

## Original Article

# Overexpression of FoxP1 is a novel biomarker of malignant human pancreatic cancer

Dapeng Qiu, Feng Han, Hao Zhuang, Qingjun Li, Xianzhou Zhang

Department of Hepato-Biliary-Pancreatic Surgery, Henan Tumor Hospital (The Tumor Hospital Affiliated to Zhengzhou University), Zhengzhou 450008, Henan, China

Received November 13, 2015; Accepted February 2, 2016; Epub June 15, 2016; Published June 30, 2016

**Abstract:** Recent studies have shown that FOXP3 plays a significant role in the pancreatic cancer cell metastasis, but whether FOXP1 plays a role in mediating tumor metastasis in pancreatic cancer has not been explored. Here, Co-immunoprecipitation assays were utilized to detect that FOXP1 was physically associated with the EMT inducer ZEB2. Most important, CAPAN-1 cells transfected with FOXP1 shRNA displayed a reversed epithelial morphology and reduced the expression of N-cadherin, while increase the expression of E-cadherin, and the PANC-1 cells transfected with FOXP1 displayed the contrary tendency. Transwell invasion assay, as well as colony formation together shown that FOXP1 promoted cell epithelial-mesenchymal transition and tumorigenesis in vitro and in vivo. Endothelial tube formation assays indicated FOXP1 might regulate the angiogenesis of pancreatic cancer through transcriptional activation of VEGF. qChIP, ChIP as well as Luciferase reporter assays suggested FOXP1 binding on the promoter of E-cadherin and VEGF. The changes in the level of protein and RNA implied that suppression of downstream E-cadherin or activation of VEGF was two distinct important mechanisms by which FOXP1 triggered EMT and angiogenesis. Further, patient samples collected shown that FOXP1 was significantly increased in pancreatic cancer samples, and its higher expression is correlated with poor prognosis, worse over all survivals. Together, our experiments revealed the mechanism for FOXP1 in facilitating tumorigenesis, angiogenesis and metastasis of pancreatic cancer cells, suggesting that FOXP1 might be a novel biomarker of malignant human pancreatic cancer and a potential therapeutic target for treating pancreatic cancer.

**Keywords:** FOXP1, ZEB2, EMT, E-cadherin, VEGF

## Introduction

Pancreatic cancer is one of the most aggressive human malignancies. Metastasis is a major clinical event which is the main cause of disease-related death, and it often has a correlation with angiogenesis, and is usually correlated with the low 5-year survival rates [1, 2].

Epithelial-mesenchymal transition (EMT) are believed to be the initial step of pancreatic cancer metastasis [3], during the process of EMT, epithelial cells with a cobblestone phenotype lost their differentiated characteristics, and acquire mesenchymal cell feature, such as spindle-shaped, motility and invasiveness [4, 5]. Epithelial-cadherin (E-cadherin; encoded by CDH1) is a member of the classical cadherins, it usually works as a tumor suppressor in the cancer progression [6, 7]. Generally downregulation of E-cadherin and upregulation of

N-cadherin are considered as the mark of EMT [8-10]. Which play important roles in the process of embryonic development as well as in tumor metastasis?

Furthermore, angiogenesis, the process of new blood vessels formation, is another important step in tumor metastasis, which involves several signaling between growth factors and endothelial cell receptors [11]. Among them, vascular endothelial growth factor (VEGF), by inducing the formation of new blood vessels, was reported by several groups [12, 13], to get an important role in angiogenesis and tumor metastasis.

FOXP1 was a member of the fork head transcription factor family, the fork head family share a conserved fork head domain, the function of which is DNA binding and mediates protein interactions. The function of FOXP3 in pan-

## FoxP1 might be a biomarker for pancreatic cancer

creatic cancer progression was well discussed [14, 15], although the sequence of FOXP1 shares high similarity with FOXP3, but the role of FOXP1 was still poorly understood.

To our particular aspects of knowledge, ZEB2/SIP1, which is a potent repressor of E-cadherin expression [16], is considered to be one of the key factors of EMT. Oppositely, vimentin, another mesenchymal marker, is reported to be up-regulated by ZEB2, which is associated with breast tumor cell EMT [17].

### Materials and methods

#### *Cells and cell culture*

Cell lines PANC-1 and CAPAN-1 cells were acquired from the American Type Culture Collection (ATCC). They were cultured in DMEM or RPMI 1640 (Gibco) respectively, supplemented with 10% fetal bovine serum (FBS) (HyClone) in a humidified atmosphere with 5% CO<sub>2</sub> at 37°C. CAPAN-1 medium was in addition supplemented with 1 mM glutamine. Cell lines hTERT-HPNE were from Shanghai institutes for Biological Sciences (CAS), and were cultured in MEGM BulletKit.

#### *Reagents*

Rabbit anti-human FOXP1 antibody, ZEB2 antibody, E-cadherin antibody, VEGF antibody,  $\beta$ -actin antibody and secondary antibody were all purchased from Santa Cruz (CA, USA).  $\alpha$ -catenin antibody, N-cadherin antibody, Vimentin antibody, were from abcam (USA). Specific shRNA targeting FOXP1, ZEB2 were from Sigma-Aldrich (CA, USA). A negative control shRNA (SCR) was also used as control (Sigma-Aldrich). Matrigel was purchased from BD Biosciences (CA, USA). Lipofectamine2000 (Invitrogen) was used for transfection.

#### *Patients and specimens*

30 pancreatic cancer samples of patients and the adjacent normal tissues were obtained in Henan Tumor Hospital from 2001 to 2008. Samples were chosen with completely clinicopathologic information. Patients who were received radiation therapy or chemotherapy prior to the surgery were excluded. The survival times were calculated based on the operation day to death, via the evaluation of metastasis or recurrence. This study has been approved by the hospital ethical committee.

#### *Quantitative real-time PCR (qRT-PCR)*

Total RNA of cell lysates were extracted with Trizol solution (Invitrogen, Carlsbad, CA, USA), and 1  $\mu$ g of total RNA were reverse transcribed to cDNA using with M-MLV Reverse Transcriptase, according to the manufacturer's instructions (TransGen Beijing, China). Real-time RT-PCR primers were as follows: FOXP1, forward 5-CTACCACAAGATGAATGGGC-3, reverse 5-CCTGTAAACATTGTGCAGCT-3; E-cadherin, forward 5-AAATCACATCCTACTGCCC-3, reverse 5-GCAACTGGAGAACCATTGTC-3; VEGF, forward 5-GACATCTTCCAGGAGTACCC-3, reverse 5-TCTTTCTTGGTCTGCATTAC-3; ZEB2, forward 5-CAGTCCAGACCAGTATTCC-3, reverse 5-AGCAAT-TCTCCCTGAAATCC-3; GAPDH, forward 5-CATTT-CCTGGTATGACAACGA-3, reverse 5-GTCTACATGGCAACTGTGAG-3, Real-time PCR was performed on an ABI 7500 sequence detection system (Applied Biosystems), using SYBR (Roche). All experiments were performed in three times. GAPDH was used as a normalization control.

#### *Co-immunoprecipitation*

For immunoprecipitation assays, cells were washed with cold PBS and lysed with cold lysis buffer on a rotator at 4°C for 45 min. Whole cell lysates were incubated with appropriate primary antibodies or normal rabbit/mouse immunoglobulin G (IgG) as negative controls on a rotator overnight at 4°C, then added protein A/G Sepharose CL-4B beads for 2 h at 4°C. Beads were then washed 5 times with lysis buffer (50 mM Tris-Cl, pH 7.4, 0.5% NP-40, 150 mM NaCl, 1 mM EDTA, 0.5% sodium deoxycholate and protease inhibitor cocktail). The immune complexes were subjected to SDS-PAGE (Invitrogen), followed by immunoblotting with secondary antibodies.

#### *Western blot*

Cells were harvested 48 h to 72 h after transfection with the indicated shRNA or recombinant plasmid. Protein concentration was measured using the bicinchoninic acid kit (BCA). Subsequently, 30  $\mu$ g proteins were run by 10% SDS-PAGE gel and then transferred on to NC membranes. 5% skim milk were used to block the NC membranes for 1 h, then incubated with primary antibodies, overnight at 4°C, including FOXP1 (1:1000), ZEB2 (1:1000), E-cadherin (1:1000),  $\alpha$ -catenin (1:1000), N-cadherin

## FoxP1 might be a biomarker for pancreatic cancer

(1:1000), Vimentin (1:500), VEGF (1:1000) and  $\beta$ -Actin (1:500). Goat anti-Rabbit secondary antibodies (1:5000), Goat anti-Mouse secondary antibodies (1:3000) and Western blotting Luminal reagent (Santa Cruz Biotechnology) were used to visualize the protein bands.

### *Colony formation assay*

After infected the related lentivirus for 3 days, a total of 1000 PANC-1 cells were seeded into 6-well plates and the medium was changed every 3 days. After 10 days of culturing, the colonies formed were collected, washed with PBS and fixed in 4% paraformaldehyde at 37°C for 15 min, after then, the colonies were stained with Coomassie for 15 min, washed and then air-dried. The colonies were counted using the microscopy (Olympus, Tokyo, Japan). The experiment was performed in triplicate.

### *ChIP and qChIP*

Chromatin immunoprecipitation (ChIP) assays were carried out according to the manufacturer's protocol (Upstate). The chromatin was incubated with 4  $\mu$ g of FOXF1 antibody, ZEB2 antibody or normal Rabbit IgG as negative control (Santa Cruz Biotechnology), at 4°C on a rotator overnight. Immunoprecipitated DNA was purified with the Qiagen PCR purification kit. qChIP was analyzed by quantitative PCR using specific primers as follows. For common ChIP assays, the final target DNA sequence was amplified and resolved on standard agarose DNA gels.

Primers used for common ChIP: E-cadherin: 5-AGGGTCACCGCTCTATG-3 (forward) and 5-CTTCCGCAAGCTCACAGG-3 (reverse), VEGF: 5-AAGGAGGAAAGTTAGTGGCTTCCCT-3 (forward) and 5-TGTTGCTGTGGTTTGGTGGA-3 (reverse); qChIP primers: E-cadherin: 5-GCAGGTCCCATAA-CCCACCTA-3 (forward) and 5-CATAGACGCGG-TGACCCTCTA-3 (reverse), VEGF: 5-AGCCTAGAG-CATGGAGCCCAGGTGA-3 (forward) and 5-CCAT-CGGTATGGTGCCTAA-3 (reverse).

### *Luciferase reporter assay*

For E-cadherin or VEGF reporter construction, the sequence of the relative promoter and partial exon was obtained by PCR. The pGL3 basic vector (Promega) was utilized to ligate these PCR products to generate pGL3-E-cadherin or pGL3-VEGF luciferase reporter construct. PANC-1 cells were transfected with promoter

luciferase reporter, Renilla luciferase plasmid, and the indicated expression constructs, using Lipofectamine LTX-Plus (Invitrogen) in 96-well plates. By addition of empty vector in each transfection, the amount of DNA was retained constant. Renilla plasmid was used as a normalization control of transfection efficiency. 48 hours after transfection, the firefly and Renilla luciferases were assayed according to the manufacturer's protocol (Promega). Each experiment was conducted in triplicate.

### *Transwell invasion assay*

The invasive ability of the CAPAN-1 cells was investigated using Transwell assay (8- $\mu$ m pore size; for 24-well plate. Millipore), matrigel used were from BD Biosciences (50  $\mu$ g/ml). First, 100  $\mu$ l matrigel was added onto the surface of the chamber, incubated in room temperature for 2 hours for solidification. A total of 100  $\mu$ l of CAPAN-1 cell suspension ( $5 \times 10^4$  cells) was in addition to the upper chamber, with serum-free medium. The lower compartment was incubated with 500  $\mu$ l of RPMI 1640 containing 10% fetal bovine serum. After incubation for 24 hours, the cells invaded into the lower surface were fixed with 2% paraformaldehyde, stained with crystal violet. Photos were taken using an inverted microscope (Olympus Corp. Japan) at 100 $\times$  magnifications.

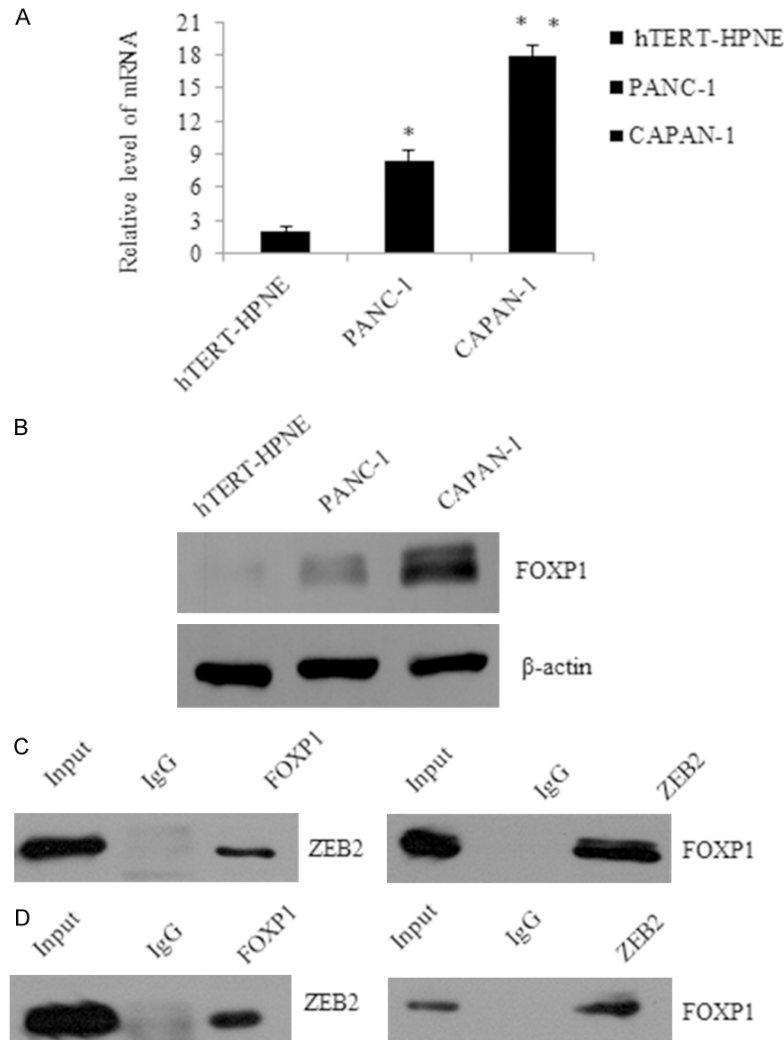
### *Tube formation assay*

Angiogenesis in vitro was performed utilizing the endothelial tube formation assay kit (Cell Biolabs, CA), according to the instruction. The extracellular matrix gel was prepared from Engelbreth-Holm-Swarm tumor cells (200  $\mu$ l/well). HUVECs (ten thousand cells/well) infected with retroviruses or cultured with CM from PANC-1 cells that were infected with the indicated retroviruses. The mixture was added onto solidified extracellular matrix gel in 600  $\mu$ l medium. After incubation for 18 h, endothelial cell tube formation was assessed and tube number was counted.

### *Statistical analysis*

All observations were confirmed by at least three independent experiments. The data was presented as mean  $\pm$  SD. One-way ANOVA was used to analyze the statistical significance of the mean values. Bivariate correlation was calculated by Spearman's rank correlation coefficients. Cox proportional hazards regression

## FoxP1 might be a biomarker for pancreatic cancer



**Figure 1.** FOXP1 is physically associated with ZEB2 in different pancreatic cancer cell lines. A. The expression level of FOXP1 was determined in hTERT-HPNE, PANC-1 and CAPAN-1. Quantitative real-time PCR were repeated three times. GAPDH was used as a normalization control. Error bars represent average  $\pm$  SD, \* $P < 0.05$ , \*\* $P < 0.01$ . B. Western blotting was used to detect the expression of FOXP1 in hTERT-HPNE, PANC-1 and CAPAN-1 cells;  $\beta$ -actin was used as a normalization control. C. Co-IP analysis of the association between FOXP1 and ZEB2. Whole cell lysates of PANC-1 were immunoprecipitated with antibodies against FOXP1, with normal IgG as the negative control, while the whole lysates as positive control. The immunocomplexes were then immunoblotted using ZEB2, then inversely. D. Whole cell lysates of CAPAN-1 were further used to detect the association between FOXP1 and ZEB2.

was utilized to test the prognostic significance of factors.  $P < 0.05$  was considered significant.

### Results

#### *FOXP1 is physically associated with ZEB2 in different pancreatic cancer cell lines*

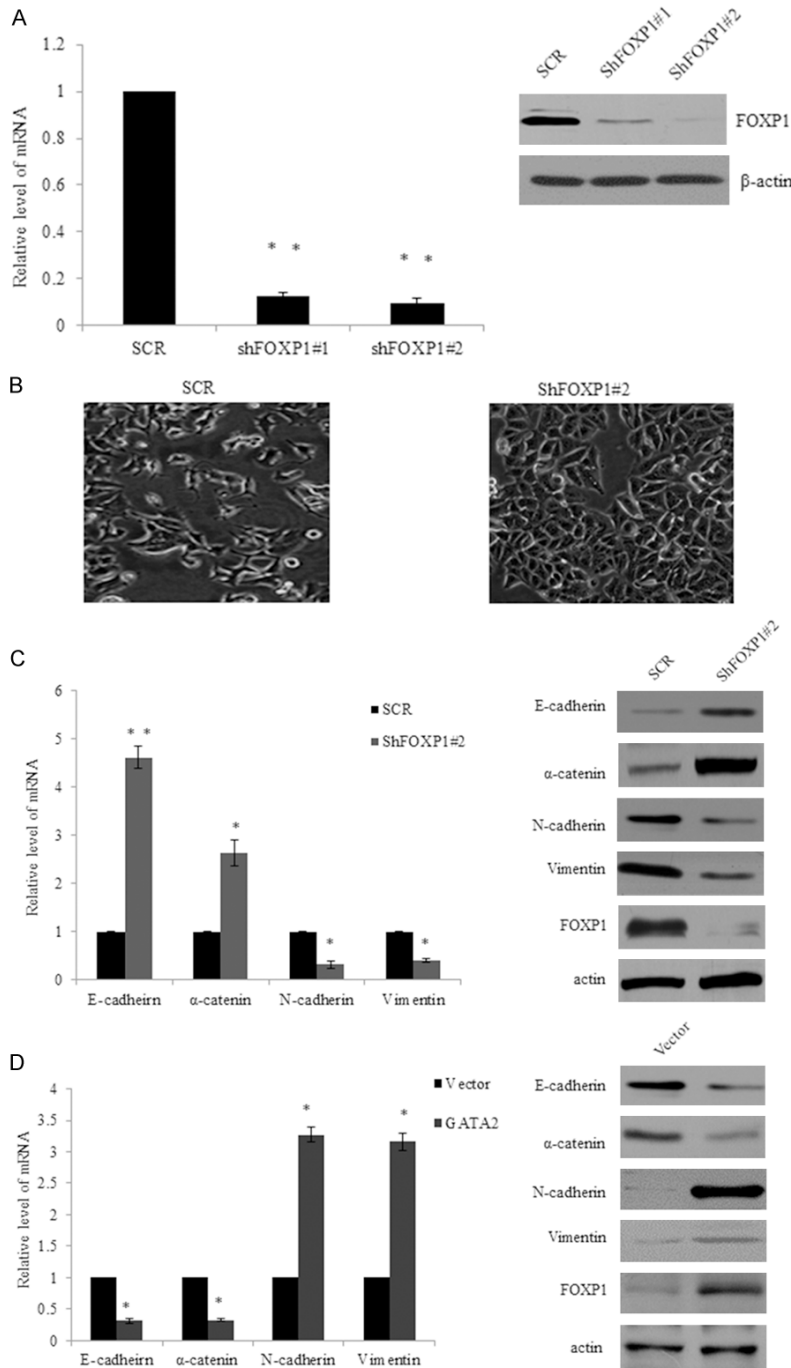
In order to investigate the role of FOXP1 during the progress of pancreatic cancer, we first

examine the expression of FOXP1 in two pancreatic cancer cell lines (PANC-1 and CAPAN-1) and the normal pancreas cell lines hTERT-HPNE by using qRT-PCR (Figure 1A) and western blot (Figure 1B). The expression of FOXP1 was significantly increased in PANC-1 and CAPAN-1, comparing with hTERT-HPNE. Then we used co-immunoprecipitation experiments to reveal that FOXP1 copurified with ZEB2 (Figure 1C and 1D), a key factor of epithelial-mesenchymal transition. Total protein extracts from PANC-1 cells were prepared, first, immunoprecipitation (IP) with anti-FOXP1 followed by immunoblotting (IB) with the anti-ZEB2 indicated that FOXP1 was co-immunoprecipitated with ZEB2 (Figure 1C left panel), again, IP with anti-ZEB2 followed by IB with anti-FOXP1 (Figure 1C right panel). To further support the in vivo interaction between FOXP1 and ZEB2, this interaction is also confirmed with endogenous proteins in CAPAN-1 cells (Figure 1D). We also detected another transcription factor, such as snail1 and twist1, both of them had no interaction with FOXP1, data were not shown.

#### *FOXP1 promotes pancreatic cancer cells EMT in vitro*

As showed in Figure 2A, CAPAN-1 cells were infected with control shRNA (SCR), shFOXP1#1, or shFOXP1#2 groups. Both mRNA level and protein level in different groups were detected. We found that mRNA level of FOXP1 was significantly reduced when cells were infected with shFOXP1#1, or shFOXP1#2 ( $P < 0.05$ . Figure 2A left panel). Concomitantly, in western blot analysis, FOXP1 was also greatly lowered in shFOXP1-infected CAPAN-1 cells as compared

## FoxP1 might be a biomarker for pancreatic cancer



**Figure 2.** FOXP1 promotes pancreatic cancer cells EMT in vitro. A. Knockdown efficiencies of FOXP1 in CAPAN-1 were confirmed by Quantitative real-time PCR (left panel) and western blotting (right panel). B. CAPAN-1 cells were treated with specific shRNA against FOXP1. Phase-contrast microscopy was used to examine the morphological alterations of these cells. C. CAPAN-1 cells were transfected with shFOXP1, the expressions of the epithelial markers such as E-cadherin,  $\alpha$ -catenin; mesenchymal markers N-cadherin, Vimentin were measured by real-time RT PCR and western blotting. Error bars represent the mean  $\pm$  S.D. \*P < 0.05; \*\*P < 0.01. D. PANC-1 cells were transfected with FOXP1 constructs. The expressions of the epithelial and mesenchymal markers were measured using real-time RT PCR and western blotting.

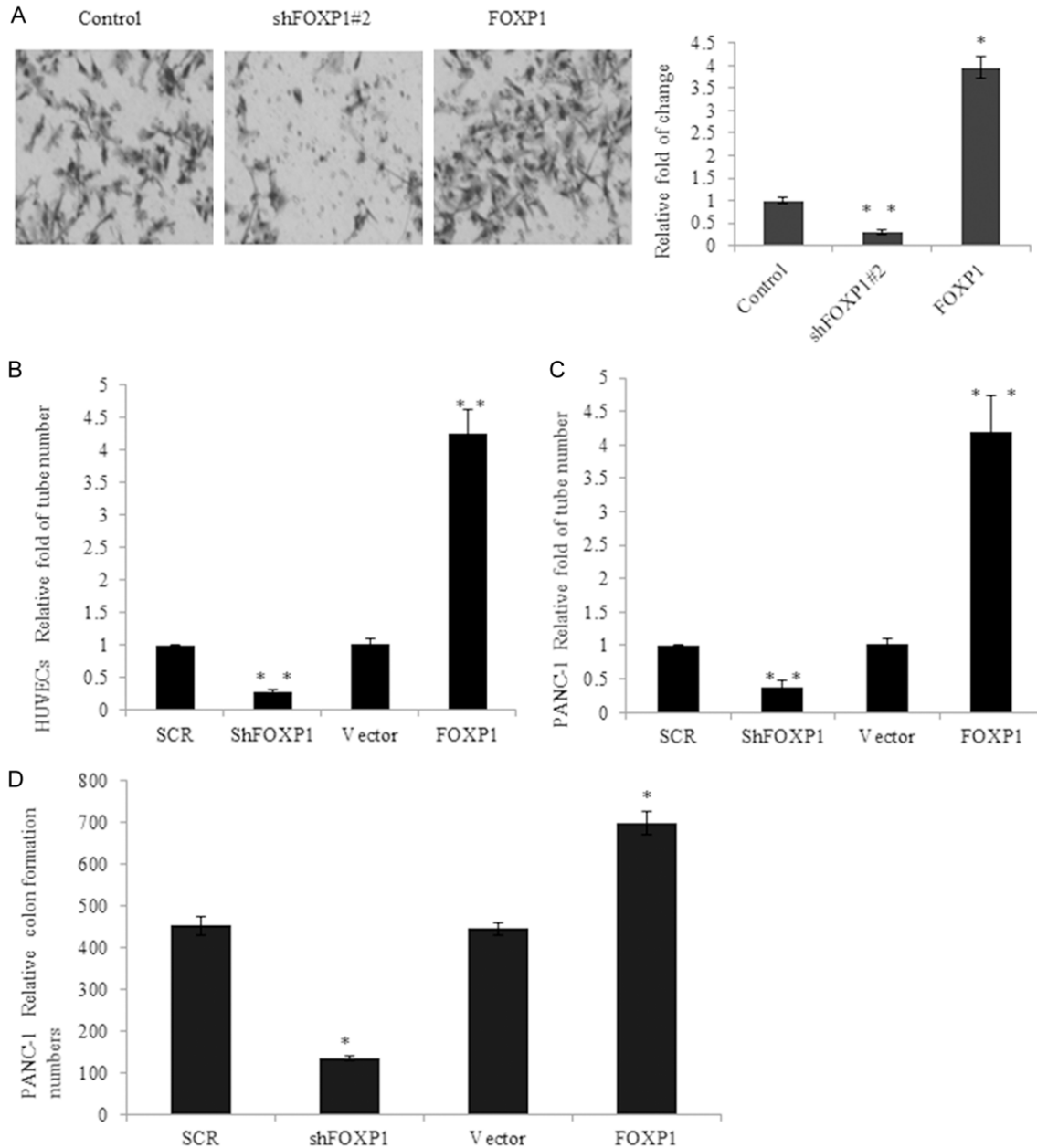
with the control shRNA-infected cells (Figure 2A right panel). The results suggested both the two relative shRNA were successfully constructed in CAPAN-1 cells. Interestingly, when CAPAN-1 were transfected with the relative shRNA, As shown in Figure 2B, while control CAPAN-1 cells maintained a spindle-like, fibroblastic morphology, knock down of FOXP1 became organized cell-cell adhesion and cobble stone-like epithelial appearance.

Considering the expression of FOXP1 in PANC-1 was lower than that of CAPAN-1, we choose FOXP1 “loss of function” experiment in CAPAN-1 cells, the expression of the epithelial markers such as E-cadherin,  $\alpha$ -catenin were up regulated; while the mesenchymal markers N-cadherin, Vimentin were raised as measured by real-time RT PCR or western blotting (Figure 2C). When FOXP1 was over-expressed in PANC-1 cells, the opposite result was shown. E-cadherin,  $\alpha$ -catenin were decreased while N-cadherin, Vimentin were up regulated (Figure 2D).

*FOXP1 enhances pancreatic cancer cells invasion and angiogenesis in vitro*

Considering EMT as the initiation step of metastasis, we further focus our attention on whether FOXP1 could also take part in the metastasis; transwell assay was performed to assess the effect of FOXP1 on cell invasion. Migrated CAPAN-1 cells infected with shFOXP1 were counted for

## FoxP1 might be a biomarker for pancreatic cancer

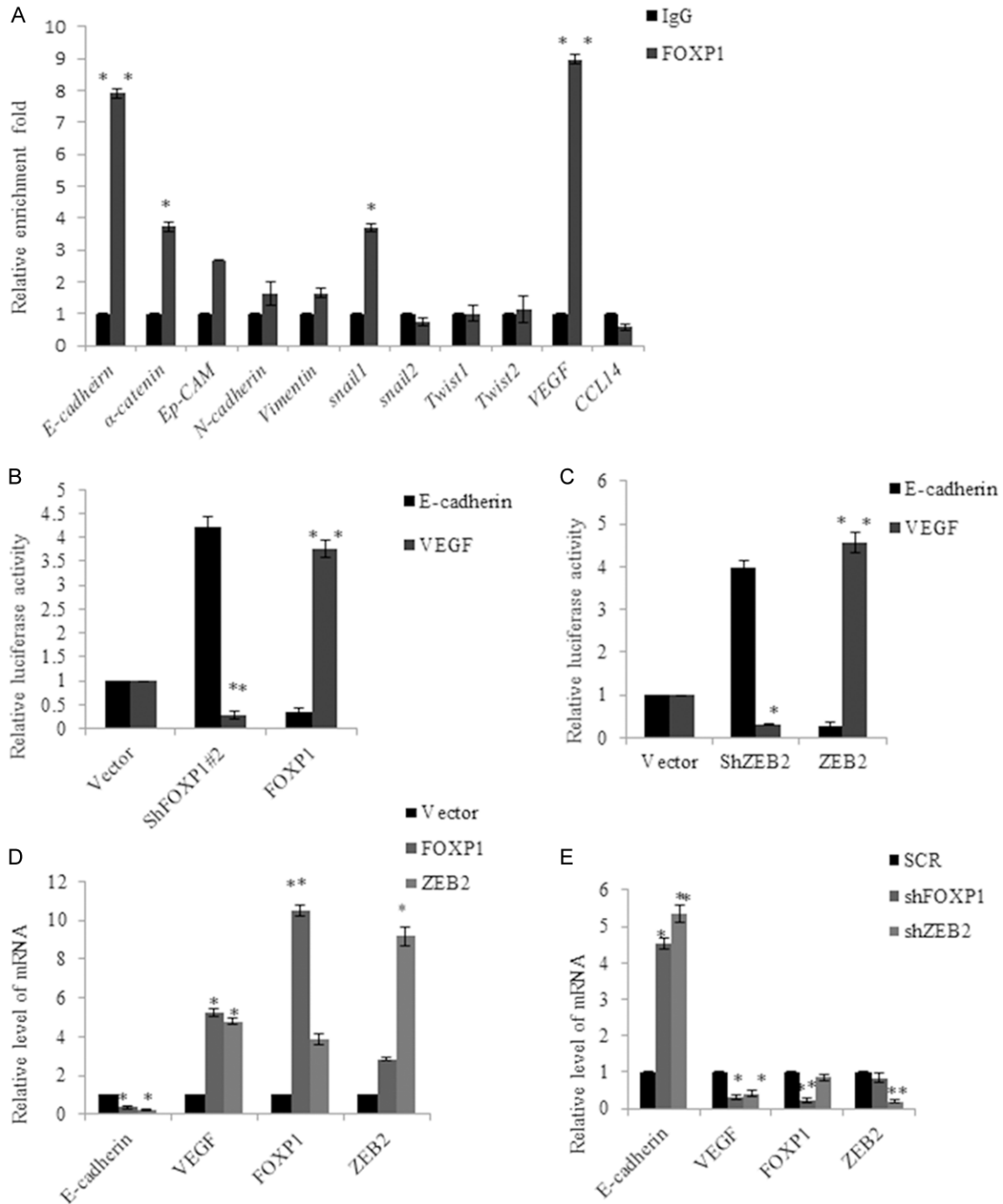


**Figure 3.** FOXP1 enhances pancreatic cancer cells invasion and angiogenesis in vitro. A. CAPAN-1 cells were transfected with control shRNA, shFOXP1, empty vector, or FOXP1 over expression construct. After 48 h of transfection, cell invasion assays were performed; the invaded cells were stained and counted. Representative photos were shown in each group. Statistically analyzed were presented as fold of change over vector. Error bars represent the mean  $\pm$  S.D. Experiments were repeated three times. \* $P < 0.05$ ; \*\* $P < 0.01$ . B. HUVECs were infected with lentiviruses carrying SCR, ShFOXP1, vector, or FOXP1 was added onto solidified extracellular matrix. After incubation, endothelial cell tube formation was assessed and the tubes were counted under light microscopy. C. HUVECs cultured with different CM from PANC-1 infected with the above mentioned lentiviruses, endothelial cell tube formation was assessed and the tubes were counted, statistically analyzed were represented. D. PANC-1 cells transfected with vector, FOXP1 or SCR, shFOXP1 were maintained in the culture media for 10 days, 1 mg/ml G418 were used to prior, the colonies were stained with crystal violet, statistically analyzed were represented.

one third than those infected with control, while FOXP1 over expression was related with 3.5 times increase of the invasion cells, which indicated FOXP1 might take a role in the metastasis of pancreatic cancer (Figure 3A).

Endothelial tube formation assays were carried out to detect whether FOXP1 take a role in the pancreatic cancer cells angiogenesis. First, Human umbilical vein endothelial cells (HUVECs) infected with lentiviruses carrying

## FoxP1 might be a biomarker for pancreatic cancer

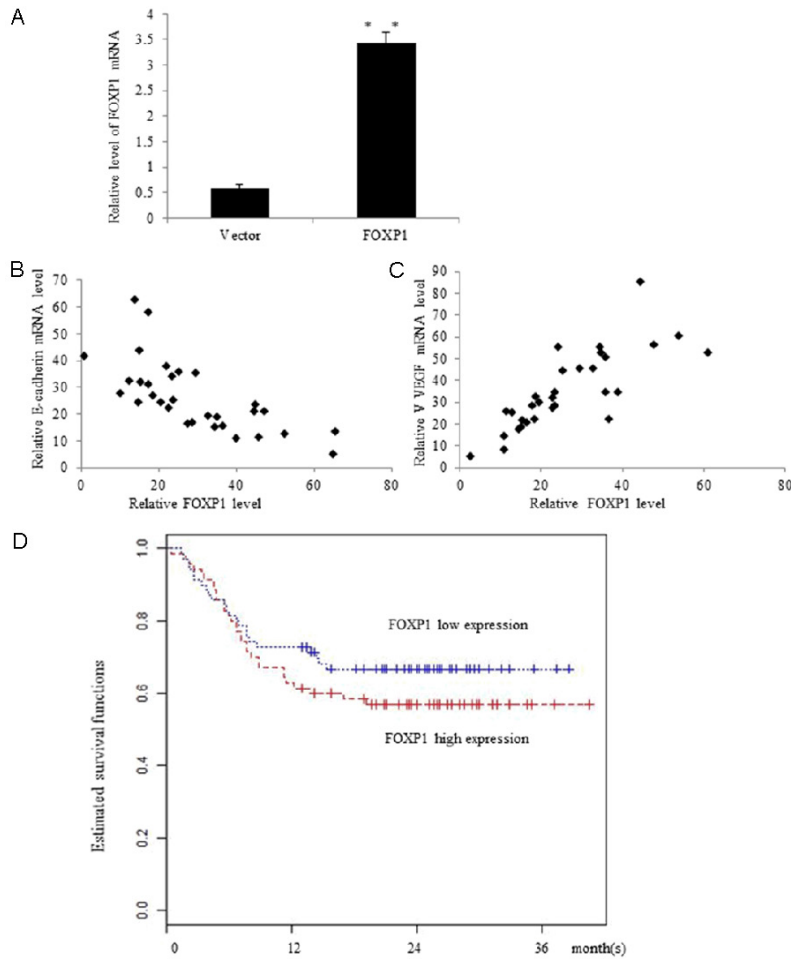


**Figure 4.** The molecular mechanism of FOXP1 in promoting pancreatic cancer cell invasion and angiogenesis. A. Recruitment of FOXP1 on E-cadherin and VEGF promoters. qChIP experiments were performed in PANC-1 cells with indicated antibodies. Each bar indicates mean  $\pm$  S.D. of three independent experiments. B. The regulation of E-cadherin promoter driven luciferase activity. PANC-1 cells were transfected with promoter luciferase constructs together with FOXP1/ZEB2 over expression constructs or relative shRNAs. Luciferase activities were measured and normalized to those of Renilla, of three independent experiments. C. The regulation of VEGF promoter driven luciferase activity. The similar experiment was carried out. D. PANC-1 cells were transfected with FOXP1 constructs or ZEB2 constructs. The mRNA levels of E-cadherin and VEGF were detected. E. The mRNA levels of E-cadherin or VEGF were detected 48 h after PANC-1 cells were transfected with shFOXP1 or shZEB2.

SCR, ShFOXP1, vector, or FOXP1, (Figure 3B), or cultured with different conditioned media from

PANC-1 that were infected with the above lentiviruses (Figure 3C). As shown, HUVECs with

## FoxP1 might be a biomarker for pancreatic cancer



**Figure 5.** FOXP1 is a potential cancer biomarker and enhances MRI Phenotyping detection. A. The expression of FOXP1 was determined by real-time PCR in 30 pairs of pancreatic cancer samples and adjacent noncancerous tissue (NT). Error bars represent standard error of the mean, Student's t-test, \* $P < 0.05$ ; \*\* $P < 0.01$ . B. Correlation between FOXP1 and E-cadherin in the resected pancreatic cancer samples from 30 patients. \* $P < 0.05$ . C. Correlation between FOXP1 and VEGF in the resected pancreatic cancer samples from 30 patients. \* $P < 0.05$ . D. Clinical data were plotted using Kaplan-Meier curves, and the 5-year survival rate was compared using the Cox log-rank test (\*\* $P < 0.001$ ). The y-axis represents the survival probability, and the x-axis represents the survival in months.

shFOXP1 or cultured in the CM with knockdown FOXP1, formed significantly fewer tubes than those in the SCR or vector group, whereas HUVECs with FOXP1 overexpression or that which were cultured in the CM with FOXP1 overexpression generated more tubes than the control.

To further explore the tumorigenic ability of FOXP1, in vitro, colony formation was performed, the average number of colonies in the SCR group or vector group was similar to each other, whereas the number of colonies in FOXP1

knockdown was only 30%, while FOXP1 overexpression was about 2.7 fold ( $P < 0.05$ ). The data indicated there was an obviously increase in colony formation because of the high expression of FOXP1 (**Figure 3D**).

*The molecular mechanism of FOXP1 in promoting pancreatic cancer cell invasion and angiogenesis*

To further understand the molecular mechanism of FOXP1 in regulating transcription, quantitative ChIP (qChIP) assays were performed in PANC-1 cells, several key genes in different pathways which were involved in metastasis were chosen to detect, such as E-cadherin,  $\alpha$ -catenin, EpCAM, N-cadherin, Vimentin, snail1, snail2, Twist1, Twist2, VEGF and CCL14. On the promoter of E-cadherin and VEGF, the bindings of FOXP1 were obviously higher than that of the normal IgG (**Figure 3A**). Since ZEB2 was reported as a repressor of E-cadherin [14] and therefore worked an inducer of EMT, furthermore, the physical interaction between ZEB2 and FOXP1 was detected in our study. It

was reasonable to suppose there was functional consistency between ZEB2 and FOXP1. To further support the argument, luciferase reporter activity assays were carried out in PANC-1 cells with E-cadherin or VEGF promoter-driven luciferase reporter under FOXP1 over expression or depletion. These experiments indicated that FOXP1 over expression or knockdown resulted in repressed or enhanced E-cadherin reporter activity, contrary to E-cadherin, on the promoter of VEGF, FOXP1 over expression or silencing led to a significant effect on the acti-



## FoxP1 might be a biomarker for pancreatic cancer

vation or repression of the VEGF reporter activity (**Figure 4B**). The up regulation or down regulation of ZEB2 got the similar results on the two promoters (**Figure 4C**).

Consistent with the promoter occupancy, in FOXP1, ZEB2 overexpressed PANC-1 cells, the mRNA (**Figure 4D**) expression of E-cadherin decreased, to the contrary. VEGF increased. Respectively, while FOXP1 or ZEB2 was knock-down in CAPAN-1 cells, in mRNA level, E-cadherin increased, VEGF decreased, which further supporting the notion that both FOXP1 and ZEB2 have dual transcriptional activities (**Figure 4E**).

*FOXP1 is a potential cancer biomarker and enhances MRI Phenotyping detection*

In order to understand the clinical significance of FOXP1 during the progress of pancreatic cancer, we collected 30 pancreatic cancer samples, and paired with adjacent noncancerous samples. The expression levels of FOXP1 were examined, using qRT-PCR. The results showed that the expression level of FOXP1 in pancreatic cancer samples was significantly higher than the noncancerous tissues ( $P < 0.01$ , **Figure 5A**). Further, we performed correlation tests in the resected patient samples. An inverse correlation was observed between FOXP1 and E-cadherin ( $R = -0.4859$ ;  $P < 0.001$ ; **Figure 5B**), as to FOXP1 and VEGF, there was a strong positive correlation ( $R = 0.6232$ ;  $P < 0.0001$ ; **Figure 5C**). Further, depending on the median FOXP1 expression level, patients were divided in to two groups, the group of FOXP1 over expression was found to have a poorer prognosis for an overall 5-year survival ( $P < 0.05$ ) (**Figure 5D**).

### Discussion

In conclusion, we found that the expression of FOXP1 in pancreatic cancer was obviously higher than that of the noncancerous samples. Furthermore, the expression of E-cadherin was negatively associated with FOXP1 in the tumor tissues, while the expression of VEGF was positively correlated with FOXP1. The concept of ZEB2 as a powerful repressor of E-cadherin has been widely accepted; here we reported not only the physical interaction between FOXP1 and ZEB2, but also the functional consistency between the two factors. We declared that FOXP1 exerts its dual transcriptional regulatory

function via activate the expression of genes implicated in angiogenesis, including VEGF and suppress the expression of E-cadherin. Further, the over expression of FOXP1 meaning for a worse overall survival than that of patients with FOXP1 low expression, it indicates FOXP1 may act as an independent prognostic factor. Especially, Tube formation assay reveal overexpression of FOXP1 may influence the depth of tumor invasion and angiogenesis which are associated with the worst prognosis.

We demonstrate that FOXP1 promotes pancreatic cancer cell angiogenesis and invasion in vitro and in vivo, implies that FOXP1 promotes EMT and enhances the invasive capacity of pancreatic cancer. Together, our experiments reveal the mechanism for FOXP1 in facilitating EMT and tumorigenesis of pancreatic cancer, uncover the distinct role of FOXP1 resulting in altered expression of the downstream target genes, suggesting that FOXP1 might be a potential therapeutic target of pancreatic cancer for treating the angiogenesis and metastasis. It is well known that the changes in the development of pancreatic cancer should not be regarded as the alternations of a small a small part of genes. Thus, we predict other targets of FOXP1 that are related to pancreatic cancer angiogenesis and invasion will be discovered in the future. Although FOXP1 and FOXP3 share high similarity sequences, the function of FOXP3 needs further discussion. So it is necessary for further exploration of the potential role of other fork head family numbers which might contribute to the pancreatic cancer.

### Disclosure of conflict of interest

None.

**Address correspondence to:** Feng Han, Department of Hepato-Biliary-Pancreatic Surgery, Henan Tumor Hospital (The Tumor Hospital Affiliated to Zhengzhou University), No. 127 dongming Road, Zhengzhou 450008, Henan, China. E-mail: xianzhouzhang007@sina.com

### References

- [1] Yamada S, Nakao A, Fujii T, Sugimoto H, Kanazumi N, Nomoto S, Kodera Y, Takeda S. Pancreatic cancer with paraaortic lymph node metastasis: a contraindication for radical surgery? *Pancreas* 2009; 38: e13-17.

## FoxP1 might be a biomarker for pancreatic cancer

- [2] Feig C, Gopinathan A, Neesse A, Chan DS, Cook N and Tuveson DA. The pancreas cancer microenvironment. *Clin Cancer Res* 2012; 18: 4266-4276.
- [3] Cano CE, Motoo Y and Iovanna JL. Epithelial-to-mesenchymal transition in pancreatic adenocarcinoma. *Sci World J* 2010; 10: 1947-1957.
- [4] Yang J and Weinberg RA. Epithelial-mesenchymal transition: At the crossroads of development and tumor metastasis. *Dev Cell* 2008; 14: 818-829.
- [5] Maier HJ, Schmidt-Strassburger U, Huber MA, Wiedemann EM, Beug H and Wirth T. NF- $\kappa$ B promotes epithelial-mesenchymal transition, migration and invasion of pancreatic carcinoma cells. *Cancer Lett* 2010; 295: 214-228.
- [6] Kalluri R and Weinberg RA. The basics of epithelial-mesenchymal transition. *J Clin Invest* 2009; 119: 1420-1428.
- [7] Adhikary A, Chakraborty S, Mazumdar M, Ghosh S, Mukherjee S, Manna A, Mohanty S, Nakka KK, Joshi S, De A, Chattopadhyay S, Sa G, Das T. Inhibition of epithelial to mesenchymal transition by E-cadherin up-regulation via repression of slug transcription and inhibition of E-cadherin degradation: dual role of scaffold/matrix attachment region-binding protein 1 (SMAR1) in breast cancer cells. *J Biol Chem* 2014; 289: 25431-25444.
- [8] Mallini P, Lennard T, Kirby J and Meeson A. Epithelial-to-mesenchymal transition: What is the impact on breast cancer stem cells and drug resistance. *Cancer Treat Rev* 2014; 40: 341-348.
- [9] Park KS, Dubon MJ and Gumbiner BM. N-cadherin mediates the migration of MCF-10A cells undergoing bone morphogenetic protein 4-mediated epithelial mesenchymal transition. *Tumour Biol* 2015; 36: 3549-56.
- [10] Kang Y, Ling J, Suzuki R, Roife D, Chopin-Laly X, Truty MJ, Chatterjee D, Wang H, Thomas RM, Katz MH, Chiao PJ, Fleming JB. SMAD4 regulates cell motility through transcription of N-cadherin in human pancreatic ductal epithelium. *PLoS One* 2014; 9: e107948.
- [11] Hogendorf P, Durczynski A, Kumor A and Strzelczyk J. Pancreatic head carcinoma and vascular endothelial growth factor (VEGF-A) concentration in portal blood: its association with cancer grade, tumor size and probably poor prognosis. *Arch Med Sci* 2014; 10: 288-293.
- [12] Jamison S, Lin Y and Lin W. Pancreatic endoplasmic reticulum kinase activation promotes medulloblastoma cell migration and invasion through induction of vascular endothelial growth factor A. *PLoS One* 2015; 10: e0120252.
- [13] Ferrara N. Binding to the extracellular matrix and proteolytic processing: two key mechanisms regulating vascular endothelial growth factor action. *Mol Biol Cell* 2010; 21: 687-690.
- [14] Hiraoka N, Onozato K, Kosuge T and Hirohashi S. Prevalence of FOXP3+ regulatory T cells increases during the progression of pancreatic ductal adenocarcinoma and its premalignant lesions. *Clin Cancer Res* 2006; 12: 5423-5434.
- [15] Jiang Y, Du Z, Yang F, Di Y, Li J, Zhou Z, Pillarsetty VG, Fu D. FOXP3+ lymphocyte density in pancreatic cancer correlates with lymph node metastasis. *PLoS One* 2014; 9: e106741.
- [16] Comijn J, Bex G, Vermassen P, Verschuere K, van Grunsven L, Bruyneel E, Mareel M, Huylebroeck D, van Roy F. The two-handed E box binding zinc finger protein SIP1 downregulates E-cadherin and induces invasion. *Mol Cell* 2001; 7: 1267-1278.
- [17] Bindels S, Mestdagt M, Vandewalle C, Jacobs N, Volders L, Noël A, van Roy F, Bex G, Foidart JM, Gilles C. Regulation of vimentin by SIP1 in human epithelial breast tumor cells. *Oncogene* 2006; 25: 4975-4985.