Overexpression and biological function of IQGAP3 in human pancreatic cancer

Weihong Xu1*, Bin Xu1*, Yiting Yao1, Xiaoling Yu1, Hua Cao1, Jun Zhang2, Jie Liu2, Huiming Sheng1

1Department of Clinical Laboratory of Tongren Hospital, School of Medicine, Shanghai Jiaotong University, Shanghai, China; 2Institute of Digestive Diseases of Huashan Hospital, Fudan University, Shanghai, China. *Co-first authors.

Received July 28, 2016; Accepted October 16, 2016; Epub December 15, 2016; Published December 30, 2016

Abstract: IQGAP3 (IQ motif containing GTPase activating protein3) belongs to IQGAP family. Recent studies have investigated that IQGAP3 was overexpressed in several tumor tissues. This study was designed to explore the expression and role of IQGAP3 in pancreatic cancer, a highly lethal disease. IQGAP3 mRNA expression was significantly increased in pancreatic cancer tissues, compared with non-cancerous tissues. Moreover, its expression was strongly associated with tumor size, differentiation, lymph node metastasis and patients’ overall survival. Gene set enrichment analysis (GSEA) on The Cancer Genome Atlas (TCGA) dataset showed that cell apoptosis, metastasis and Cdc42 pathways were strongly associated with IQGAP3 expression in pancreatic cancer patients. Knocking down of IQGAP3 in two pancreatic cancer cell lines with high level of IQGAP3 (BXPC-3 and SW1990) significantly inhibited cell proliferation, migration and invasion, and induced cell apoptosis. Moreover, silencing of IQGAP3 also affected the expression of cell apoptosis-, metastasis- and Cdc42 pathway-related proteins. Cdc42 knockdown had similar inhibitory effects on the cellular behavior of BXPC-3 cells. In conclusion, IQGAP3 may act as an oncogene in pancreatic cancer through regulating Cdc42 expression. Our data suggest IQGAP3 might be a novel diagnostic marker and therapeutic target for this cancer.

Keywords: IQGAP3, apoptosis, metastasis, Cdc42

Introduction

Pancreatic cancer is a highly lethal disease with a 5-year overall survival rate of 5% [1]. Globally, pancreatic cancer is the seventh most common cause of cancer deaths with an annual death of about 330,000 people despite advances in surgical techniques and systemic treatment [2]. Because of the extraordinary tumor invasion and early systemic dissemination of pancreatic cancer [3], most patients diagnosed with this disease cannot receive curative resection, thus contributing to the high mortality rate of this disease. This emphasizes the need for the identification of novel diagnostic markers and therapeutic targets to expedite the diagnosis and the treatment.

IQGAP3 (IQ motif containing GTPase activating protein3), isolated in 2007, belongs to IQGAP family [4]. IQGAP family is well conserved among different species from yeast to mammals. These proteins contain calponin-homology domain (CH), internal repeats (IR), tryptophan repeat motif (WW), isoleucine-glutamine (IQ) domain and RasGAP-related domain (GRD) [5]. IQGAP3 is the least studied member of the IQGAP family. By interacting with its target proteins, IQGAP3 functions in the regulation of the proliferation of both normal [4, 6, 7] and cancer cells [8, 9], migration of cancer cells [9], as well as invasiveness of squamous cell carcinoma (SCC) [10]. Elevated levels of IQGAP3 were reported in several tumor tissues [9, 11] (bone marrow, breast, large intestine, lung, ovary, stomach and colon). However, the expression and role of IQGAP3 in pancreatic cancer is still poorly understood.

In the current study, we compared IQGAP3 expression between pancreatic cancer and paired non-cancerous tissues. The effects of IQGAP3 knockdown on the proliferation, migration and invasion of pancreatic cancer cells were then evaluated. We also tried to explore the involved possible mechanism.
our study showed that IQGAP3 was overexpressed in pancreatic cancer tissues and IQGAP3 is a potential oncogene for pancreatic cancer.

**Materials and methods**

**Tissue collection**

The tumor samples (n=100) and non-tumorous samples (n=35) were obtained from patients with pancreatic cancer, who were admitted at Department of Clinical Laboratory of Tongren Hospital, Shanghai Jiatong University (Shanghai, China) from January 2007 to July 2009. Tissues were snap frozen immediately after surgery. None of the enrolled patients had been treated with radiotherapy, chemotherapy, or other related anti-tumor therapies prior to surgery. Overall survival (OS) time was calculated from the date of the surgery until death. Use of these clinical specimens was approved by Research Ethics Committee of Tongren Hospital (Shanghai, China). Written informed consent was obtained from all patients.

**Cell lines**

Human pancreatic cancer cell lines HPAC, CAPAN-1, ASPC-1, BXPC-3, PANC-1 and SW1990 were obtained from the American Type Culture Collection (Rockville, MD, USA). Cells were cultured in RPMI 1640 (Life Technologies, Inc., Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS, Hyclone, Logan, UT, USA), 4 mM L-glutamine, 100 units/ml of penicillin and 100 μg/ml streptomycin. Incubation was carried out at 37°C under 5% CO₂ in air.

**RNA isolation and quantitative real-time PCR**

Total RNA was extracted from tissue samples or cultured cells by using Trizol reagent (Invitrogen, Carlsbad, CA USA) according to the manufacturer’s instructions. IQGAP3 mRNA expression levels were determined by quantitative real-time PCR using SYBR Green Master Mix (TOYOBO, Osaka, Japan) on an ABI 7300 machine (Applied Biosystems, Foster City, CA, USA), with GAPDH as an internal control. The primers used here were listed as follows: IQGAP3 (NM_178229.4), 5'-GCAGAATGTTGCC-TATCAG-3' and 5'-CGGAAATGTAAGCCAGTTG-3'; GAPDH (NM_001256799.1), 5'-CACCCACTCC-TCCACCTTTG-3' and 5'-CCACCACCTGTTGCTG-TAG-3'. All reactions were performed with the following cycling parameters, 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 45 s. IQGAP3 expression normalized by internal control was calculated using the formula 2⁻ΔΔCT.

**Bioinformatics analysis**

Microarray gene-expression profiles for pancreatic tumor and adjacent non-tumor tissues with pancreatic ductal adenocarcinoma (PDAC) were downloaded from the NCBI Gene Expression Omnibus (GEO). The dataset (GSE28735 [12]) consisted of 45 pairs of pancreatic tumor and adjacent non-tumor tissues. RNA-seq data of The Cancer Genome Atlas project (TCGA) Pancreatic adenocarcinoma (PAAD) cohort were downloaded from TCGA website (https://tcga-data.nci.nih.gov/tcga/). IQGAP3 expression among pancreatic cancer and adjacent normal tissues were analyzed by student’s t test.

Gene Set Enrichment Analysis (GSEA) was performed with GSEA version 2.0 from the Broad Institute at MIT as previously described [13, 14]. PAAD cohort of TCGA was analyzed to determine the signaling pathways related to IQGAP3 expression in pancreatic cancer. In our analysis, the gene sets of fewer than 15 genes were excluded. Using a permutation test 1,000 times, the significant cut-off level was P=0.05.

**RNA interference**

Double-stranded small interference RNA (siRNA) targeting human IQGAP3, human Cdc42 (GGACGGAUUGAUUCCACAU) and negative control siRNA were synthesized by Genepharma Co., Ltd (Shanghai, China). BXPC-3 and SW1990 Cells were transfected with siIQGAP3 and siNC using Lipofectamine 2000 (Invitrogen) as described by the manufacturer. At 48 h post transfection, they were then harvested or reseeded for further assays.

**Antibodies and western blot assays**

Primary antibodies were obtained from the following companies: (1) IQGAP3, Caspase 3, Capase 9, Cyclin D1, Cyclin E, Twist and Src, Abcam (Cambridge, MA, USA); (2) Bax, Santa Cruz Biotech. (Santa Cruz, CA, USA); (2) Bad, E-cadherin, β-catenin, Snail, Rac1, Cdc42 and GAPDH, Cell Signaling Biotech. (Danvers, MA,
Cells were lysed with ice-cold radioimmunoprecipitation assay buffer (RIPA buffer, Beyotime) in the presence of a Protease Inhibitor Cocktail (Pierce, Rockford, IL, USA). Extracts equivalent to 30–40 μg of total protein were separated on SDS-polyacrylamide gel electrophoresis and transferred onto polyvinylidene difluoride (PVDF) membranes and analyzed by Western blotting using enhanced chemiluminescence system (Bio-Rad, Richmond, CA, USA).

Cell proliferation assay

Cell proliferation was assessed using Cell Counting Kit-8 (CCK-8) Assay Kit (Dojindo Lab, Kumamoto, Japan). Briefly, cells transfected with siIQGAP3 or siNC, along with non-treated cells (WT), were plated into 96-well plates at a density of 3,000 cells/well. After 0, 24, 48 and 72 h, CCK-8 solution was added and cells were incubated at 37°C for 1 h. Optical density values (OD) were measured at a wavelength of 450 nm with a microplate reader (Epoch, BioTek, Luzern, Switzerland).

Cell apoptosis analysis

The percentage of cells undergoing early apoptosis was determined by double stained with Annexin V-fluorescein isothiocyanate (FITC) and PI. Cells were harvest at 48 h post transfection, stained with Annexin V-FITC/PI apoptosis kit (KeyGen, Nanjing, China) in the dark for 20 min and analyzed on FACScan (BD Biosciences). The early apoptotic cells are represented in the lower right quadrant of the FACS histogram.

Cell migration and invasion assay

Migration assay was carried out in a 24-well Chemotaxis chamber (8-µm, Corning Life Sciences, Corning, NY, USA) according to the manufacturer’s protocol. At 24 h post transfection, cells were collected, suspended in serum-free medium and seeded into the upper chamber (3 × 10⁴ cells/well). Then medium with 10% FBS were added into the lower chamber as a chemoattractant. Cells remaining in the upper chamber were removed with a cotton swab after 24 h of incubation. Cells migrated to the bottom of the membranes were fixed with 10% formalin and stained with 0.2% crystal violet. The degree of migration was expressed as the average number of cells in five 200 × fields. Cell invasion assay was done in the same manner as migration assay, except that the upper chamber was pre-coated with 100 μL of 1 mg/mL Matrigel (BD Biosciences).

Establishment of stable knockdown cells and in vivo tumorigenicity assay

IQGAP3 short-hairpin RNA (shRNA, shIQGAP3) and a negative control construct (shNC) created in the same vector system (pLKO.1, Addgene, Cambridge, MA, USA). The lentivirus were then packaged in 293T cells [15]. BXPC-3 cells were transduced with the appropriate lentiviruses, and stable cell lines were selected using puromycin (Sigma).

The animal study protocol was approved by the Animal Experimentation Ethics Committee of Shanghai Jiaotong University School of medicine. Eighteen BALB/c nude mice aged 4-5 weeks old (SLAC Animal, Shanghai, China) were housed under specific pathogen-free conditions and maintained under constant humidity and temperature. BXPC-3 cells (2 × 10⁶) stably transduced with shIQGAP3 or shNC were collected and subcutaneously injected into the flank of nude mice. Tumor volume (mm³) was monitored every three days by using the following formula: volume =1/2 × (largest diameter) × (smallest diameter)². 30 days after cell inoculation, the mice were sacrificed by CO₂ inhalation euthanasia and the xenograft tumors were resected. During the experiment, all the mice were monitored every day. No mice died before the end of the experiment.

Statistical analysis

Statistical analysis was carried out using GraphPad Prism 6 (GraphPad Software, San Diego, CA, USA). The correlation between clinicopathological features and IQGAP3 mRNA expression were assessed using the Fisher’s exact test. Overall survival was analyzed with Kaplan-Meier survival curves and log-rank test. In vitro experiments were replicated three times. Data are expressed as the mean ± standard deviation (SD). The two-tailed Student’s t-test was used to calculate the statistical significance of difference between groups. P values <0.05 were considered statistically significant.
Expression of IQGAP3 mRNA is increased in pancreatic cancer tissues

We re-analyzed pancreatic adenocarcinoma (PAAD) cohort of The Cancer Genome Atlas (TCGA, https://tcga-data.nci.nih.gov/tcga/) and a pancreatic ductal adenocarcinoma (PDAC) dataset of the NCBI Gene Expression Omnibus (GEO, GSE28735 [12]). The results showed that IQGAP3 mRNA was significantly higher in pancreatic cancer tissues than that in non-cancerous tissues on both datasets (Figure 1A and 1B). We then examined the mRNA levels of IQGAP3 on surgical samples collected from pancreatic cancer patients admitted at Department of Clinical Laboratory of Tongren Hospital using quantitative real-time PCR. The results confirmed the overexpression of IQGAP3 mRNA in pancreatic cancer tissues (Figure 1C).

According to IQGAP3 mRNA levels in tumor tissues, the pancreatic patients (n=100) were evenly divided into IQGAP3 lower expression group and higher expression group. Fisher’s exact text was performed to evaluate whether there is correlation between IQGAP3 mRNA expression and clinicopathological characteristics in pancreatic cancer patients. IQGAP3
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Figure 2. GSEA identified cell apoptosis, metastasis and Cdc42 pathways as regulatory targets of IQGAP3 in TCGA PAAD dataset. NES, normalized enrichment score.

expression was correlated with tumor size, differentiation, and lymph node metastasis (Table 1). Kaplan-Meier survival analysis was performed to study the clinical outcome of pancreatic cancer patients with lower or higher IQGAP3 expression. As shown in Figure 1D, the patients with higher IQGAP3 expression had shorter survival time than those with lower IQGAP3 expression (P=0.0002). These results demonstrated that IQGAP3 was overexpressed in pancreatic cancer tissues and strongly associated with poor prognosis of patients.

IQGAP3 is positively correlated with apoptosis, metastasis and Cdc42 pathways

Gene Set Enrichment Analysis (GSEA) is a conventional approach to identify pathways related to gene expression. To elucidate whether IQGAP3 was involved in pancreatic tumorigenesis, we performed GSEA on TCGA PAAD dataset. As shown in Figure 2A-C, the cell apoptosis, metastasis and Cdc42 pathways were identified with the significant association with IQGAP3 expression in the TCGA dataset, which suggested that IQGAP3 may play a key role in pancreatic tumorigenesis.

Knocking down of IQGAP3 expression in pancreatic cancer cell lines by siRNA transfection

To further investigate the role of IQGAP3 in pancreatic cancer, siRNA duplexes targeting human IQGAP3 (siIQGAP3) or control scrambled siRNA (siNC) were transfected into BXPC-3 and SW1990, which had high IQGAP3 expression (Figure 3A). The results in Figure 3B showed that transfection of siIQGAP3, but not siNC, caused significant knockdown of IQGAP3 in BXPC-3 cells relative to GAPDH expression. Transfection of siIQGAP3 in SW1990 cells caused similar knockdown of IQGAP3 relative to GAPDH expression (Figure 3C).

IQGAP3 regulates pancreatic cancer cell proliferation

In light of recent reports that IQGAP3 regulates cancer cell proliferation [5, 6], we examined the role of IQGAP3 in determining the rate of proliferation of pancreatic cancer cells. CCK-8 assay was carried out on both BXPC-3 and SW1990 cells following siRNA transfection (Figure 4A). The proliferation of both cell lines was evidently inhibited by IQGAP3 knockdown at 48 h (P<0.01) and 72 h (P<0.001) post transfection.
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On the contrary, overexpression of IQGAP3 in PANC-1 cells, which had low IQGAP3 expression, significantly promoted cell proliferation (Figure S1). These findings suggest that IQGAP3 greatly enhanced the proliferative ability of pancreatic cancer cells.

Next, we examined whether knocking down of IQGAP3 could reduce tumor growth in vivo. BXPC-3 cells stably transduced with shIQGAP3 or shNC were established and subcutaneously injected in nude mice. Tumors formed from shIQGAP3 stable cells grew slower than the shNC group. At 30 days after cell implantation, tumor weight was significantly decreased in shIQGAP3 group compared with that in shNC group (Figure 4B). These in vivo data strengthened and confirmed our in vitro results that IQGAP3 contributes to pancreatic tumorigenesis.

Silencing of IQGAP3 induces cell apoptosis

According to the results of GSEA, cell apoptosis pathway was closely associated with IQGAP3 expression in pancreatic cancer patients (Figure 2A). We then carried out Annexin V/PI dual staining on pancreatic cancer cells at 48 h after transfection with siIQGAP3 and siNC. Significant early apoptosis was observed in siIQGAP3-transfected BXPC-3 and SW1990 cells, while siNC-transfected cells showed no evidence of apoptosis (Figure 4C).

Knockdown of IQGAP3 inhibits cell migration and invasion

GSEA results showed that metastasis pathway was closely correlated with IQGAP3 expression by GSEA analysis (Figure 2B). Thus, we assumed that IQGAP3 could affect the migration and invasive ability of pancreatic cancer cells. As shown in Figure 4D, 4E, cells with IQGAP3 knockdown showed markedly decreased migration and invasive ability compared with control cells in both pancreatic cancer cells, whereas reverse results were obtained in IQGAP3 overexpression experiments (Figure S1B).

IQGAP3-related pathways in pancreatic cancer

To further validate the GSEA results, we detected the protein expression of cell apoptosis (Bax, Bad, Caspase 3 and Caspase 9), metastasis (Twist, Snail, E-cadherin and β-catenin) and Cdc42 (Cdc42, Rac1 and Src) pathways-related proteins in IQGAP3 silenced pancreatic cancer cells. Knockdown of IQGAP significantly increased the expression levels of Bax, Bad, Caspase 3, Caspase 9 and E-cadherin, but...
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A

BXPC-3

B

BXPC-3

C

SW1990

D

BXPC-3

siNC

silIQGAP3

Annexin V

Annexin V

Migration assay

SW1990

siNC

silIQGAP3

Migration assay
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decreased the expression levels of Twist, Snail, β-catenin, Cdc42, Rac1 and Src in both BXPC-3 and SW1990 cells after the downregulation of IQGAP3 (Figure 5).

Wang et al. have shown that IQGAP3 interacts directly with activated Cdc42 by in vitro binding assay and IQGAP3 is a potential effector of Cdc42 [4]. We then tried to investigate the effects of Cdc42 on the cellular behavior of human pancreatic cancer cells. As shown in Figure 6, knocking down of Cdc42 expression in BXPC-3 cells significantly suppressed the proliferation, cell motility and invasiveness of BXPC-3 cells. These data suggested that IQGAP3 might exert its function in pancreatic cancer cells through down-regulating Cdc42.

Discussion

Three members of the IQGAP family have been described in humans [4, 16, 17]. Elevated expression of IQGAP1 has been reported in several human malignancies, including oligodendroglioma [18], lung cancer [19], colon cancer [20] and hepatocellular carcinoma [21], and IQGAP1 is proposed to be an oncogene. IQGAP2 expression was down-regulated in gastric cancer [22] and hepatocellular carcinoma [21], and IQGAP2 is a potential tumor suppressor. IQGAP3 expression was also found increased in several tumor tissues [9, 11] (bone marrow, breast, large intestine, lung, ovary, stomach and colon). Here, the present study demonstrated that IQGAP3 was overexpressed in pancreatic cancer tissues (Figure 1). Importantly, higher expression of IQGAP3 was associated with larger tumor size, poorer differentiation, higher incidence of lymph node metastasis (Table 1) and shorter overall survival. These findings confirmed the involvement of IQGAPs with tumorigenesis.

To investigate the role of IQGAP3 in pancreatic cancer, its expression was knocking down in two pancreatic cancer cell lines with high expression level of IQGAP3 (Figure 3). Silencing of IQGAP3 expression significantly suppressed tumor cell growth, migration, invasion and xenograft tumor growth, and remarkably induced cell apoptosis (Figure 4). On the contrary, cell proliferation, migration and invasion of PANC-1, which had relative low IQGAP3
Figure 5. Expression of cell apoptosis, metastasis and Cdc42 pathways-related proteins was determined by Western blotting. Western blotting was replicated three times. *P<0.05, **P<0.01 and ***P<0.001 vs siNC.
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expression level, was increased by IQGAP3 overexpression (Figure S1). These results were consistent with previous studies on other cancer cells [8-10] and indicated the ontogenetic role of IQGAP3 in pancreatic cancer.

Further, we tried to investigate the underlying mechanisms how IQGAP3 exerts its function. GSEA indicated that IQGAP3 expression was associated with the cell apoptosis, metastasis and Cdc42 pathways (Figure 2). Bax and Bad are pro-apoptotic members of Bcl-2 family, which induce programmed cell death [23]. Caspase 3 and Caspase 9 are well-known mediators of apoptosis [24, 25]. Here, IQGAP3 knockdown remarkably increased the protein levels of Bax, Bad, Caspase 3 and Caspase 9, which indicated a relation between IQGAP3 function and the regulation of cell apoptosis in pancreatic cancer cells. Epithelial-mesenchymal transition (EMT) is involved in metastasis of tumors. E-cadherin is a main factor of EMT [26]. Knockdown of IQGAP1 significantly up-regulated E-cadherin level in thyroid cancer SW1736 cells [27], while IQGAP2 increased E-cadherin expression in prostate DU145 cells [28]. In this study, we found that silencing of IQGAP3 induced the expression of E-cadherin. Liu et al. has proved the interaction of IQGAP1 interacted with E-cadherin [27]. Whether IQGAP3 interacts with E-cadherin needs further investigation. Moreover, the expression of three known inducers of EMT (Twist, Snail1 and β-catenin) was significantly reduced by IQGAP3 knockdown. Thus, we hypothesize that EMT pathway might be involved in the function of IQGAP3. Furthermore, Wang et al. have shown that IQGAP3 interacts directly with activated

Figure 6. Effects of Cdc42 knockdown on cell proliferation, migration and invasion. IQGAP3 interacted to Cdc42. A: Cdc42 expression was suppressed by Cdc42 siRNA (siCdc42) transfection in BXPC-3 cells as indicated by Western blotting. B: Cdc42 knockdown notably suppressed cell proliferation as determined by CCK-8 assay. C: Cdc42 knockdown significantly inhibited the motility and invasiveness of BXPC-3 cells. Western blotting, proliferation and migration/invasion assays were replicated three times. ***P<0.001 vs siNC.
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Cdc42 in vitro and serves as a downstream effector of Cdc42 [4]. Cdc42 is a member of Rho GTPases, involving in a variety of cell functions, including cell cytoskeleton organization, cell proliferation, survival, adhesion and migration [29]. Here, we found that IQGAP3 knockdown significantly decreased Cdc42 protein level (Figure 5). Cdc42 knockdown significantly inhibited cell proliferation (Figure 6A), cell migration and cell invasion (Figure 6C). A previous study has showed that IQGAP3 interacts with activated Ras, another member of Rho GTPases in epithelial cells [30]. Whether the interaction occurs in pancreatic cancer cells needs further investigation. Our data suggest that IQGAP3 works as an oncogene through affecting both apoptosis and metastasis of pancreatic cancer cells, although further investigations are required to revealed the mechanisms how IQGAP3 regulates these pathways.

In conclusion, our study demonstrated for the first time that IQGAP3 expression was elevated in pancreatic cancer. IQGAP3 may serve as an oncogene for pancreatic cancer through regulating cell apoptosis, metastasis and Cdc42 pathways.

Acknowledgements

This study was supported by Subproject of the 863 Program [2014AA022304], Coordination Science? Research Project of Shanghai Municipal Commission of Science and Technology [2013SY059], Scientific Research Project of Changning District, Shanghai Science and Technology Commission [CNKW2014204] and National Natural Science Foundation of China [81672083].

Disclosure of conflict of interest

None.

Address correspondence to: Drs. Weihong Xu and Huiming Sheng, Department of Clinical Laboratory of Tongren Hospital, School of Medicine, Shanghai Jiaotong University, Shanghai, China. E-mail: whxutr@sina.com (WHX); hmshengsh@sina.com (HMS)

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Figure S1. Effects of IQGAP3 overexpression on cell proliferation, migration and invasion. (A) Full-length IQGAP3 were cloned into the expression vector pCDNA3 (Invitrogen). PANC-1 cells were transfected with IQGAP expressing plasmid and control Vector and cell proliferation was assessed by CCK-8 assay. (B, C) Migration (B) and Invasion (C) assay were performed in Transwell chambers. ***P<0.001.