signaling pathways

## Introduction

Pancreatic cancer (PC) is a highly life-destructive human digestive system carcinoma characterized by the evident parallel between its incidence and mortality. The overall five-year survival was reported to be less than 6% in PC patients in USA even after the therapeutic interventions alone or in combination (such as surgery, radiation, chemotherapy) [1]. Although PC occurs usually in the elderly population, family history, smoke, chronic pancreatitis, obesity and diabetes mellitus are believed to be the main risk factors for PC occurrence and development [2].

Epithelial-mesenchymal transition (EMT) is a critically biological process that participates in implantation, embryo formation, organ deve-

lopment and fibrosis, wound healing, tissue regeneration as well as cancer progression [3]. Mounting evidences demonstrated that EMT played an essential role in a variety of *in vitro* cancer types including breast cancer [4], colorectal cancer [5], ovarian cancer [6] and PC [7]. EMT endows epithelial cell with a series of mesenchymal phenotype which contain enhanced migratory capacity, elevated resistance to apoptosis, and increased production of extracellular matrix (ECM) components [8]. Currently, it is difficult to take a direct observation of EMT; however, detecting abnormal mRNA and/or protein expressions of epithelial/mesenchymal markers (such as downregulation of E-cadherin or upregulation of N-cadherin) is a common way to investigate EMT in PC progression [9, 10].

EMT is a complex process and can be divided into three classes based on distinct biological settings. It has been revealed that at least twelve core signaling pathways were associated with EMT induction in PC including TGF- $\beta$  pathway [8, 11, 12]. TGF- $\beta$  is a member of an extremely complex family of structurally related polypeptide growth factors governing tremendous cellular processes consisting of cell cycle arrest, apoptosis and tumor progress [13]. Once binding to TGF- $\beta$  at the cell surface, TGF- $\beta$ R1 and TGF- $\beta$ R2 is dimerized, phosphorylated and activated, initiating the TGF- $\beta$  signaling [14]. TGF- $\beta$  pathway was reported to be either Smad-dependent or -independent [15].

Response gene to complement 32 (RGC-32), a gene first cloned by Badea et al. in 1998, was induced by complement involving in cell cycle activation [16]. RGC-32 can be found comprehensively in heart, brain, liver, skeletal muscle, placenta, kidney, and pancreas [17]. Overexpression of RGC-32 has been observed in colon cancer and many other tumors [18]. In a previous work, we demonstrated that RGC-32 could mediate TGF- $\beta$ -induced EMT in human PC cells [7]. However, the underlying mechanism remained largely unknown. In the present study, the involvements of three non-Smad signaling pathways, i.e. PI3K-Akt, Erk-MAPK and p38-MAPK in TGF- $\beta$ -induced EMT mediated by RGC-32 were further explored.

## Materials and methods

### *Cell culture and treatments*

The BxPC-3 human pancreatic cancer cell line was provided by the Institute of Liver Disease, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, Hubei, China. BxPC-3 cells were cultured in RPMI-1640 (GIBCO, NY, United States) culture medium containing 10% fetal bovine serum (FBS, Gibco), 300 mg/L glutamine, 100 U/mL penicillin and 100  $\mu$ g/mL streptomycin at 37°C in a humidified atmosphere of 95% air and 5% CO<sub>2</sub>. The cells were seeded in a 6-well plate at a density of 3 × 10<sup>5</sup> cells/well. After reaching 70% of confluency, the media was removed and the cells were washed with PBS twice. Then, BxPC-3 cells were incubated in serum-free RPMI-1640 supplemented with 10 ng/mL TGF- $\beta$ 1 and 10  $\mu$ M U0126/SB203-580/LY294002 (Beyotime, Shanghai, China) for another 72 h. The cells treated with TGF- $\beta$ 1

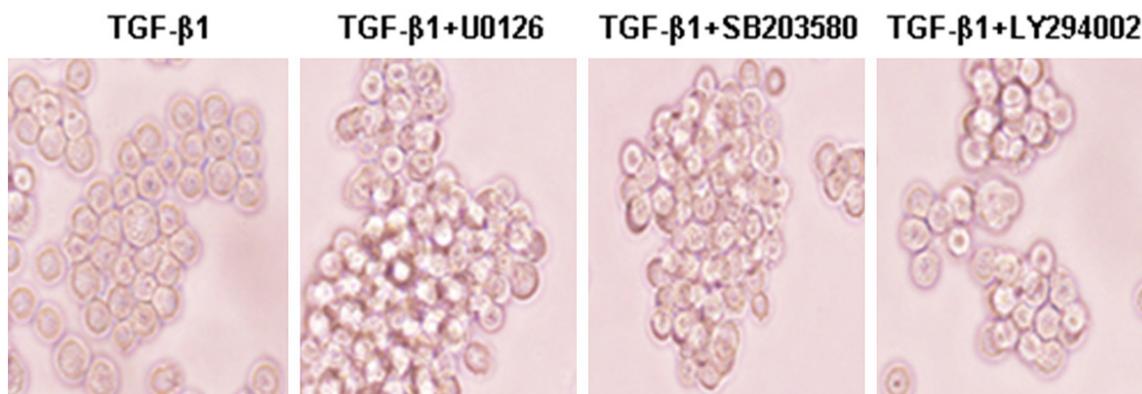
alone were set as control. The morphology of cells was visualized with a phase contrast microscope (× 200, Nikon, Japan) and imaged with digital photography.

### *Plasmid construction, short hairpin interfering RGC-32 (shRGC-32) synthesis and transfection*

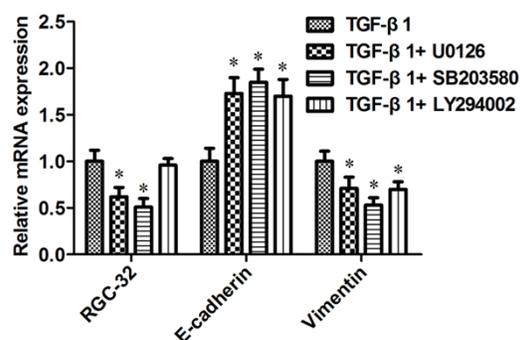
The experimental procedures were reported in a previous study with minor modifications [7]. Briefly, RGC-32 cDNA was amplified from mRNA extracted from BxPC-3 cells and then cloned into pcDNA3.1/myc-His C expression vector (Invitrogen, USA) between Hind III and BamH I restriction sites. The cloned cDNA was sequenced for verification. The shRGC-32 sequence was CAGAUUCACUUUUAUAGGAUUCCUAUA-AAGUGAAUCUG. The scrambled shRNA (shCtrl) sequence was CGCCTCTCTCTAGTGAGATTC-AAGAGAATCTACTAAGAGAGAGGCCT and used as the negative control. Both shRNA sequences were synthesized by Ribobio (Guangzhou, China). After plasmid construction, BxPC-3 cells were seeded in a 6-well plate at a density of 3 × 10<sup>5</sup> cells/well and incubated to reach 95% of confluency. Then, the cells were transiently transfected with lipofectamine 2000 (Invitrogen, USA) according to the manufacturer's recommendations. Freshly prepared plasmid DNA (= 4.0  $\mu$ g) and lipofectamine 2000 (= 10  $\mu$ l) diluted separately in Opti-MEM I medium (Gibco, USA) were co-incubated for 30 min at 37°C. Then, the solution was added into each well and mixed with the cells for another 5 h at 37°C. Subsequently, the medium was replaced with RPMI-1640 medium containing 10% FBS and incubated for another 72 h. For shRGC-32 transfection, BxPC-3 cells were seeded in a 6-well plate at a density of 1 × 10<sup>5</sup> cells/well and incubated to reach 50% of confluency. The cells were transfected with 50 nM shRGC-32/shCtrl using lipofectamine 2000 at 37°C in a 5% CO<sub>2</sub> incubator (Thermo, USA) according to the manufacturer's instructions. After 6 h of transfection, the cells were transferred to freshly prepared RPMI-1640 for another 72 h.

### *Quantitative reverse transcription polymerase chain reaction (qRT-PCR)*

The performance was in accordance with a previous work with a few modifications [7]. Briefly, total RNA was extracted from BxPC-3 cells by TRIzol reagent (Invitrogen, USA) according to the manufacturer's instructions and was resuspended in nuclease-free water. An aliquot of



**Figure 1.** Representative images of EMT after the treatments of TGF- $\beta$ 1, TGF- $\beta$ 1 + U0126, TGF- $\beta$ 1 + SB203580 and TGF- $\beta$ 1 + LY294002 in BxPC-3 cells. U0126, SB203580 and LY294002 are three specific inhibitors of Erk-MAPK, p38-MAPK and PI3K-Akt pathways. The cells treated with TGF- $\beta$ 1 was set as the control.



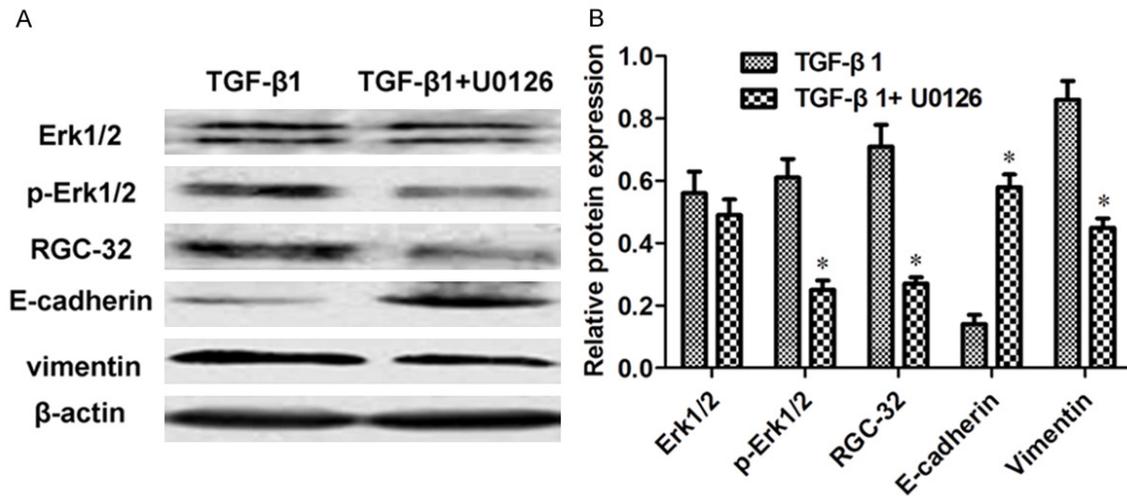
**Figure 2.** qRT-PCR results revealed the effects of TGF- $\beta$ 1, TGF- $\beta$ 1 + U0126, TGF- $\beta$ 1 + SB203580 and TGF- $\beta$ 1 + LY294002 on relative mRNA expressions of RGC-32, E-cadherin and Vimentin after 72-h of incubations in BxPC-3 cells. GAPDH was set as the reference gene. \* $P < 0.05$ , compared with the cells treated with TGF- $\beta$ 1 alone.

total RNA (= 2  $\mu$ g) was transcribed into cDNA followed by incubations with 25  $\mu$ L of reverse transcription reaction mixture containing 5  $\mu$ L of 5  $\times$  RT Reaction Buffer, 3  $\mu$ L of dNTPs (10 mmol/L), 1  $\mu$ L of Oligo (dT), 1  $\mu$ L of M-MLV (Promega, USA), 1  $\mu$ L of Rnasin (Fermentas, USA) and indicated amount of DEPC water. The conditions of reverse transcription reaction were 70°C for 5 min, 37°C for 1 h and 85°C for 10 min. The primers of RGC-32, E-cadherin, vimentin and GAPDH were synthesized by Sangon (Shanghai, China) and the sequences were as follows: RGC-32 forward: 5'-AGAAG-GCTGGGGCTCATTTG-3', reverse: 5'-AGGGGCC-ATCCACAGTCTTC-3' E-cadherin forward: 5'-AA-TGCCGCCATCGCTTAC-3', reverse: 5'-CCACCA-GGGTATACGTAGGGA-3'; Vimentin forward: 5'-CCGCTTCGCCAACTACATC-3', reverse: 5'-GGTT-

AGCTGGTCCACCTGCC-3'; GAPDH forward: 5'-AGAAGGCTGGGGCTCATTTG-3', reverse: 5'-AG-GGGCCATCCACAGTCTTC-3'. GAPDH was used as the reference gene. The qPCR was performed with StepOne real-time PCR systems (ABI, USA) in a reaction volume of 20  $\mu$ L containing 2  $\mu$ L of cDNA, 0.8  $\mu$ L of forward primer (10 nM), 0.8  $\mu$ L of reverse primer (10 nM), 10  $\mu$ L of SYBR Green Real-time PCR Master Mix (Toyobo, Japan) and 6.4  $\mu$ L of ddH<sub>2</sub>O. The procedure was followed by 95°C for 60 s, 40 cycles of 95°C for 15 s and 60°C for 30 s. The analysis of qPCR was carried out using the 2<sup>- $\Delta\Delta$ Ct</sup> method as described before [19].

#### Western blot

Western blot assay was performed as described previously [7]. Briefly, Total proteins were extracted from BxPC-3 cells using EpiQuik Total Histone Extraction Kit (Epigentek, Farmingdale, NY, USA). The protein was quantified using a BCA Protein Quantification Kit (Vazyma, Nanjing, Jiangsu, China) according to the manufacturer's instructions. Then, 80  $\mu$ g of cell protein was separated on a 12% SDS-PAGE and transferred to nitrocellulose membranes (Millipore, Billerica, MA, USA). The nitrocellulose membranes were blocked at room temperature for 2 h in blocking buffer (5% skim milk in TBST) and then respectively incubated with RGC-32 antibody (1:200), E-cadherin antibody (1:400) and vimentin antibody (1:1000, ProteinTech Group, Inc., USA) overnight at 4°C.  $\beta$ -actin antibody (1:1000, ProteinTech Group, Inc., USA) was set as the control. After three times of washing with TBST, nitrocellulose membranes were incubated in HRP-conjugated goat anti-rabbit second-



**Figure 3.** (A) Representative images of western blot and (B) relative protein quantifications displayed the effects of TGF-β1 and TGF-β1 + U0126 on the levels of Erk, p-Erk, RGC-32, E-cadherin and Vimentin proteins after the BxPC-3 cells was incubated with TGF-β1 for 72 h. β-actin was set as the control. \*,  $P < 0.05$ , compared with the cells treated with TGF-β1 alone.

ary antibody (1:3000, Boster, Wuhan, China) for 1 h at room temperature. After three times of washing with TBST, the protein samples were visualized by Super Signal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific Inc, USA) according to the manufacturer's instructions. The blots were scanned and densitometric analysis was performed by Image J software (National Institutes of Health, USA).

#### Statistical analysis

All experiments were performed triplicate in three independent occasions. The data were presented as mean  $\pm$  standard deviation. The data between two groups were analyzed by the two-tailed Student's t-test, while the data among groups were analyzed by one-way analysis of variance (ANOVA). Statistical analyses were conducted by SPSS 17.0 (SPSS, Chicago, IL, USA). A value of  $P < 0.05$  was considered statistically significant.

## Results

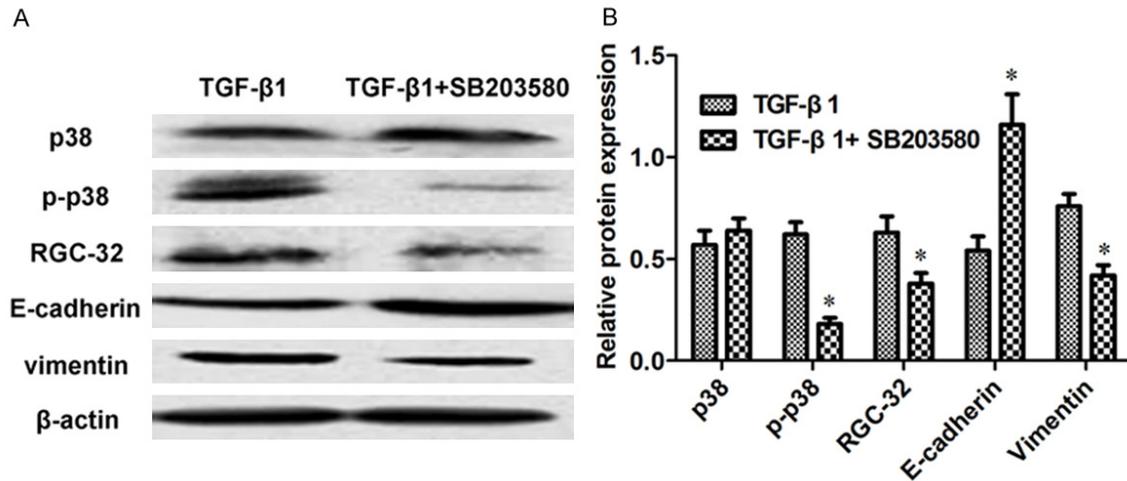
### Signaling pathway inhibitors interfered with EMT

Compared with the control treated with TGF-β1, the BxPC-3 cells co-incubated with TGF-β1 and signaling pathway inhibitors (U0126, SB203580 and LY294002) displayed evident EMT reversion in cell morphology from a mesenchymal-like spindle-cell shape with increased

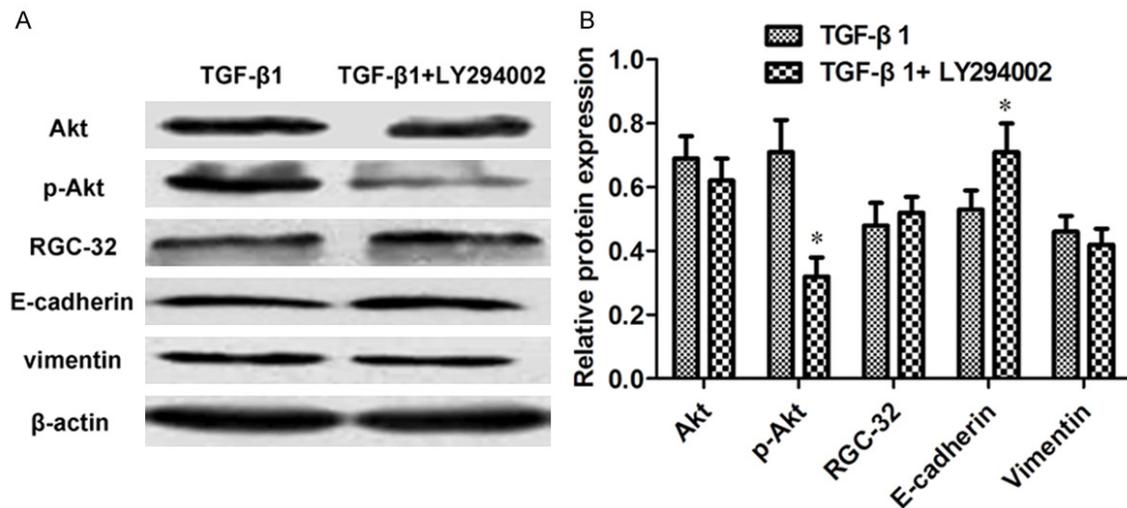
intercellular space to a more epithelial-like appearance with close cell-cell connection (**Figure 1**). The PCR results showed that the mRNA expressions of RGC-32 and Vimentin were downregulated by 1.41-1.96 fold ( $P < 0.05$ ) except the cells treated with TGF-β1 + LY294002 with a decrease of 1.04-fold. The mRNA expressions of E-cadherin were upregulated by 1.70-1.85 fold ( $P < 0.05$ , **Figure 2**). Compared with the cells treated with TGF-β1 alone, the protein expressions of phosphorylated Erk1/2 (p-Erk1/2), RGC-32 and Vimentin decreased by 2.44-, 2.63- and 1.91-fold ( $P < 0.05$ ), while that of E-cadherin increased by 4.14-fold ( $P < 0.05$ ) after the combination of TGF-β1 and U0126 (**Figure 3A** and **3B**). As for TGF-β1 + SB203580, the protein levels of phosphorylated p38 (p-p38), RGC-32, Vimentin and E-cadherin experienced 3.44-, 1.66- and 1.81-fold of decreases and 2.15-fold of increase respectively ( $P < 0.05$ , **Figure 4A** and **4B**). Following the combination of TGF-β1 and LY294002, the protein level of phosphorylated Akt (p-Akt) had a decrease of 2.22-fold, while that of E-cadherin owned an increase of 1.34-fold ( $P < 0.05$ , **Figure 5A** and **5B**).

### Silenced RGC-32 inhibited EMT and the expressions of snail and Zeb1 proteins

The technique of siRNA was employed to silence the expression of RGC-32. It could be observed that siCtrl + TGF-β1 promoted EMT



**Figure 4.** The analysis of proteins related with p38-MAPK pathway. (A) Representative images of western blot and (B) relative protein quantifications displayed the effects of TGF-β1 and TGF-β1 + SB203580 on the levels of p38, p-p38, RGC-32, E-cadherin and Vimentin after the BxPC-3 cells was incubated with TGF-β1 for 72 h. β-actin was set as the control. \*,  $P < 0.05$ , compared with the cells treated with TGF-β1 alone.

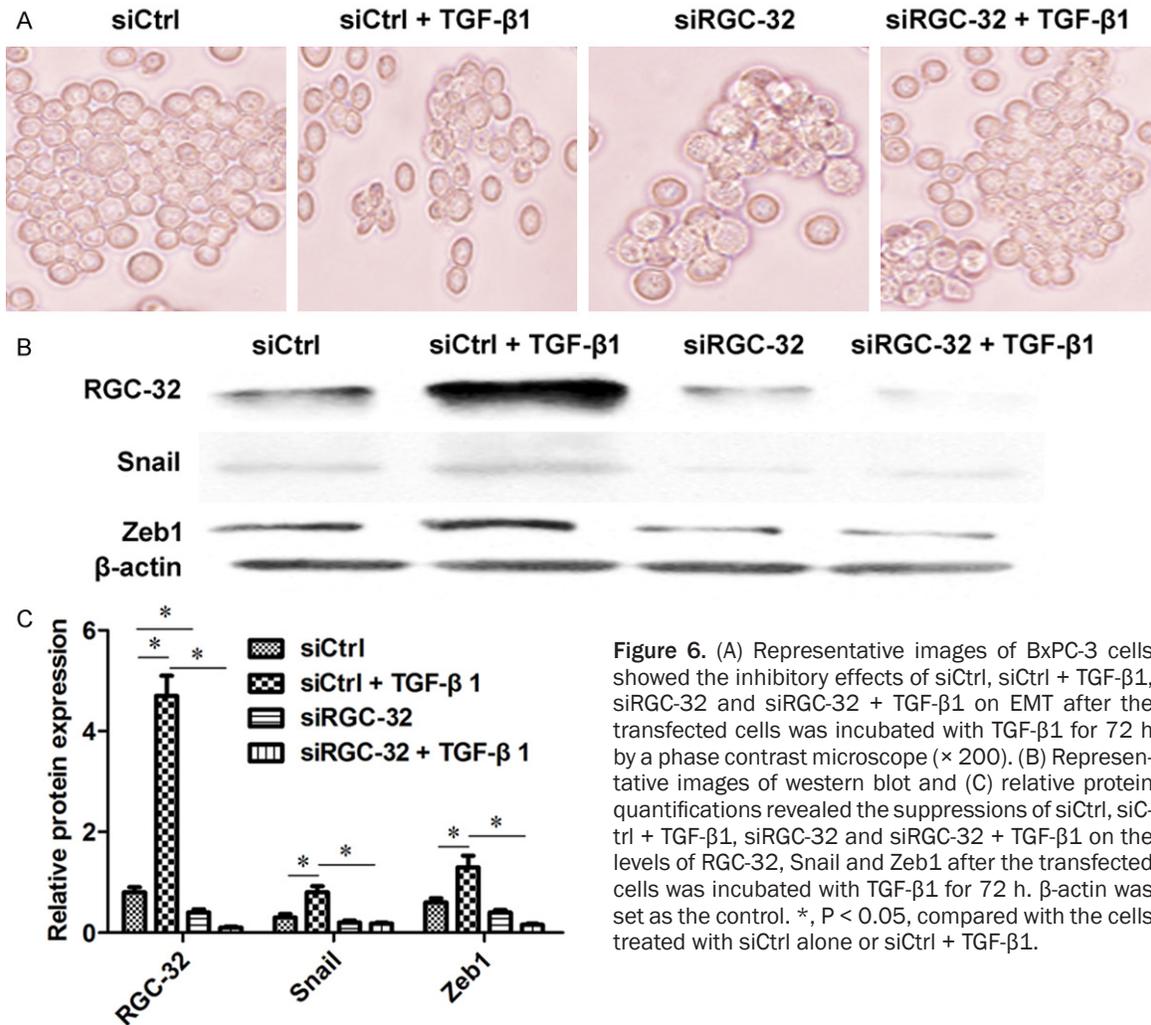


**Figure 5.** The analysis of proteins related with PI3K-Akt pathway. (A) Representative images of western blot and (B) relative protein quantifications displayed the effects of TGF-β1 and TGF-β1 + LY294002 on the levels of Akt, p-Akt, RGC-32, E-cadherin and Vimentin after the BxPC-3 cells was incubated with TGF-β1 for 72 h. β-actin was set as the control. \*,  $P < 0.05$ , compared with the cells treated with TGF-β1 alone.

development, while siRGC-32 + TGF-β1 inhibited EMT progression compared with the control transfected with siCtrl (**Figure 6A**). Compared with siCtrl alone, the protein levels of RGC-32, snail and Zeb1 acquired 5.88-, 2.67- and 2.17-fold increases ( $P < 0.05$ ) after co-treatment of siCtrl + TGF-β1. When siRGC-32 was used in combination with TGF-β1, the RGC-32, snail and Zeb1 levels obtained 47-, 4.44- and 8.13-fold of decreases ( $P < 0.05$ ) compared with siCtrl + TGF-β1 (**Figure 6B** and **6C**).

## Discussion

During the past decade, growing evidence indicated that RGC-32 could induce and promote EMT in lung cancer cells, renal tubular cells, renal proximal tubular cells, etc. [17, 20, 21]. In a previous study, RGC-32 was observed to enhance TGF-β-induced EMT in a human PC cell line BxPC3 which was deficient in Smad4 gene [7]. In canonical Smad signaling pathway, Smad2 and Smad3 proteins activated by phos-



**Figure 6.** (A) Representative images of BxPC-3 cells showed the inhibitory effects of siCtrl, siCtrl + TGF-β1, siRGC-32 and siRGC-32 + TGF-β1 on EMT after the transfected cells was incubated with TGF-β1 for 72 h by a phase contrast microscope ( $\times 200$ ). (B) Representative images of western blot and (C) relative protein quantifications revealed the suppressions of siCtrl, siCtrl + TGF-β1, siRGC-32 and siRGC-32 + TGF-β1 on the levels of RGC-32, Snail and Zeb1 after the transfected cells was incubated with TGF-β1 for 72 h.  $\beta$ -actin was set as the control. \*,  $P < 0.05$ , compared with the cells treated with siCtrl alone or siCtrl + TGF-β1.

phorylated TGF-βR1 will form a complex with Smad4 protein to regulate EMT-related genes. Smad4, a tumor suppressor gene, is in charge of suppressing epithelial cell growth via TGF-β signaling pathway [22]. Although it is functional in multiple cancers, Smad4 is believed to lose activity in more than 50% PC cases resulting in poor prognosis [23]. Therefore, it was suggested that RGC-32 might mediated TGF-β-induced EMT via non-Smad pathways. Deciphering the roles of non-Smad pathways in EMT progression during PC processes will provide new insights into the plasticity of cellular phenotypes and possible therapeutic interventions.

It is a common strategy to employ inhibitors to investigate relevant signaling pathways. U0126, SB203580 and LY294002 have been widely used to block Erk-MAPK, p38-MAPK and PI3K-Akt pathways which are the three exten-

sively studied non-Smad signaling pathways in cancer development [24-26]. Compared with the control treated with TGF-β, the three inhibitors could hinder the EMT development induced by TGF-β in treated PC cells. Subsequently, we analyzed the expressions of several critical genes and proteins related with the three pathways to further evaluate their roles in TGF-β-induced EMT mediated by RGC-32.

Erk-MAPK is one of the most clarified MAPK pathways and has been reported to be related with cell proliferation, differentiation, migration, senescence and apoptosis [27]. Erk proteins are a family of serine/threonine kinases including ERK 1 (MW 44 KD) and ERK2 (MW 42 KD) and can be activated by MEK1/2 [28]. Erk-MAPK pathway was believed to be involved in the regulations of prostate cancer cell proliferation and migration by chloride intracellular

channel 1, the proliferations of renal-cell carcinoma by  $\beta$ -Elemene and hepatocellular carcinoma cell by lysyl oxidase propeptide [29-31]. In this study, the levels of phosphorylated Erk1/2 (p-Erk1/2), RGC-32, E-cadherin (epithelial markers) and vimentin (mesenchymal markers) were altered significantly, indicating an involvement of Erk-MAPK pathway in TGF- $\beta$ -induced EMT mediated by RGC-32.

p38 proteins are also a group of serine/threonine kinases including four members, i.e. p38 $\alpha$ , p38 $\beta$ , p38 $\gamma$  and p38 $\delta$  [32]. p38-MAPK is another important MAPK pathway and induces a wide variety of biological responses including cell cycle, apoptosis, embryonic development and cancer progression [33]. p38-MAPK pathway could regulate TGF- $\beta$ -induced EMT in mammary epithelial cells [34], adult rat hepatocytes [35], etc. In present study, except the expression of p38, the significant changes of phosphorylated p38 (p-p38), RGC-32, E-cadherin and vimentin implied a participation of p38-MAPK pathway in TGF- $\beta$ -induced EMT mediated by RGC-32.

PI3K ubiquitous in the cytoplasm has a regulatory subunit (p85) and a catalytic subunit (p110). PI3K-Akt signaling pathway critical for cell proliferation and EMT possesses the activities of both phosphatidylinositol kinase and serine/threonine protein kinase [36]. Increasing evidence revealed that PI3K-Akt pathway was closely associated with TGF- $\beta$ -induced EMT [37-39]. To our surprise, only the levels of p-Akt and E-cadherin changed significantly. These results suggested that PI3K-Akt pathway seemed not to be implicated in TGF- $\beta$ -induced EMT mediated by RGC-32.

Snail and Zeb1 proteins belong to zinc finger transcriptional factors and act as inducers of EMT by downregulating E-cadherin and upregulating Vimentin [40]. Both proteins support tumor growth and progression in different cancer types via Erk-MAPK/p38-MAPK/PI3K-Akt pathway [41, 42]. After silencing RGC-32, Snail and Zeb1 levels decreased significantly, inferring that Snail and Zeb1 proteins might be downstream targets of RGC-32.

Of note, TGF- $\beta$  has been demonstrated to activate autophagy in a manner dependent on both Smad and non-Smad pathways [43]. We also noticed that Smad3 and Smad2 were assumed

to form transcriptional complexes with other transcriptional factors in favor of tumor initiation and progression without Smad4 [44]. Therefore, both Smad and non-Smad pathways might be associated with TGF- $\beta$ -induced EMT mediated by RGC-32.

In conclusion, we demonstrated that Erk-MAPK and p38-MAPK pathways might play more important roles than PI3K-Akt pathway in TGF- $\beta$ -induced EMT mediated by RGC-32. The results in the present study could expand our insight in the molecular mechanisms of TGF- $\beta$ -induced EMT mediated by RGC-32 and provide new therapeutic targets in PC.

### Acknowledgements

This study was supported by National Natural Science Foundation of China (No. 81302152), Project of Health and Family Planning Commission of Jiangxi Province (No. 20155207) and 2017 Natural Science Foundation of Jiangxi Province (Project title: The research on the effect of RGC-32 in hypoxia-induced EMT in pancreatic cancer and the underlying mechanism).

### Disclosure of conflict of interest

None.

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