

## RESEARCH COMMUNICATION

# Knockdown of Radixin by RNA Interference Suppresses the Growth of Human Pancreatic Cancer Cells in Vitro and in Vivo

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### Abstract

Radixin, encoded by a gene on chromosome 11, plays important roles in cell motility, invasion and tumor progression. However, its function in pancreatic cancer remains elusive. In this study, radixin gene expression was suppressed with a lentivirus-mediated short-hairpin RNA (shRNA) method. We found that radixin shRNA caused down-regulation of radixin in PANC-1 cells, associated with inhibition of pancreatic cancer cell proliferation, survival, adhesion and invasive potential in vitro. When radixin-silenced cells were implanted in nude mice, tumor growth and microvessel density were significantly inhibited as compared to blank control cells or nonsense shRNA control cells. Thrombospondin-1 (TSP-1) and E-cadherin were up-regulated in radixin-silenced PANC-1 cells. Our results suggest that radixin might play a critical role in pancreatic cancer progression, possibly through involvement of down-regulation of TSP-1 and E-cadherin expression.

**Keywords:** Pancreatic neoplasms - small interference RNA - radixin

*Asian Pacific J Cancer Prev*, 13, 753-759

### Introduction

Radixin, encoded by chromosome 11 (11 exons), is a member of the ERM (ezrin–radixin–moesin) family; it functions as a membrane-cytoskeletal crosslinkers in actin-rich cell surface structures and is thereby thought to be essential for cortical cytoskeleton organization, cell motility, adhesion and proliferation (Hoeftlich and Ikura, 2004). ERM proteins display a similar structural organization. They share extensive homology in their amino-terminal domain, which is called the four-point one, ezrin, radixin, moesin (FERM) domain (Gautreau et al., 2002). The last 34 residues of the ~100 amino acid carboxy-terminal domain (CTD) of radixin consists of a (filamentous) F-actin-binding site. Spanning these globular domains lies an  $\alpha$ -helix-rich domain, termed the  $\alpha$ -domain. Recent biophysical studies indicate that the radixin  $\alpha$ -domain is an extremely long (240Å in length from N- to C-terminus), linear monomer with an enhanced number of electrostatic, salt bridge interactions predicted to contribute synergistically to its thermal stability (Hoeftlich et al., 2003). Activated radixin has been shown to join actin filaments to CD43, CD44, and ICAM1-3 cell adhesion molecules and various membrane channels and receptors, such as the Na<sup>+</sup>/H<sup>+</sup> exchanger-3 (NHE3), cystic fibrosis transmembrane conductance regulator (CFTR) and the  $\beta$ 2-adrenergic receptor (Tsukita and Yonemura, 1999; Bretscher et al., 2002; ).

Radixin is expressed in a variety of normal and

neoplastic cells, including many types of epithelial and lymphoid cells (Ramoni et al., 2002; Suda et al., 2011). In pancreatic carcinomas, a high-level radixin expression is associated with high metastatic potential. Patients with pancreatic ductal adenocarcinoma (PDAC) with radixin expression exhibited poorer prognosis compared to those without radixin expression and radixin protein was more likely to be present in cancers with lymph node metastasis (Cui et al., 2009). Furthermore, ezrin, another member of the ERM family, silencing by small hairpin RNA could reverse the metastatic behavior of human pancreatic cancer cells (Meng et al., 2010). The observed effects of ezrin overexpression and silencing on the cell malignant transformation indicate a role for ezrin in regulating tumor metastasis and progression (Curto et al., 2004). However, the mechanisms of radixin-mediated tumor development still require further elucidation. In this study, we investigated the effect of radixin on the growth and invasion ability of the pancreatic cancer cell line PANC-1.

### Materials and Methods

#### *Cells and antibodies*

The pancreatic adenocarcinoma cell line PANC-1 was purchased from the American Type Culture Collection (Manassas, VA) and cultured in DMEM (GIBCO, Grand Island, NY) supplemented with 10% fetal calf serum (FCS) and 1% L-glutamine (Invitrogen, Karlsruhe, Germany) and maintained at 37°C in 5% CO<sub>2</sub>. Rabbit

polyclonal anti-radixin antibody was purchased from Upstate technology (Lake Placid, NY). Mouse monoclonal anti-E-Cadherin, anti-TSP-1 antibodies were purchased from Cell Signaling Technology (Beverly, MA). The mouse monoclonal antibody GAPDH was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The secondary antibodies were purchased from Saier Biotechnology (Tianjing, China).

#### RNA interference

Sequences of radixin (GeneBank, No. L02320) shRNA were inserted into the pSIL-GFP lentivirus RNAi expression system. The shRNA containing vectors were transfected together into 293T cells with pHelper1.0 and the lentiviral helper plasmid pHelper2.0 to generate the respective lentiviruses. Viral stocks were collected from the transduced 293T cells and were used to infect PANC-1 cells. The sequence of radixin nonsense shRNA was: ACTACCGTTGTTATAGGTG; the sequence of radixin shRNA was GACGACAAGTTAACACCTAAA (Kubo et al., 2008). The mRNA and protein levels were measured 72 h after cells being infected.

#### Quantitative RT-PCR

Total cellular RNA was extracted with M-MLV-RTase (Promega, Madison, WI) according to the manufacturer's protocols. The resulting cDNA was used for PCR using the SYBR-Green Master PCR Mix (Applied Biosystem, Carlsbad, CA) in triplicates. Primers for qRT-PCR were as follows: radixin forward primer: CGAGGA AGAACGTGTAACCGAA; radixin reverse primer: TCTTGGTTTTTCATCTCTGGCTTG; E-Cadherin (GeneBank, No. Z13009) forward primer: CGGGAATGCAGTTGAG GATC; E-Cadherin reverse primer: AGGATGGTGTAAGCGATGGC; TSP-1 (GeneBank, No. X04665) forward primer: TGGAACTATGGGCTTGAGAAAAC; TSP-1 reverse primer: CACTGATGCAAGCACAGAAAAGA. PCR and data collection were performed on the TP800 qPCR System (Takara, Shiga, Japan). All quantitations were normalized to an endogenous  $\beta$ -actin control.  $\beta$ -actin forward primer: CACCACCATGTACCCTGGCA;  $\beta$ -actin reverse primer: GCTGTCACCTTC ACCGTTCC. The relative quantitation value for each target gene compared to the calibrator for that target is expressed as  $2^{-(Ct-Cc)}$  (Ct and Cc are the mean threshold cycle differences after normalizing to  $\beta$ -actin).

#### Western blotting

Western blotting was performed as previously described (Guo et al., 2009). Briefly, cell lysates (30  $\mu$ g protein) resolved on 10% SDS-PAGE were transferred to a polyvinylidene difluoride membrane (Millipore, Bedford, MA). For immunoblotting, antibodies against radixin, E-Cadherin, TSP-1 and  $\beta$ -actin were used.

#### MTT Assay

MTT assay was performed as previously described (Chen et al., 2010). Viable cells ( $5 \times 10^4$  cells/ml) were plated into 96-well plates (100  $\mu$ l complete medium/well) and cultured at 37°C in 5% CO<sub>2</sub>. At different time

points, MTT reagent (DingGuo Biotech, Beijing, China) was added (20  $\mu$ l per well) and incubated at 37°C for 4 h. The reaction was stopped with addition of 100  $\mu$ l DMSO and the optical density was determined at OD570 nm on a multi-well plate reader.

#### Cell Cycle Assay

Cell cycle analysis was performed by flow cytometry as described previously (Meng et al., 2010).  $5 \times 10^5$  cells were plated in 60-mm dishes and cultured for 2 days. Afterwards, cells were collected by trypsinization, fixed with 95% ethanol, washed with PBS, resuspended in 1 mL of 0.01 M PBS with RNase and 50  $\mu$ g/mL propidium iodide, incubated for 20 minutes in the dark at room temperature and analyzed by flow cytometry using a FACS Calibur (Becton Dickinson, Bedford, MA).

#### Detection of apoptotic cells

Cells were stained with fluorescein isothiocyanate (FITC) labeled annexin-V, and simultaneously with propidium iodide (PI) stain, to discriminate intact cells (annexin-/PI-) from apoptotic cells (annexin+/PI-) and necrotic cells (annexin+/PI+). A total of  $1.0 \times 10^6$  cells were washed twice with ice-cold PBS and incubated for 30 min in a binding buffer (1  $\mu$ g/ml PI and 1  $\mu$ g/ml FITC labeled annexin-V) respectively. FACS analysis for annexin-V and PI staining was performed by flow cytometer.9

#### Adhesion assay

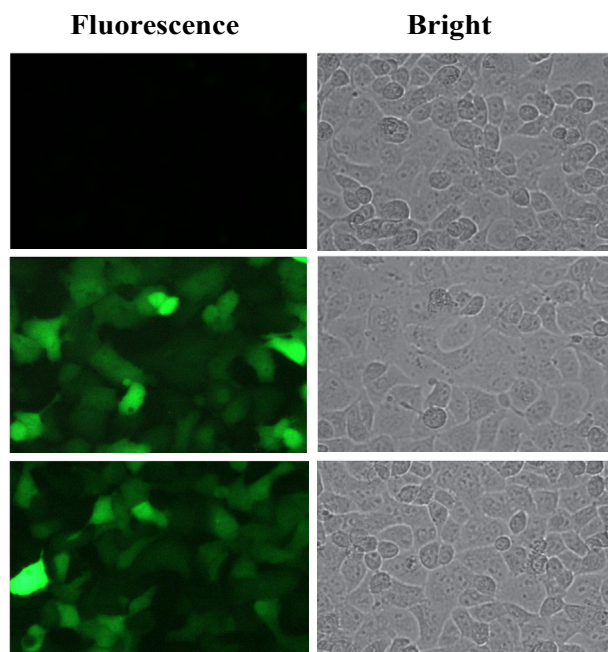
96-well plates were coated overnight at 4°C with 100  $\mu$ l of matrigel and blocked with 0.5% BSA for 1 hour at room temperature. Then  $4 \times 10^5$  cells were seeded in each well and incubated 37°C. After incubation, unbound cells were removed along with the culture medium. Remaining adherent cells were colored for 15 minutes with 0.5% violet crystal and fixed with 20% methanol. After washed with PBS, adherent cells were lysed with 1% SDS. Adhesion was quantified with spectrophotometric optical density measurement at 570 nm.

#### Invasion assay

Invasion assay was performed as previously described (Albini et al., 1978). In short, cells were harvested and washed with serum free medium.  $1 \times 10^5$  cell suspension was then added into the matrigel transwells, with 600  $\mu$ l of culture medium containing 10 ng/ml ECM as the chemoattractant in the lower chamber. Cells that invaded through the matrigel membrane were stained and counted after 24 h incubation at 37°C.

#### Tumor growth in nude mice

Female BALB/c nude mice (body weight, 15 to 17 g) were bred under specified pathogen-free conditions (26°C, 70% relative humidity and a 12-h light/12-h dark cycle) in a germ-free environment with free access to food and water. The logarithmically growing cells were trypsinized and resuspended in D-Hanks solution.  $5 \times 10^6$  cells in 0.2 ml were injected subcutaneously into the left flank of mice. Experimental and control groups had at least 8 mice each. Tumors were measured twice weekly with microcalipers, and the tumor volume was



**Figure 1. Efficient Delivery of ShRNA into PANC-1 Cells.** High efficiency of transfection with fluorescent shRNA (green) in PANC-1 cells was easily identified for 48 h post-transfection. CON: blank control; NC: nonsense shRNA; KD: radixin shRNA

calculated according to the formula:  $V(\text{volume}) = LW^2 \times \pi/6$  where “L” represents the greatest length and “W” represents the perpendicular width (Butler et al., 1986). The animals were sacrificed after 7 weeks. The tumors were excised and weighed. The tumor specimens were fixed in 4% formaldehyde, embedded in paraffin and cut in 4  $\mu\text{m}$  sections for immunohistochemical analysis.

*Quantification of tumor microvessel density and in situ TUNEL assay for apoptotic cells*

Tumor microvessel densities (MVD) were quantified by anti-CD31 immunohistochemistry as previously described (Singh et al., 2010). Apoptotic cells in tumor samples were identified by terminal deoxyribonucleotidyl transferase dUTP nick end fluorescein labeling (TUNEL) assay according to the manufacturer’s instructions (In situ Cell Death Detection Kit, Roche, Indianapolis, IN). The number of apoptotic cells was evaluated by counting the positive (brown-stained) cells in 10 random fields ( $\times 200$ ).

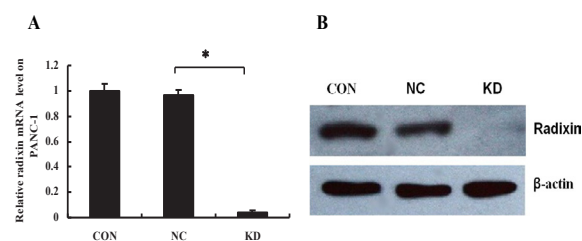
*Statistical analysis*

Each experiment was performed three to four times. All of the data were expressed as mean  $\pm$  SD. Statistical analysis was performed with SPSS 13.0 software. (SPSS Inc, Chicago, IL). Comparisons between groups were conducted using one-way analysis of variance (ANOVA). Differences were considered statistically significant at  $p < 0.05$ .

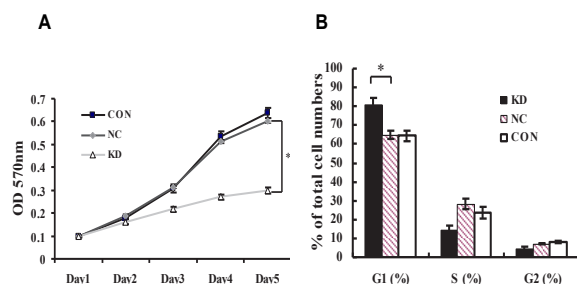
**Results**

*Reduction of radixin mRNA and protein expression by shRNA in vitro*

To investigate the biological role of radixin in PANC-1 cells, we knocked down radixin transcript by employing



**Figure 2. Stable Silencing of Radixin in PANC-1 Cells.** (A) The radixin mRNA level was downregulated by radixin shRNA in PANC-1 cells. (B) The radixin protein level was downregulated by radixin shRNA in PANC-1 cells. Results represent the mean  $\pm$  SD of three independent experiments. \* $p < 0.05$



**Figure 3. Down-Regulation of Radixin Inhibits Proliferation of Pancreatic Cancer Cells.** (A) Proliferation of PANC-1 cells were inhibited when treated with radixin shRNA by MTT assay. (B) The proportion of PANC-1 cells increased with G1 phase as a consequence of decreased radixin expression. Results represent the mean  $\pm$  SD of three independent experiments. \* $p < 0.05$

shRNA technology. shRNA to radixin was constructed into pSIL-GFP vector using lentivirus transfection system, as shown in Figure 1. The lentivirus transfection system successfully down-regulated radixin expression at both mRNA level and protein level in comparison with blank controls or nonsense shRNA controls (Figure 2).

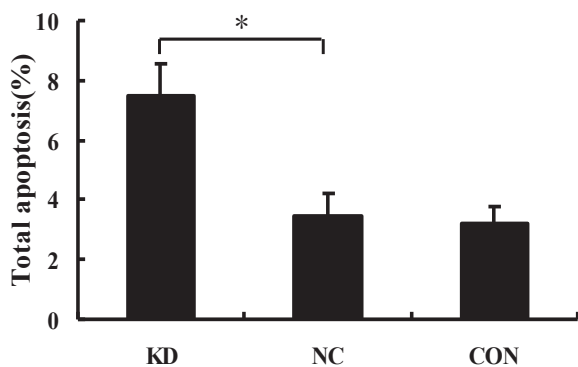
*Radixin down-regulation inhibited the proliferation of PANC-1 cells*

Using the radixin knockdown cells, we first tested the change of cell growth, using the MTT assay. The results showed a significant decrease of cell growth and indicated that cell proliferation was inhibited after transfection with radixin shRNA (Figure 3A). The flow cytometry assay, in further detail, showed that the proportion of cells in the G1 phase was significantly increased and the proportion of cells in the G2 and S phases was decreased after transfection with radixin shRNA (Figure 3B). These results indicated that radixin could enhance the growth ability of PANC-1 cells.

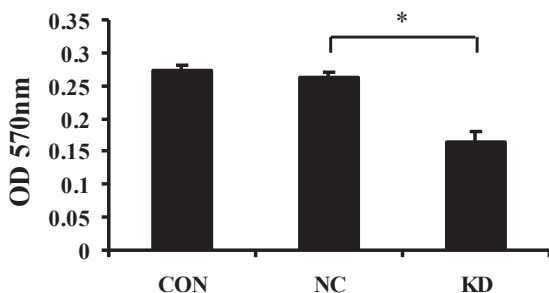
*Radixin down-regulation promoted the apoptosis of PANC-1 cells*

To determine the effects of downregulation of radixin on the radixin-induced apoptosis in PANC-1 cells, the rate of apoptosis was evaluated by flow cytometry analysis.

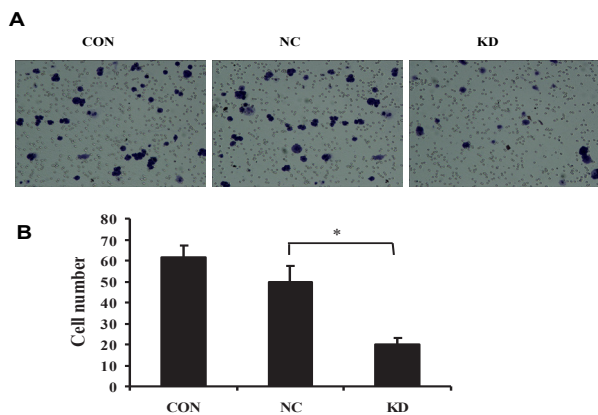
As shown in Figure 4, the effects of radixin-induced apoptosis were investigated in cells treated with radixin shRNA as well as cells in blank controls and cells treated with nonsense shRNA. We found that the significantly



**Figure 4. Down-Regulation of Radixin Promotes Apoptosis of Pancreatic Cancer Cells.** The data present the average number of apoptotic cells ( $\pm$  SD) in three independent experiments. \* $p < 0.05$



**Figure 5. Down-Regulation of Radixin Inhibits the Adhesion of Pancreatic Cancer Cells.** The number of adherent living cells was significantly lower in the KD group than in the NC group and CON group. \* $p < 0.05$ .

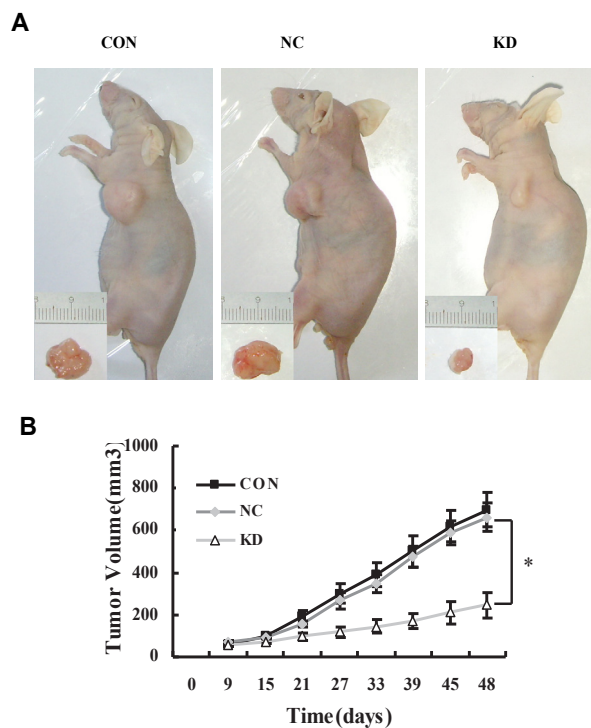


**Figure 6. Down-Regulation of Radixin Inhibits the Invasion of Pancreatic Cancer Cells.** (A) Matrigel-coated transwell chambers were used to detect cell invasion and representative fields were photographed. (B) The decrease in the numbers of invasive cells in the KD cells compared to those of the NC cells was statistically significant. Results represent the mean  $\pm$  SD of three independent experiments. \* $p < 0.05$

elevated percentage of apoptosis in the cells transfected with radixin shRNA compared with the NC cells ( $p < 0.05$ ), while there was no significant difference between blank controls and cells transfected with nonsense shRNA ( $p > 0.05$ ).

*Radixin down-regulation inhibited the adhesion and invasion of PANC-1 cells*

In the cell adhesion in vitro experiment, the number of adherent living cells was significantly lower in the

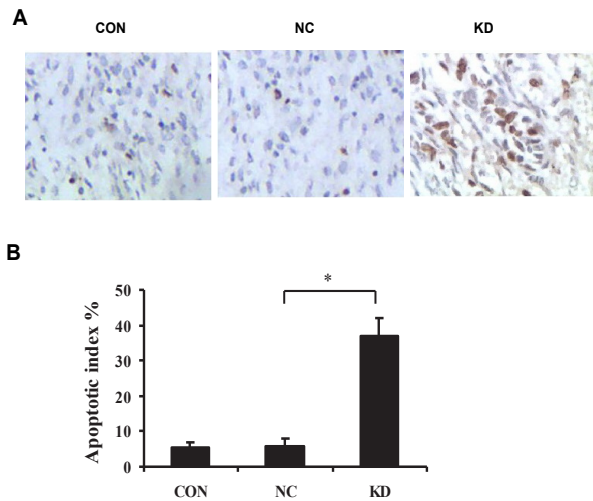


**Figure 7. Down-Regulation of Radixin Reduces Pancreatic Cancer Tumor Growth in Vivo.** Pancreatic cancer cells (CON, NC or KD) were subcutaneously injected into the left flank region of nude mice (A). Tumor volume were measured twice weekly with a caliper and were calculated by using the formula  $\pi/6 \times (\text{smaller diameter})^2 \times (\text{larger diameter})$ . (B) Tumor volume from day 0 to day 48 (n=8). Growth of KD were significantly reduced ( $p < 0.05$ ) compared with NC or CON group. \* $p < 0.05$

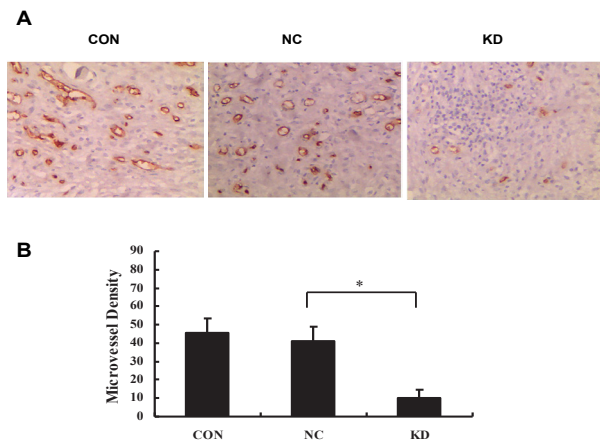
radixin shRNA group (KD) than in the nonsense shRNA group (NC) and blank control group (CON) (Figure 5). These results suggest that radixin knockdown induces the loss of cell adhesion to the matrix. Since two critical steps are involved in metastasis, that is adhesion and invasion, we next examined whether radixin can affect the invasion activity of pancreatic cancer cells by the matrigel invasion assay. Cell invasive activity was also dramatically decreased in the KD cells compared to that in the NC cells or CON cells. The average cell number invading to the lower chamber for 24 hours was  $20.25 \pm 2.62/\text{HPF}$  in the KD cells, compared to  $71.50 \pm 17.06/\text{HPF}$  in the NC cells. The quantitative analysis showed that the number of KD cells invading to the lower chamber was decreased by 71.69% compared to that of the NC cells ( $p < 0.05$ ), while there was no significant difference between NC cells and CON cells ( $p > 0.05$ ) (Figure 6).

*Radixin down-regulation resulted in reduced tumor growth and angiogenesis*

To specifically determine the role of radixin in tumor development, KD, NC or CON cells were injected into the left flank region of nude mice (n=8/group). All of the mice developed tumors (Figure 7A). Seven weeks post-tumor injection, tumor volumes were measured. Subcutaneous tumors formed from KD cells were significantly smaller compared with the NC or CON cells (KD,  $696.25 \pm 82.32\text{mm}^3$ ; NC,  $661.74 \pm 67.23 \text{mm}^3$ ; CON,  $246.20 \pm$



**Figure 8. Down-regulation of radixin increases apoptosis in vivo.** (A) Representative photographs of the tumor sections examined by TUNEL assay. (B) The data present the average number of apoptotic cells  $\pm$  SD. \* $p < 0.05$



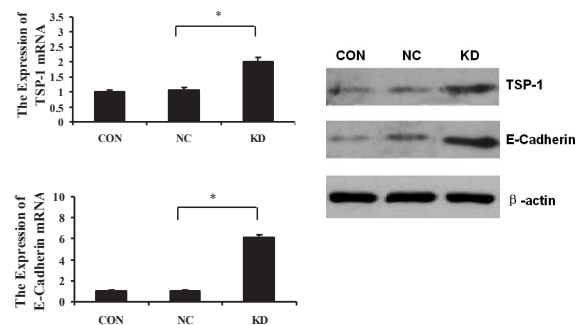
**Figure 9. Down-Regulation of Radixin Decreases Microvessel Density in Vivo.** (A) Immunohistochemical staining for microvessels with anti-CD31. The representative pictures are shown at 200 $\times$ magnification. (B) Microvessel density was quantitated microscopically with a 5 $\times$ 5 reticle grid at 400 $\times$ magnification. The values are mean  $\pm$  SD. \* $p < 0.05$

60.42 mm<sup>3</sup> Figure 7B). Additionally, KD tumors had significantly increased numbers of TUNEL-positive cells as compared with NC or CON tumors ( $p < 0.05$ ) (Figure 8).

One possible mechanism for decreased tumor growth is attenuated neovascularization. In order to determine whether down-regulation of radixin could potentially disrupt the neovascularization, we examined vascularity in tumors. Staining on KD tumor sections showed a 5.1 fold decrease in the number of blood vessels as compared to NC tumors (Figure 9).

#### *Thrombospondin-1 (TSP-1) and E-Cadherin were regulated in radixin-silenced PANC-1 cells*

In order to determine the mechanism(s) by which radixin regulates tumor growth and progression, we examined potential radixin-regulated molecules. As we know, E-cadherin plays a central role in epithelial cell-



**Figure 10. Thrombospondin-1 (TSP-1) and E-Cadherin were Regulated in Radixin-Silenced PANC-1 Cells.** (A) The TSP-1 and E-Cadherin mRNA levels were up-regulated by radixin shRNA in PANC-1 cells. (B) The TSP-1 and E-Cadherin protein levels were up-regulated by radixin shRNA in PANC-1 cells. Results represent the mean  $\pm$  SD of three independent experiments. \* $p < 0.05$

cell adhesion and maintenance of epithelial cell colony integrity (Takeichi, 1993). TSP-1 is a potent inhibitor of neovascularization that limits tumor growth (Castle et al., 1997; Gutierrez et al., 2003). In this study, Western blot analysis and quantitative RT-PCR revealed that TSP-1 and E-Cadherin were dramatically up-regulated in the cells transfected with radixin shRNA (Figure 10). Those results suggest that down-regulation of TSP-1 and E-Cadherin expressions might participate in the radixin-mediated tumor growth and progression.

## Discussion

Radixin is a member of the ERM (ezrin-radixin-moesin) family, it shares the common membrane-binding N-terminal FERM domain with band-4.1 family members (Turunen et al., 1994). By organizing membrane-cytoskeleton-associated complexes and creating specialized membrane domains, the ERM proteins regulate cellular activities such as survival, adhesion, and migration/invasion, all of which are important during tumor development and progression (Bretscher et al., 2002; McClatchey, 2003). Mice lacking radixin are characterized by a breakdown of hepatocyte apical microvilli which ultimately results in mild liver injury similar to human conjugated hyperbilirubinemia in Dubin-Johnson syndrome (Kikuchi et al., 2002). High level ERM expressions were observed in many tumor cell lines, such as breast carcinoma and osteosarcoma cell line (Khanna et al., 2004; Revillion et al., 2008). Radixin overexpression was also been observed in pancreatic cancer tissues and associated with tumor malignant transformation and metastatic potential (Cui et al., 2009); however, its role and mechanisms remain elusive.

Our results showed that knocking down radixin resulted in an inhibition of cell proliferation and survival. In addition, we also observed decreased adhesion and invasion of radixin knock-down PANC-1 cells. We established experimental mice models and showed that knock-down of radixin regulated primary tumor growth, angiogenesis and survival. The results were consistent with our observations in vitro. Hence, the results of

our study suggest an important association between the expression of radixin and pancreatic cancer growth and invasion.

Increased cell proliferation and decreased cell death play a pivotal role in tumor progression. Any decreased tumor growth of radixin knock-down cells may be due to their decreased response to cell proliferation, adhesion, survival or invasion. The present in vitro data reveals that knocking down radixin results in a significant decrease in proliferation. Another important finding of this study was the enhanced apoptosis of radixin knock-down cells. An apoptotic property of these cells was observed both in vitro and in tumor tissues. Our results are consistent with the previous finding in hippocampal pyramidal cells (Paolini et al., 1998).

The invasion of cells into the surrounding tissue is a multi-step action that requires cell-cell contact, cell motility and degradation of the extracellular matrix by matrix metalloproteinases. Malignant tumor cells display a varying degree of resistance to detachment, generating cell-extracellular matrix interactions that are related with the adhesion complex, and this property contributes to tumorigenesis and metastasis (Frisch and Francis, 1994). Our in vitro study showed significant difference in adhesion and invasion between radixin knock-down and control cells. Radixin is a cytoskeletal protein that might affect the assembly of cytoskeletal elements at the cytoplasmic face of the membrane and the nuclear skeleton, which would then facilitate cell invasion. These results are in agreement with another report where ERM stable knockdown by siRNA reduced in vitro migration and invasion of human SGC-7901 cells (Ou-Yang et al., 2011). Angiogenesis is another essential step for tumor growth and metastasis. Our immunohistochemistry results support that primary tumor vasculature contributed to in vivo differences between radixin knock-down and nonsense shRNA control tumors.

E-cadherin was first discovered in 1995 by Berx (Berx et al., 2008). It is a calcium-dependent cell-cell adhesion molecule with pivotal roles in epithelial cell behavior, tissue development, and suppression of cancer growth (Stemmler, 2008; van Roy and Berx, 2008). TSP-1 is a potent inhibitor of neovascularization that limits tumor growth (Castle et al., 1997; Gutierrez et al., 2003), and stimulates apoptosis and growth inhibition in neovascular endothelial cell (Jimenez et al., 2000; Lawler et al., 2001). TSP-1 is also reported to inhibit invasion (Moon et al., 2005). Our results showed that E-cadherin and TSP-1 were markedly increased in the radixin knock-down cells. Therefore, down-regulation of TSP-1 and E-Cadherin expressions may be involved in radixin-induced growth and invasion of pancreatic cancer cells. However, further research is necessary in order to understand the detailed mechanism.

In conclusion, we successfully silenced the radixin protein and mRNA expression in PANC-1 cells, and found that changes in the radixin protein and mRNA level were correlated with changes in the proliferation, apoptosis, adhesion and invasion, which are all tumor cells features. Based on these results, we propose that radixin might play a critical role in pancreatic cancer progression. Down-

regulation of TSP-1 and E-Cadherin expressions may be involved in this role. These results indicate that blocking radixin function may represent a novel and effective strategy for preventing pancreatic cancer progression.

## Acknowledgements

The authors declare no competing financial interests.

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