USP5 promotes tumorigenesis and progression of pancreatic cancer by stabilizing FoxM1 protein

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ABSTRACT
Increased ubiquitin-specific protease 5 (USP5) has been associated with tumorigenesis of malignancy including glioblastoma, melanoma and hepatocellular carcinoma. However, the role of USP5 in tumorigenesis of pancreatic ductal adenocarcinoma (PDAC) has not been studied yet. In this study, we demonstrated that USP5 was significantly upregulated in a panel of PDAC cell lines and correlated with FoxM1 protein expression. USP5 knockdown inhibited proliferation of PANC-1 and SW1990, two PDAC cell lines. In the mouse xenografted pancreatic tumor model, suppression of USP5 significantly decreased tumor growth, correlated with down regulation of FoxM1. Additionally, we found that overexpression of USP5 stabilized the FoxM1 protein in PDAC cells. Overexpression of USP5 extended the half-life of FoxM1. Knockdown of USP5 in PANC-1 cells decreased FoxM1 protein level while the proteasome inhibitor MG-132 treatment restored FoxM1 expression. We also found that endogenous USP5 was coimmunoprecipitated with an endogenous FoxM1 from PANC-1 cells while FoxM1 was also coimmunoprecipitated with USP5. Furthermore, we also confirmed that USP5 regulated proliferation of PDAC via FoxM1 by rescuing the inhibitory effect of USP5 knockdown with ectopic expression of FoxM1 in USP5-depleted cells. Taken together, our study demonstrates that USP5 plays a critical role in tumorigenesis and progression of pancreatic cancer by stabilizing FoxM1 protein, and provides a rationale for USP5 being a potential therapeutic approach against PDAC.

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1. Introduction
Pancreatic ductal adenocarcinoma (PDAC) is the most aggressive human malignancy with a 5-year overall survival of 6% [1]. It remains largely incurable disease because of poor prognosis and lack of chemotherapy efficacy [2]. Therefore, a better understanding of molecular mechanisms underlying PDAC would be helpful to identify both biomarkers for early diagnosis and molecular targets for therapy.

Ubiquitination plays an important role in post-translational modification of cellular proteins which are involved in multiple diseases including cancers [3]. Modification of ubiquitin-ubiquitin and ubiquitin-protein is regulated by a dynamic two-way process that can be reversed by deubiquitinating enzymes (DUBs) [4]. Previous studies suggest that DUBs may be a potential therapeutic target for the treatment of cancer [5,6]. As a member of the ubiquitin specific protease (USP) family of DUBs, ubiquitin specific peptidase 5 (USP5) makes major contribution to regulate unanchored polyubiquitin disassembly and protein deubiquitination by binding to a protein and double-strand DNA repair [7]. Previous studies indicated that USP5 had many cellular targets and stabilizes multiple proteins, such as p53 [8,9] and Cav3.2 protein [10,11]. Recently, it has been shown that USP5 controlled activation of apoptotic signaling during eye development [12,13], and its expression was correlated with several tumorigenesis such as hepatocellular carcinoma and glioblastoma [14,15].

Forkhead box protein M1 (FoxM1) is a transcription factor that plays a crucial role in cell proliferation, differentiation, and transformation [16–18]. Increasing evidence indicated that FoxM1 overexpression was correlated with most human malignancies, such as pancreatic cancer, glioblastoma, hepatocellular carcinoma and breast cancer, and plays an important role in tumorigenesis,
invasion, and metastasis [19–24]. A recent study showed that USP5 directly interacted with FoxM1 as a substrate in glioma cells [25]. However, little is known about the pathogenic mechanisms of USP5 and FoxM1 involved in PDAC tumorigenesis and progression. In the present study, we identified that USP5 was significantly overexpressed in a panel of PDAC cell lines and positively associated with FoxM1 expression. We found that inhibition of FoxM1 significantly decreased tumor growth, correlated with down regulation of FoxM1. Additionally, we found that overexpression of USP5 stabilized the FoxM1 protein in PDAC cells and FoxM1 rescued the inhibitory effect of USP5 knockdown in USP5-depleted cells. Hence, these data suggest that USP5 plays a critical role in tumorigenesis and progression of pancreatic cancer by stabilizing the FoxM1 protein.

2. Materials and methods

2.1. Cell culture, cloning, transfection and infection

The human pancreatic ductal epithelial (HPDE) cell and human pancreatic cancer cell lines PANC-1, HPF2, CFPAC, SW1990, HUPT3 and BXP3 were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). The cells were maintained in Dulbecco’s Modified Eagle Medium (DMEM) or RPMI-1640 supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/ml), and streptomycin (10 μg/ml) at 37 °C in a humidified incubator under 5% CO2 condition. Two kinds of shRNAs targeting human USP5 and a control shRNA were subcloned into the pLKO lentiviral vector. Expression constructs were transfected into cells using jetPRIME transfection reagent (Polyplus transfection). Lentiviruses were produced in HEK293T packaging cell lines according to the routine procedure [26]. Transduction was performed into PANC-1 cells and SW1990 cells in 6-well plate. The cells were then selected in puromycin for 2 weeks before experiments.

2.2. Cell viability assay

Cell viability was assayed using the CellTiter-Glo luminescent cell viability kit (Promega) according to the manufacturer’s instruction. The cells were placed on 96-well plates at the density of 1 × 10^3 cells and incubated at 37 °C for 1, 2, 3, 4, 5 and 6 days. Briefly, 50 μl of the CellTiter-Glo reagent was added directly to each well for 10-min incubation. The plate was read by GloMax® 96 microplate luminometer (Promega) to monitor the luminescence signal.

2.3. RNA preparation and quantitative real-time PCR (qPCR)

RNA was extracted using the Trizol reagent (Invitrogen), and qPCR was carried out as described previously. Each RNA samples were reversely transcribed into cDNA using miScript reverse transcription kit (Qiagen). RT-PCR was then carried out in ROCHE lightcycler® 480 qPCR system (Roche) using Realmastermix (SYBR Green I) (Bio-Rad) according to a previous study [27]. Primers for the USP5 were: 5’-CGGGACAGGCTTGAA-3’, 5’-TCGCTCAATGTGACTGAAGTCCA-3’ [9].

2.4. Western blotting analysis

Cells were lysed in RIPA buffer supplemented with the protease inhibitors and phosphatase inhibitors (Roche). Lysates were then separated by SDS–PAGE and the proteins were subsequently transferred to polyvinylidene fluoride membranes (Millipore). The membranes were immunoblotted with the indicated antibodies. The following antibodies and their dilutions were used in the study: anti-USP5 (Proteintech, 10473-1-AP, 1:1000), anti-FoxM1 antibody (Abcam, ab180710, 1:1000), and Anti-beta III Tubulin antibody (Abcam, ab18207, 1:10,000).

2.5. Colony formation assay

Cells were seeded into 6-well plates at a density of 1000 cells per well and cultured for 7 days until visible clones appeared. Cell colonies were stained using the Giemsa solution and counted under a microscope. The experiment was performed in triplicate. The colony formation rate was calculated using the following equation:

\[ \text{Colony formation rate} = \left( \frac{\text{Number of colonies}}{\text{Number of seeded cells}} \right) \times 100\% \]

2.6. Protein stability measurement

In protein half-life assays, 100 mg/ml cycloheximide (CHX) (Sigma) was added to cell culture to block protein synthesis. Cells were collected at indicated time points and the protein levels were measured and quantified by western blotting and phosphor imager. The half-life of proteins was calculated from three independent experiments.

2.7. Immunoprecipitation

To identify the interactions between endogenous USP5 and FoxM1, USP5 or FoxM1 was immunoprecipitated from PANC-1 cells by anti-USP5 (Proteintech, 10473-1-AP, 1:250) or anti-FoxM1 antibody (Abcam, ab180710, 1:250) using the Pierce™ co-immunoprecipitation kit according to the manufacturer’s protocol.

2.8. Immunohistochemistry (IHC) analysis

The xenografts tissues were removed from nude mice and fixed with 4% paraformaldehyde. Paraffin-embedded sections (thickness, 4-μm) were prepared in a routine procedure [28]. The sections were then processed for immunohistochemical staining as previously reported [28]. The sections were deparaffinized and treated with the 3% H2O2 solution. After blocking with normal goat serum for 30 min, the sections were incubated with anti-USP5 (1:200, Proteintech, 10473-1-AP), anti-ki67 (1:500, Abcam, ab15580) antibody at 4 °C overnight. Then the sections were incubated with the secondary antibody followed by the diaminobenzidine-peroxidase reaction and counterstaining with hematoxylin. The results were observed with light microscopy (Leica Microsystems) and evaluated using Image-Pro Plus 6.0 (Media Cybernetics).

2.9. Mouse xenograft model

To further evaluate the role of USP5 in PDAC progression, six male BALB/c nude mice of each group were injected subcutaneously with PANC-1 cells stably expressing non-targeting (shCtrl) or shUSP5 (shUSP5) at a concentration of 1 × 10^7 cells into left flanks. Tumor volumes of subcutaneous implantation models were measured and calculated three times per week using calipers to calculate tumor volumes according to formula V = ab^2/2. All mice were sacrificed and the xenografts were resected 48 days after treatment and tumor weights were measured.

2.10. Statistical analysis

All data are expressed as means ± SD. Unpaired and two-tailed
3. Results

3.1. **USP5 and FoxM1 protein were coordinately overexpressed in a panel of PDAC cell lines**

QRT-PCR and Western blot were performed to analyze USP5 and FoxM1 expression in HPDE and human pancreatic cancer cell lines including PANC-1, HPAF2, CFPCA, SW1990, HUPT3 and BXPC3. Compared to the HPDE control, USP5 mRNA and protein expressions were significantly higher in PANC-1, CFPCA, SW1990, HUPT3 and BXPC3 cells, but not in HPAF2 (Fig. 1A and C). Strikingly, FoxM1 protein abundance in PANC-1, SW1990, HUPT3 and BXPC3 cells correlated well with USP5 abundance. One cell line CFPCA contained abundant USP5 but little FoxM1 (Fig. 1C, lane 4). The amount of USP5 protein in the PDAC cell lines correlated largely with USP5 mRNA abundance, suggesting that elevated USP5 in PDAC is due to transcriptional upregulation (Fig. 1A and C). In contrast, FoxM1 protein and mRNA levels correlated poorly (Fig. 1B and C).

3.2. **Knockdown of USP5 inhibited PDAC proliferation in vitro**

To identify the role of USP5 in PDAC progression, we designed two shRNA sequences (shUSP5-1 and shUSP5-2) and cloned them into the lentiviral vector. The knockdown efficiency was about 65% by shUSP5-2 (P < 0.05 by one-way ANOVA; Fig. 2A and B). Then we generated USP5 knockdown stable cell line of PANC-1 and SW1990. The cell viability assay revealed that cell proliferation was significantly inhibited in USP5 knockdown cells at day 6 (P < 0.05 by two-way ANOVA; Fig. 2C and D). Furthermore, the colony formation assays also showed that USP5 knockdown inhibited proliferation of PANC-1 and SW1990, two PDAC cell lines.

3.3. **Knockdown of USP5 inhibited PDAC growth in vivo**

To further evaluate the role of USP5 in PDAC progression, six male BALB/c nude mice of each group were injected subcutaneously with PANC-1 cells stably expressing non-targeting (shCtrl) or shUSP5 (shUSP5) at a concentration of 1 × 10^7 cells into left flanks respectively. Compared to the control mice, USP5 knockout significantly decreased tumor volume and weight (Fig. 3A–C). Furthermore, IHC analyses showed that USP5 knockdown significantly decreased the expression of USP5 and the cell proliferation marker Ki-67 in the xenografts of mice (Fig. 3D and E). Western blot revealed that the expression of USP5 and FoxM1 was significantly down regulated in USP5 knockdown mice.

3.4. **USP5 stabilized FoxM1 protein in PDAC and regulated cell proliferation via FoxM1 proteins**

Previous studies have shown that FoxM1 played critical roles in proliferation, invasion, metastasis, epithelial-to-mesenchymal transition and stem cell self-renewal in pancreatic cancer [9,29–31]. Further investigations discovered that USP5 directly interacted with FoxM1 by the Co-IP assays [25]. Therefore, we hypothesized that FoxM1 was involved in USP5-mediated tumor progression of PDAC. As expected, knockdown of USP5 in PANC-1 cells decreased FoxM1 protein level (Fig. 4A) but did not affect FoxM1 mRNA level (Fig. 4B). The proteasome inhibitor MG-132 treatment restored FoxM1 expression after USP5 knockdown.

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Fig. 1. **USP5 and FoxM1 protein were coordinately overexpressed in a panel of PDAC cell lines.** USP5 and FoxM1 expression at mRNA (A, B) and protein levels (C) were detected by qPCR and Western blot in a panel of pancreatic cancer cell lines. Data presented are from three independent experiments (*P < 0.05 vs. control group).
Fig. 2. Knockdown of USP5 inhibited proliferation and colony formation of the pancreatic cancer cell. (A, B) The efficiency of USP5 knockdown was detected by qPCR and western blotting. PANC-1 cells were infected with lentivirus expressing shUSP5 (2 different targeting vectors named shUSP5-1 and shUSP5-2) or control shRNA. After 48 h of transfection, cells were collected for detection of USP5 expression level. (C, D) PANC-1 and SW1990 cells stably expressing shUSP5 or control shRNA were placed on 96-well plates with the density of 4 × 10^3 cells and incubated at 37 °C for 6 days. Cell viability was assayed using CellTiter-Glo Luminescent Cell Viability Assay at 1, 2, 3, 4, 5 and 6 days. Data presented are from three independent experiments (*P < 0.05 vs. control group). (E-H) PANC-1 and SW1990 cells stably expressing shUSP5 and shCtl were seeded into 6-well plates at a density of 1000 cells per well and cultured for 7 days until visible clones appeared. Cell colonies were stained using Giemsa solution and counted under a microscope. Data presented are from three independent experiments (*P < 0.05 vs. control group).
Moreover, when USP5 was transfected and overexpressed in PANC-1 cells, FoxM1 degradation was inhibited. The result showed that overexpressed USP5 extended the half-life of FoxM1 over 240 min longer in the cells transfected with a control vector (Fig. 4C). In the next study, we confirmed that endogenous USP5 coimmunoprecipitated with an endogenous FoxM1 in PANC-1 cells while FoxM1 was also coimmunoprecipitated with USP5 (Fig. 4D and E). To identify the role of FoxM1 in rescuing the inhibitory effects of USP5 knockdown, FoxM1 was transfected into USP5-depleted cells. The inhibition of colony formation in USP5-depleted cells was also rescued by FoxM1 over expression (P < 0.05 by one-way ANOVA; Fig. 4F–G). The results suggested that USP5 regulated cell proliferation via the FoxM1 by rescuing the effects of USP5 knockdown in PANC-1 cells with ectopic expression of FoxM1.

4. Discussion

Increasing evidence has shown that the USP family members are potential therapeutic targets in cancer treatment [6]. Although USP5 overexpression was reported in glioblastoma, melanoma and hepatocellular carcinoma, the molecular mechanisms underlying USP5 during PDAC tumorigenesis are still elusive. Here, we demonstrated that USP5 was significantly upregulated in a panel of PDAC cell lines and correlated with FoxM1 protein expression. The amount of USP5 protein in the PDAC cell lines correlated largely with USP5 mRNA abundance, suggesting that elevated USP5 in PDAC is due to transcriptional upregulation. In contrast, FoxM1 protein and mRNA levels correlated poorly. USP5 knockdown led to a significant reduction in proliferation of PANC-1 and SW1990 cells. In the mouse xenografted pancreatic tumor model, knockdown of USP5 significantly suppressed tumor growth, correlated with downregulation of FoxM1. These data indicate that USP5 plays a significant role in PDAC tumorigenesis.

As a member of the Forkhead superfamily of transcription factors, FoxM1 promotes cancer cell proliferation and cell cycle progression which contribute to the hallmarks of cancer [29,32]. Overexpression of FoxM1 has been reported in most cancers, including PDAC, breast, colorectal and lung cancer, correlated with tumor progression and poor prognosis [24]. Dysregulated
expression and activation of FoxM1 was suggested promoting the warburg effect and tumorigenesis in pancreatic cancer patients via transcriptional regulation of LDHA expression [33]. Recent studies have shown that FoxM1 is regulated by ubiquitination and deubiquitination process [34,35]. Overexpression of USP5 reduces the endogenous ubiquitination of FoxM1 and increases FoxM1 expression [25]. In the current study, the protein levels of FoxM1 were up-regulated following USP5 overexpressed. To investigate whether USP5 prolonged the half-life of FoxM1, we transfected PANC-1 cells with USP5 and measured FoxM1 expression level in the presence of the translational inhibitor CHX. The result showed that overexpressed USP5 extended the half-life of FoxM1 to over 240 min longer than that of FoxM1 from the cells transfected with a control vector. Knockdown of USP5 in PANC-1 cells decreased

Fig. 4. USP5 stabilized FoxM1 protein in PDAC. (A) Western blot analysis of FoxM1 protein in PANC-1 cells transfected with shCtrl or USP5 shRNAs, where indicated, treated with 10 mM MG-132 for 4 h. (B) QRT-PCR analysis of FoxM1 mRNA in PANC-1 cells transfected with shCtrl or USP5 shRNAs. (C) Western blot analysis of endogenous FoxM1 in PANC-1 cells transfected with vector only (CTL) or USP5 expressing plasmid. Cells were treated with 25 mg/ml CHX for the times indicated. FoxM1 was quantified by densitometry (right panel). (D, E) FoxM1 (D) or USP5 (E) was immunoprecipitated from PANC-1 cells respectively. Control immunoprecipitations were with nonspecific IgG, followed by western blotting of the precipitated proteins with anti-USP5, anti- FoxM1. (F-G) FoxM1 significantly rescues the inhibition of the colony formation in USP5-depleted cells. PANC-1 cells stably expressing shUSP5 or shCtrl were transfected with vector or FoxM1 expressing plasmid. Then the cells were seeded into 6-well plates at a density of 1000 cells per well and incubated at 37 °C for 7 days until visible clones appeared. Data presented are from three independent experiments (*P < 0.05 vs. shUSP5/FoxM1+/− group).
FoxM1 protein level but did not affect FoxM1 mRNA level, indicating that FoxM1 was regulated at the post-transcriptional level. The proteasome inhibitor MG-132 treatment restored FoxM1 expression after the USP5 knockdown, suggesting that FoxM1 protein destabilization was via proteasome-mediated degradation. Therefore, the results indicated that USP5 stabilized FoxM1 in PAC-1 cells. Furthermore, we confirmed that endogenous USP5 was coimmunoprecipitated with an endogenous FoxM1 from PAC-1 cells while FoxM1 was also coimmunoprecipitated with USP5, which indicated that FoxM1 is a substrate for USP5, consistent with the previous study [25]. We confirmed that USP5 regulated proliferation of PDAC via FoxM1 by rescuing the inhibitory effect of USP5 knockdown with ectopic expression of FoxM1 in USP5-depleted cells.

In conclusion, our study indicates that USP5 plays a critical role in tumorigenesis and progression of pancreatic cancer by stabilizing the FoxM1 protein. Thereby, it provides a rationale for USP5 to be a potential therapeutic approach against PDAC.

Conflict of interest

The authors declare no conflict of interest.

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