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PIAS4 is an activator of hypoxia signalling via VHL suppression during growth of pancreatic cancer cells

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Background: The PIAS4 protein belongs to the family of protein inhibitors of activated STAT, but has since been implicated in various biological activities including the post-translational modification known as sumoylation. In this study, we explored the roles of PIAS4 in pancreatic tumorigenesis.

Methods: The expression levels of PIAS4 in pancreatic cancer cells were examined. Cell proliferation and invasion was studied after overexpression and gene silencing of PIAS4. The effect of PIAS4 on hypoxia signalling was investigated.

Results: The protein was overexpressed in pancreatic cancer cells compared with the normal pancreas. Gene silencing by PIAS4 small interfering RNA (siRNA) suppressed pancreatic cancer cell growth and overexpression of PIAS4 induced expression of genes related to cell growth. The overexpression of PIAS4 is essential for the regulation of the hypoxia signalling pathway. PIAS4 interacts with the tumour suppressor von Hippel-Lindau (VHL) and leads to VHL sumoylation, oligomerization, and impaired function. Pancreatic cancer cells (Panc0327, MiaPaCa2) treated with PIAS4 siRNA suppressed expression of the hypoxia-inducible factor hypoxia-inducible factor 1 alpha and its target genes JMJD1A, VEGF, and STAT3.

Conclusion: Our study elucidates the role of PIAS4 in the regulation of pancreatic cancer cell growth, where the suppression of its activity represents a novel therapeutic target for pancreatic cancers.

Pancreatic adenocarcinoma (PDAC) has become one of the leading causes of death in industrialised countries (Vincent *et al*, 2011). It is usually diagnosed at late stages, where curative surgery provides poor prognosis, and other forms of therapy including chemotherapy lack efficacy (McCarthy, 2009; Colucci *et al*, 2010). Continuing efforts have been made to identify both biomarkers for early diagnosis and molecular targets for therapy (Natoni *et al*, 2005; Adachi *et al*, 2010). Emerging studies show that protein sumoylation by small ubiquitin-related modifier (SUMO) proteins is an important post-translational modification in regulating the

biological activities of proteins (Jackson, 2001). Sumoylation involves sequential actions by E1-activating enzyme, E2-conjugating enzyme, and E3 protein ligase (Wilkinson and Henley, 2010). Both positive and negative effects on protein functions by sumoylation have been reported (Depaux *et al*, 2007; Karamouzi *et al*, 2008). PIAS4 (protein inhibitor of activated STAT protein 4) is an E3-type SUMO ligase. It interacts with and suppresses the biological activity of the tumour suppressor protein von Hippel-Lindau (VHL) in renal clear-cell carcinoma (Sachdev *et al*, 2001). Sumoylation of VHL mediated by PIAS4 results in the formation

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of VHL oligomers, which in turn reduces its tumour suppressor activity against hypoxia-inducible factor 1 alpha (HIF1 α) (Cai *et al*, 2010; Cai and Robertson, 2010). Hypoxia has an essential role in tumourigenesis and metastasis of solid tumours including PDAC (Denko, 2008; Wilson and Hay, 2011). We found that PIAS4 is highly expressed in PDAC cell lines and tumours, and small interfering RNA (siRNA) against PIAS4 suppressed their proliferation. Our study demonstrates for the first time that PIAS4 may also constitute a therapeutic target against pancreatic cancer.

MATERIALS AND METHODS

Cell culture. Fourteen pancreatic cancer cell lines (AsPc1 BxPc3, Panc1, Panc0203, Panc0327, Panc0403, Panc0504, Panc0813, Panc1005, CFPAC1, CaPan2, MiaPaCa2, PL45, and SU8686) were obtained from the American Type Culture Collection (Manassas, VA, USA). All the cell lines were maintained in either DMEM or RPMI-1640 supplemented with 10% FBS. For the induction of hypoxia, cells were incubated in temperature-controlled hypoxic culture chambers at 1% O₂, 5% CO₂, and 94% N₂.

Small interfering RNAs. Scrambled control siRNA and PIAS4-targeted siRNA mixtures (pool of 4) were purchased from Cell Signalling (Boston, MA, USA). Non-target negative control (NC) and exon-specific targeted siRNAs (siEXON2 and siEXON6 for exons 2 and 6, respectively) were from IDT (Coralville, IA, USA). Duplex sequences were NC: 5'-CGUUAUCGUAUAAUACGCGUAT-3', 5'-CAGCAAUUAGCGCAUUAUUGCGCAUA-3'; siEXON2: 5'-GCUCUACGGAAAGUACUUAACGGA-3', 5'-UC CGUUUAGUACUUCCGUAGAGCAC-3'; siEXON6: 5'-ACC UCACUCACCUCAUGUACCUGT-3', 5'-GACAGGUACAUGAG GUGAGUGAGGUUG-3'). RNAiMAX (used for siRNA transfection) and Lipofectamine 2000 (used for plasmid DNA overexpression) were from Invitrogen (Carlsbad, CA, USA).

Public databases. Oncomine (Compendia Bioscience, Ann Arbor, MI, USA) and the Human Protein Atlas were used for analysis and visualisation.

MTT assays. A total of 3000 pancreatic cancer cells were plated in 96-well plates and cultured for various durations as indicated. Cell viability was assayed using MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) and calculated with BioDataFit GraphPad Prism software (Castro Valley, CA, USA).

Colony formation and invasion assays. For colony assays, equal number of cells was seeded in 24-well plates at 24 h after siRNA transfection and 72 h later, the colonies were stained with 0.1% crystal violet in 50% methanol and quantified by measurement of optical density at 540 nm after dissolving the cells in 20% acetic acid. For migration assays, cells were serum-starved overnight and equal numbers of cells were plated on cell culture inserts with 8 μ m membrane (Boyden chambers from Millipore, Billerica, MA, USA) in 24-well plates. After 24 h incubation, cells that did not pass through the filter were removed with a cotton swab, and the culture inserts were stained with 0.1% crystal violet in 50% methanol and photographed.

Real-time reverse transcription PCR. Total RNA from cell lines, as well as from tissue per IRB protocol, was extracted using Invitrogen Trizol and cleaned up with Qiagen RNase kit (Hilden, Germany). All RNAs after extraction were treated with DNase to remove genomic DNA. First strand cDNA synthesis kit from Fermentas (Waltham, MA, USA) was used to prepare cDNA from 2 to 5 μ g RNA. Real-time PCR was performed using SYBR Green PCR ready-mixed reagents from Fermentas in Applied Biosystems 7500 Fast PCR system (Foster City, CA, USA). All PCR reactions were performed in triplicates. PCR products were

checked for specificity on a 2% agarose gel. Expression levels of GAPDH were used as internal controls to calculate relative target gene expression levels.

Immunoprecipitation assays. Cells were co-transfected with PIAS4 and different VHL expression vectors by Invitrogen Lipofectamine 2000. At 48 h after transfection, cells were lysed with lysis buffer containing 50 mM Tris, pH 7.6, 5 mM EDTA, 300 mM NaCl, 1 mM dithiothreitol, and 0.1% Nonidet P-40 with Fermentas protease inhibitors. For immunoprecipitation, the cell lysates were incubated with antibody at 4 °C for 2 h, and the immunoprecipitated complexes were bound to protein A/G-Sepharose beads from Invitrogen at 4 °C overnight. The beads were washed four times with lysis buffer, and proteins were eluted in sample buffer and resolved on 10% SDS-PAGE for western blot analysis. Von Hippel-Lindau expression vectors included pCSVHL-WT, pCSVHL-W88S, pCSVHL-Y112H, and pCSVHL-R167Q.

Western blot analysis. Protein lysates were prepared using the Fermentas ProteoJET Mammalian Cell Lysis Reagent according to the manufacturer's protocol. Twenty micrograms of protein lysates were used in SDS-PAGE followed by western blot transfer. Antibodies (phospho-NF κ B, phospho-GSK3 β , phospho-STAT3; phospho-ERK, MYC, Cyclin D1, α -tubulin, VHL; GAPDH; PIAS4, HIF1 α) were purchased from Cell Signalling (Boston, MA, USA), Santa Cruz (Santa Cruz, CA, USA), Sigma-Aldrich (St. Louis, MO, USA), and Novus (Littleton, CO, USA). SuperSignal West Dura or West Femto chemiluminescent substrates from Pierce (Rockford, IL, USA) were used to detect the immunocomplexes.

Reporter assays. For Myc reporter assays, cells at subconfluent density in 12-well plates were co-transfected with 1 μ g of Myc-LUC, 25 ng of Renilla luciferase pRL-TK transfection control and 1 μ g of either PIAS4 expression vector (pCMV-PIAS4) or empty vector (pCMV). The Myc reporter construct (Myc-LUC) contains 700 bp of c-Myc promoter cloned upstream of the firefly luciferase cDNA. Reporter activities were determined using the Dual Luciferase Assay Kit from Promega (Fitchburg, WI, USA). Results were calculated from triplicates of two independent experiments. For hypoxia-response element reporter assays, HRE-LUC was used, which contains three repeats of the hypoxia-response element upstream of the thymidine kinase minimal promoter driving the luciferase reporter. The cells were co-transfected with HRE-LUC and pCMV-PIAS4, and either pCSVHL-WT, pCSVHL-W88S, pCSVHL-Y112H, pCSVHL-R167Q or pCS, with Renilla luciferase pRL-TK as a transfection control.

RESULTS

Expression levels of PIAS4 are elevated in pancreatic cancer cells compared with normal pancreas. The expression of PIAS4 in 12 pancreatic cancer cell lines and 11 pancreatic tumours, as well as 6 normal pancreas samples was assessed by real-time reverse transcription PCR (RT-PCR). Elevated levels of PIAS4 occurred in pancreatic cancer cell lines and tumours compared with normal pancreas with a mean increase of three-fold in pancreatic cancer cell lines and six-fold in fresh pancreatic tumours ($P=0.0154$) (Figure 1A). Immunohistochemistry analysis showed that PIAS4 was strongly expressed in pancreatic tumours compared with surrounding pancreatic parenchyma (Figure 1B). This observation was corroborated by mining public databases. The Oncomine expression data showed upregulation of PIAS4 transcript in pancreatic ductal adenocarcinoma (Supplementary Figure 1; Buchholz *et al*, 2005). The Human Protein Atlas reported positive PIAS4 staining in both the cytosol and membrane of pancreatic tumours and negative staining in normal pancreas (Supplementary

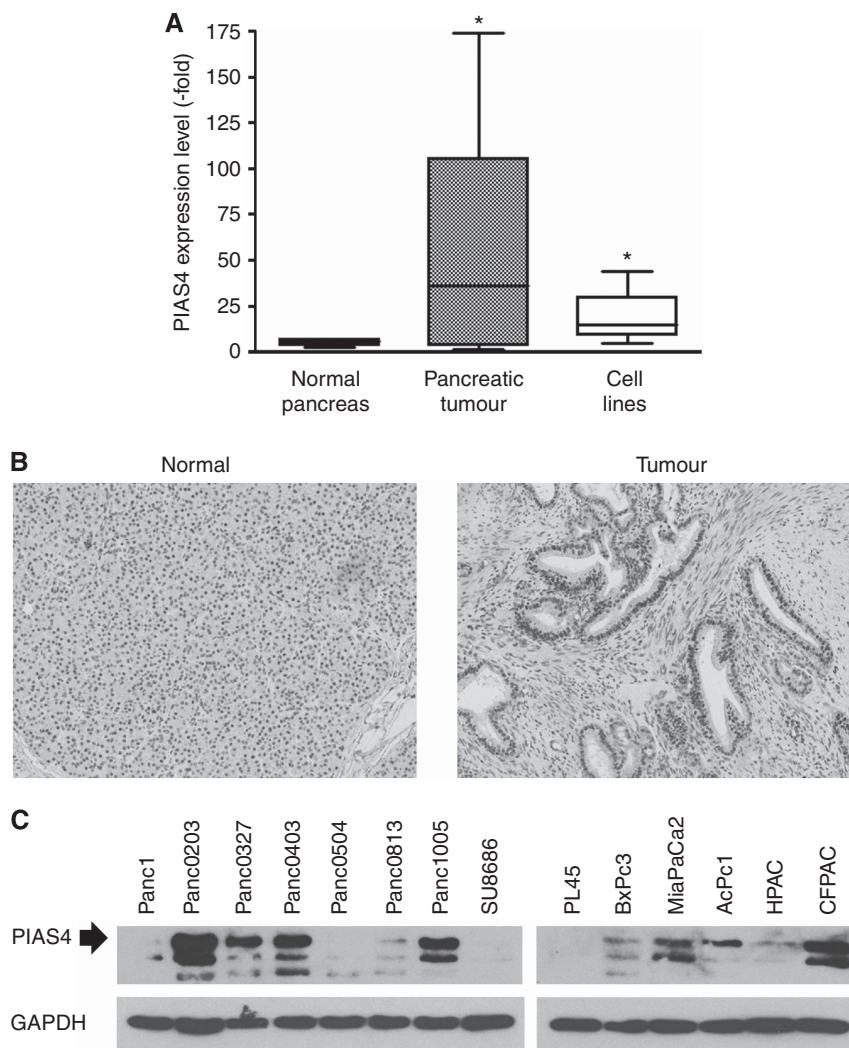


Figure 1. Expression of PIAS4 in human pancreatic cancer cell lines, pancreatic tumours, and normal pancreas. **(A)** Relative RNA expression levels of PIAS4 as a mean of 12 pancreatic cancer cell lines, 11 tumor samples, and 6 normal pancreas. The relative *PIAS4* RNA expression level in each sample was normalised to that of GAPDH. *compared with the normal pancreas, $P=0.0154$. **(B)** Immunohistochemistry analysis of PIAS4 in normal pancreas and pancreatic tumor. **(C)** Western blot analysis of PIAS4 protein expression in 14 pancreatic cancer cell lines. The arrow indicates the band corresponding to the PIAS4 protein. GAPDH was used as a loading control.

Figure 2; Uhlen *et al*, 2010). The expression of PIAS4 protein was further quantified in 14 pancreatic cancer cell lines by western blot analysis. High or moderate expression of PIAS4 was detected in Panc0203, Panc0327, Panc0403, Panc1005, MiaPaCa2, and CFPAC human pancreatic cancer cell lines (Figure 1C).

Gene silencing by PIAS4 siRNA suppressed cell growth in human pancreatic cancer cells; whereas PIAS4 overexpression induced cell growth genes.

To test the role of endogenous PIAS4 in cell proliferation, two cell lines (Panc0327 and Panc1005) with high and two (AsPc1 and BxPc3) with low PIAS4 expression were used for siRNA transfection first with PIAS4 siRNA mixture containing a pool of four siRNAs and compared with scrambled control siRNA. Liquid culture proliferation assays showed that pancreatic cancer cells transfected with PIAS4 siRNA had slower cell growth compared with two controls (wt: wild-type; ctrl siRNA: control siRNA) (Figure 2A). Also, colony assays of Panc0327 and Panc1005 showed decreased colony number in cells transfected with PIAS4 siRNA compared with either wild-type cells or cells transfected with control scrambled siRNA (ctrl siRNA) (Figure 2B). In addition, we tested two extra siRNA targeting either exon 2 (siEXON2) or exon 6 (siEXON6) of PIAS4 in these

pancreatic cancer cells. We found that pancreatic cancer cell lines transfected with both of these siRNAs suppressed cell proliferation compared with either NC (non-target siRNA NC) or wild-type cells (Figure 2C).

Exogenous PIAS4 was expressed in a low PIAS4-expressing pancreatic cancer cell line (Panc1) to examine its effect on pancreatic cancer cells. Proteins related to cell cycle and cell proliferation (Cyclin D1, MYC, phosphorylated ERK, and phosphorylated GSK3 β) were increased in Panc1 cells transfected with pCMV-PIAS4 compared with cells transfected with the empty vector (Figure 2D). In addition, the phosphorylation level of NF κ B/p65 was elevated after overexpression of PIAS4 in Panc1 cells, suggesting the activation of the NF κ B signalling pathway by PIAS4 (Figure 2D). Also, MYC reporter assays showed more than 15-fold induction of the MYC promoter activity in pancreatic cancer cells transfected with the PIAS4 expression vector compared with cells transfected with the empty vector (Figure 2E).

Hypoxia-inducible factor 1 alpha is regulated by PIAS4 via sumoylation of VHL. Because pancreatic cancer frequently occurs in a low oxygen environment, we examined the expression levels of PIAS4 and HIF1 α mRNA under hypoxic conditions (1% O $_2$).

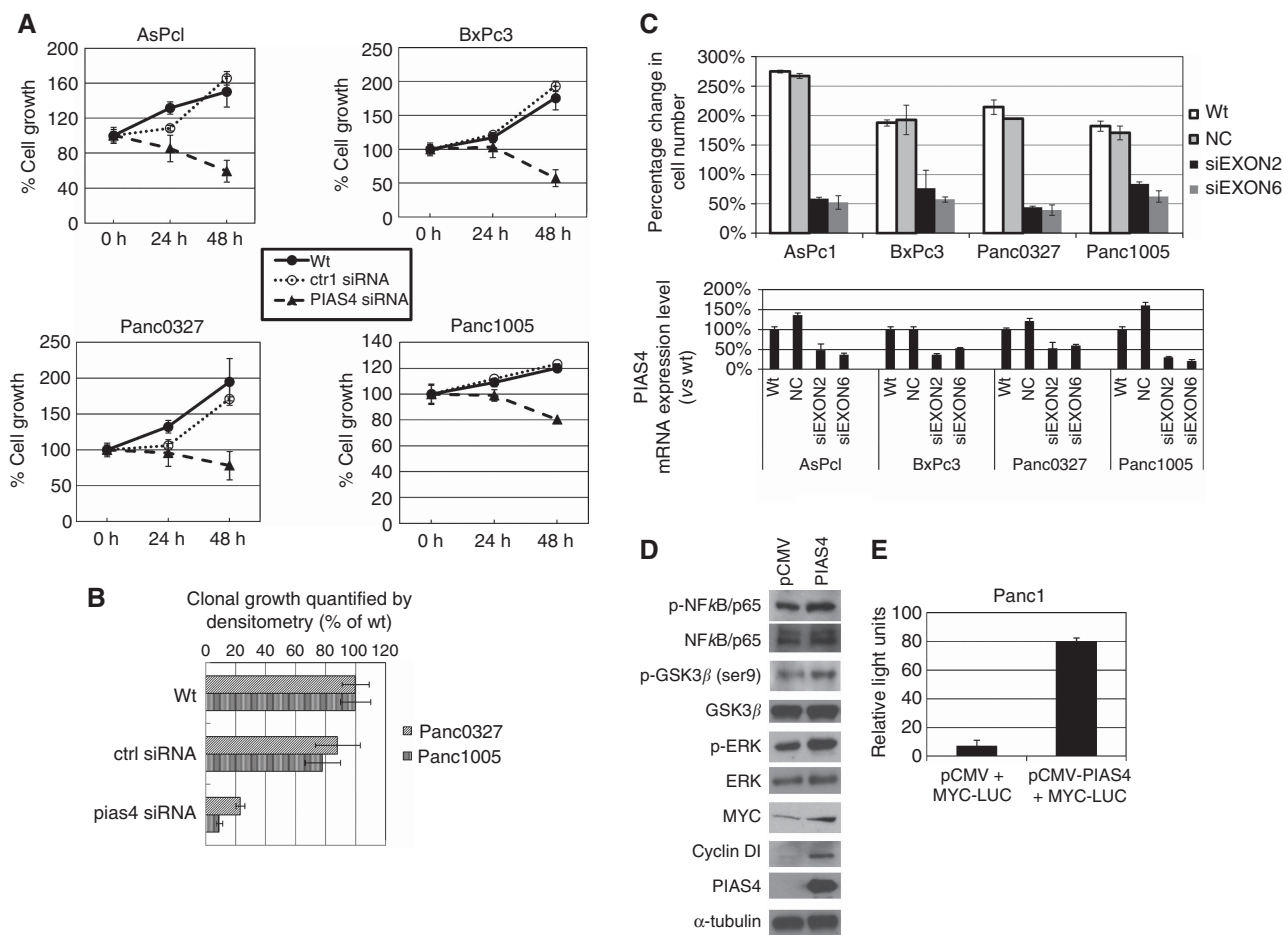


Figure 2. Effect of silencing and overexpression of PIAS4. **(A)** Four pancreatic cancer cell lines (AsPc1, BxPc3, Panc0327, and Panc1005) were transfected with either mock transfection (wild-type, wt), control siRNA (ctrl siRNA) or pooled PIAS4 siRNA. MTT assays show the effect of PIAS4 gene silencing on cell growth. Results represent the mean of three independent experiments with triplicate wells per experimental point. **(B)** Colony assays were photographed 72 h after seeding and stained with crystal violet. Bar graph shows the quantification of colonies by densitometry after colonies were solubilised and measured at OD 540 nm. **(C)** MTT assays show the effect of PIAS4 gene silencing (targeting either exon 2 (siEXON2) or exon 6 (siEXON6), or a negative control (non-target siRNA NC)) on cell growth. Real-time PCR was used to validate knock down of PIAS4 mRNA in these cells (lower panel). **(D)** Panc1 cells were transfected with either the PIAS4 overexpression construct pCMV-PIAS4 or empty vector pCMV. Alpha-Tubulin was used as a loading control in western blot analysis. **(E)** Panc1 cells were co-transfected with a Myc reporter (Myc-LUC) and pCMV-PIAS4 or pCMV. Myc reporter activity was normalised to Renilla activity and calculated as relative light units. Results represent the mean \pm s.d. of two experiments in triplicate wells.

Real-time RT-PCR showed that PIAS4 and HIF1 α were induced 15- to 20-fold by hypoxia in Panc0327 and Panc1005 within 4 h (Figure 3A). The induction levels were less in BxPc3 and AsPc1 pancreatic cancer cell lines at 4- to 8-fold but still significant (Figure 3A). We investigated the protein expression levels of HIF1 α and PIAS4, as well as STAT3, a modulator of cell proliferation mediated by HIF1 α , in BxPc3 cells under hypoxia. After 2 h exposure to 1% O₂, acute induction of HIF1 α was associated with the induction of phosphorylated STAT3; and this induction of both proteins was diminished after 48 h of 1% O₂ exposure (Figure 3B). The PIAS4 protein level was also induced after 2 h exposure to hypoxia, and the expression levels remained elevated under chronic hypoxia conditions (48 h, 1% O₂) (Figure 3B). In addition, we explored the role of PIAS4 in NF κ B, AKT, and MAPK pathways under hypoxic condition. Increase in phosphorylation levels of AKT and ERK was associated with the induction of HIF1 α and phosphorylated STAT3 after 2 h exposure to 1% O₂; and levels of these activated proteins decreased after 48 h of 1% O₂ exposure. On the other hand, NF κ B exhibited significant basal phosphorylation which decreased after acute and chronic hypoxia (Figure 3B).

A previous study in renal cell carcinoma cells showed that PIAS4 siRNA increased the degradation of HIF1 α by activation of VHL. Initially, we investigated the effect of PIAS4 siRNA on levels of HIF1 α at normoxia conditions. Knockdown of PIAS4 in MiaPaCa2 pancreatic cancer cells decreased levels of HIF1 α as well as expression of the HIF1 α target gene VEGF (Figure 4A). Surprisingly, VHL expression level was also suppressed by PIAS4 siRNA in these cells (Figure 4A), suggesting another mechanism of VHL regulation by PIAS4. Because pancreatic cancer cells are highly invasive, the effect of PIAS4 knockdown was studied using Boyden chamber assays. The MiaPaCa2 cells transfected with PIAS4 siRNA exhibited both suppression of cell proliferation on adherent cells and decreased cell invasion through Boyden chambers compared with wild-type and control siRNA transfected cells (Figure 4B). Furthermore, we examined the effect of PIAS4 siRNA on the HIF1 α pathway under hypoxic conditions (1% O₂) in MiaPaCa2 cells. Real-time RT-PCR showed that the induction of both VEGF and another HIF1 α target gene, JMJD1A, were suppressed after PIAS4 siRNA transfection in MiaPaCa2 cells compared with the two control conditions (wild-type untransfected and control siRNA transfected) (Figure 4C).

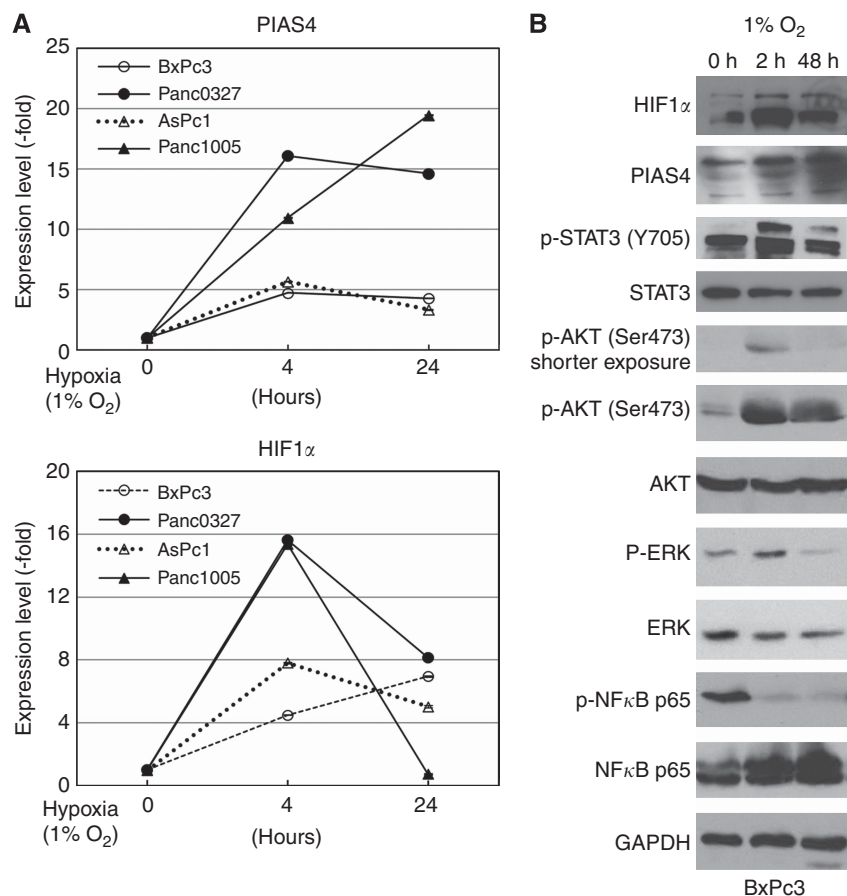


Figure 3. Induction of expression of PIAS4 and HIF1 α under hypoxic conditions. Pancreatic cancer cell lines were incubated in 1% O₂ for the durations as indicated and examined for induction of gene expression. **(A)** Real-time quantitative RT-PCR measurement of PIAS4 expression (top panel) and HIF1 α (bottom panel) in four cell lines (BxPc3, Panc0327, AsPc1, and Panc1005). **(B)** Western blot analysis of protein expression in BxPc3 cells under hypoxic conditions (1% O₂) for 2 and 48 h. GAPDH was used as loading control.

Next, we examined the effect of PIAS4 siRNA on the protein expression levels of HIF1 α and STAT3 in pancreatic cancer cells (MiaPaCa2 and Panc0327) in hypoxic conditions (1% O₂). In the two control conditions (wild-type and control siRNA), the induction of HIF1 α protein was readily detectable after exposure to hypoxia for 2 h (Figure 5A and B). The expression levels of the induced HIF1 α protein continued to be robustly expressed even after 48 h exposure to hypoxia in these cells with control siRNA (Figure 5A and B). In PIAS4 siRNA knockdown cells, the induction levels of HIF1 α were either decreased as in MiaPaCa2 cells (Figure 5A) or the HIF1 α induction was markedly delayed with prominent expression only after 48 h exposure to hypoxia in Panc0327 cells compared with controls (Figure 5B). The phosphorylation level of STAT3 was prominently diminished in PIAS4 siRNA transfected cells, and chronic hypoxia (48 h) was unable to induce STAT3 phosphorylation in these cells (Figure 5A and B).

The protein level of HIF1 α can be regulated by VHL-mediated degradation; and E3 ubiquitin ligase activity of VHL can be affected by its levels of sumoylation. In MiaPaCa2 pancreatic cancer cells treated with PIAS4 siRNA, the levels of SUMO1 in total cell lysates were decreased as shown by western blot analysis (Figure 6A) when PIAS4 was knocked down by >90% (Figure 6B). Immunoprecipitation experiments showed that PIAS4 and VHL interacted, and as expected this interaction was diminished after PIAS4 siRNA was transfected into MiaPaCa2 cells (Figure 6C). When the same western blot was probed with VHL antibodies,

the sumoylation levels of VHL were increased under hypoxic conditions in the two control cells (wild-type and ctrl siRNA transfected) but the sumoylation was suppressed in PIAS4 siRNA-treated cells (Figure 6D). Although the real-time PCR results demonstrated decreased levels of VHL after siRNA treatment (Figure 4A), no decrease in the levels of VHL protein occurred in these cells (Figure 6D).

Prior investigators described (Cai *et al*, 2010) that the binding domain of PIAS4 for VHL was between amino acids 54 and 120. We used two VHL expression vectors containing mutations in this region (pCSVHL-W88S; pCSVHL-Y112H) and one mutation outside of this region (pCSVHL-R167Q) to examine the binding between VHL and PIAS4. Co-expression of PIAS4 and wild-type VHL again showed binding between them and this interaction was lost when wild-type VHL was replaced with VHL containing mutations in the PIAS-binding region (W88S and Y112H) (Figure 7A). The binding was retained when the VHL mutation occurred outside of the PIAS4-binding region (R167Q) (Figure 7A). Interaction between VHL and PIAS4 was able to regulate HIF1 α activity in a reporter assay containing the hypoxia-response element (HRE-LUC) (Figure 7B). The loss of the interaction between VHL and PIAS4 subsequently suppressed the induction of HIF1 α activity (Figure 7B). In addition, we found that mutation of VHL at amino acid 167 also suppressed the hypoxia-response element (reporter assays) (Figure 7B), although it did not disrupt the interaction between PIAS4 and VHL.

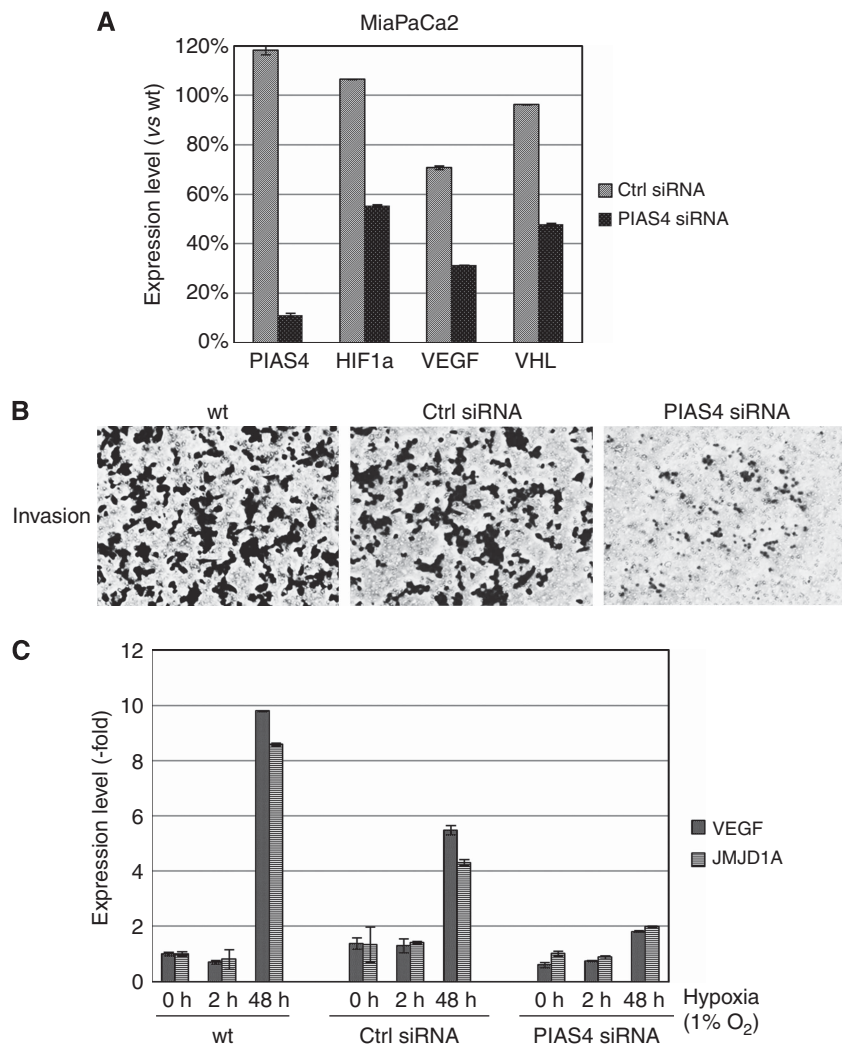


Figure 4. Effect of PIAS4 siRNA on expression of HIF α target genes and on cell invasion. MiaPaCa2 pancreatic cancer cells were transfected with either control siRNA or PIAS4 siRNA. **(A)** Relative RNA expression levels of PIAS4, HIF α , VEGF, and VHL in transfected cells (real-time quantitative RT-PCR). Expression of each gene in each sample was normalised to GAPDH. **(B)** Cellular invasion assays. After transfection, equal numbers of cells were applied and allowed to migrate overnight through Boyden chambers. Unmigrated cells were removed and migrated cells were fixed and stained with crystal violet for photography (dark staining represents migrating cells). Results are representative of two independent experiments, each with duplicates. **(C)** Relative RNA expression of VEGF and JMJD1A under hypoxic condition (1% O₂) for 2 and 48 h (real-time quantitative RT-PCR). After transfection, cells were incubated in a hypoxic chamber (1% O₂) and assayed for gene expression normalised to GAPDH.

DISCUSSION

In this study, we demonstrated for the first time that PIAS4 is a potential anticancer target in pancreatic cancer cells by regulating HIF1 α signalling through interaction and sumoylation of VHL. PIAS4 belongs to the PIAS protein family, which includes PIAS1, PIAS2 (or PIASX), PIAS3, and PIAS4 (or PIAS γ). They share sequence homology and were initially characterised as protein inhibitors of activated STAT proteins (Jackson, 2001). Subsequent studies showed that the PIAS family of proteins modulated transcription by different mechanisms, such as regulation of DNA-binding activity of transcription factors, recruitment of either corepressors or coactivators or engage in post-translational modification by their SUMO E3 ligase activity (Bischof *et al*, 2006; Wilkinson and Henley, 2010). In the non-small cell lung carcinoma cell line H1299, PIAS4 interacted with p53 and inhibited p53-mediated transactivation of its target genes BAX and p21, although it did not inhibit apoptosis (Nelson *et al*, 2001). In the prostate cancer cells LNCaP, PIAS4 interacted with the

DNA-binding domain of the androgen receptor (AR) and acted as a transcriptional corepressor of AR which is central to prostate cancer development and progression (Gross *et al*, 2004). Conditional expression of PIAS4 induced apoptosis in a human chronic myeloid leukaemia cell line KCL22 (Ohmine *et al*, 2001). However, in synovial sarcomas, PIAS4-mediated sumoylation resulted in overexpression of nuclear receptor coactivator 3, which is essential for tumour formation (Sun *et al*, 2011). In hepatocellular carcinoma HepG2 cells, PIAS4 modulates sumoylation of NEMO (NF- κ B essential modulator), and NF- κ B activation in response to DNA damage and knockdown of PIAS4 by siRNA in these cells inhibited NF- κ B activation (Mabb *et al*, 2006).

Our examination of PIAS4 expression in a panel of 14 pancreatic cancer cell lines and tumours indicated that dysregulation of PIAS4 frequently occurred in pancreatic cancers. Substantial increase in the PIAS4 protein expression levels was found in tumour tissues compared with normal pancreatic epithelium. By gene silencing of PIAS4 with siRNA, we demonstrated that inhibition of PIAS4 with either pooled siRNAs or individual siRNA targeting specific exons in four cell lines was

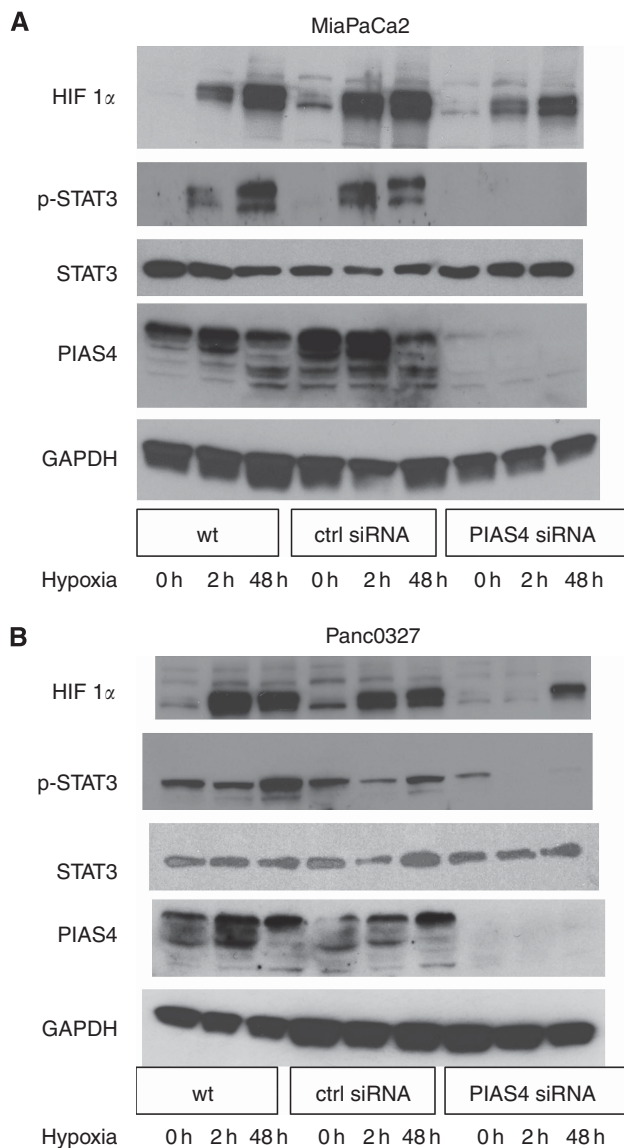


Figure 5. Effects of PIAS4 siRNA on HIF1 α and phosphorylated STAT3 under hypoxic conditions. (A) MiaPaCa2 and (B) Panc0327 cells were transfected with either control siRNA or PIAS4 siRNA and incubated in a hypoxic chamber (1% O₂) for 2 and 48 h. Total protein lysates were harvested for western blot analysis. GAPDH antibody was used as loading control.

associated with decrease in cell number. Also, knockdown of PIAS4 in pancreatic cancer cells suppressed cell invasiveness as measured by Boyden chamber assays. Forced expression of PIAS4 in one cell line (Panc1) resulted in induction of genes related to cell growth such as Myc. A Myc reporter assay demonstrated that PIAS4 activated the Myc promoter. C-Myc is a target of the LEF/TCF transcription factor (Koenig *et al*, 2006). PIAS4 may either directly stimulate the Myc promoter or acts as a coactivator of LEF/TCF, which subsequently enhances transcription of Myc. In a previous study in COS cells, PIAS4 colocalised with TCF in the nucleus, sumoylated TCF, and activated TCF transcription (Ihara *et al*, 2005). In addition, we found that overexpression of PIAS4 caused an increase, albeit subtle, in phosphorylation of NF κ B/p65 in Panc1 pancreatic cancer cells. We were unable to detect phosphorylated IKK β or I κ B α , which suggests that this signalling pathway is not robust in Panc1 cells. Previous studies showed that upon DNA damage, PIAS4 caused NF- κ B activation in HepG2

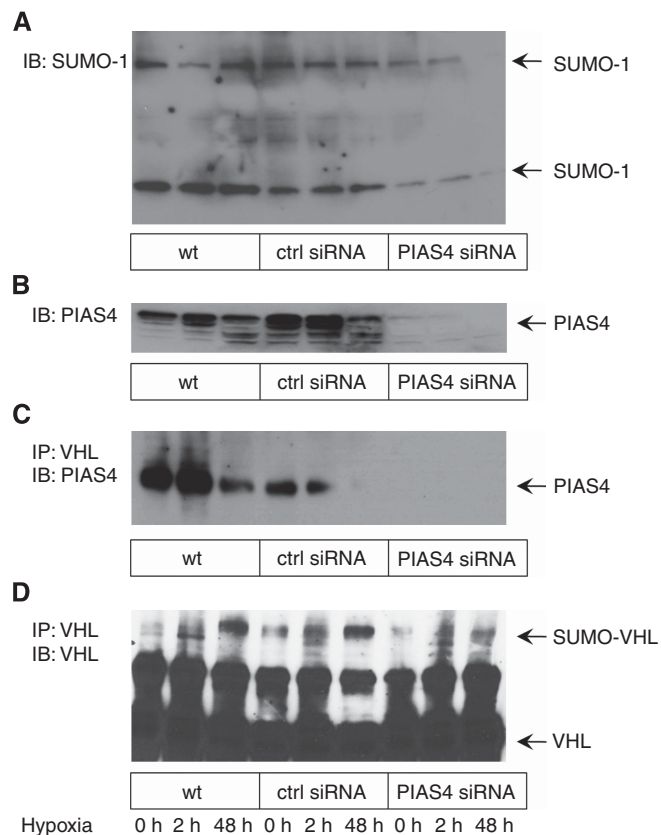


Figure 6. Effects of PIAS4 siRNA on sumoylation, and interaction of PIAS4 with VHL. MiaPaCa2 cells were transfected with either control siRNA or PIAS4 siRNA, and cultured in 1% O₂ for 0, 2, and 48 h. (A, B) Western blot analysis (IB) of total cell lysates for expression levels of sumoylation (SUMO-1) and PIAS4. (C, D) Cell lysates were immunoprecipitated (IP) with VHL antibodies and western blotted (IB) with either PIAS4 or VHL antibodies. ctrl siRNA, control siRNA.

cells (Mabb *et al*, 2006), but inhibited NF- κ B activity in skin squamous cell carcinomas (Albor and Kulesz-Martin, 2007).

Pancreatic adenocarcinoma exists in a hypoxic microenvironment (Couvelard *et al*, 2008), and by real-time RT-PCR, we showed that PIAS4 was inducible together with HIF1 α under hypoxic conditions (1% O₂). Induction of PIAS4 positively correlated with levels of HIF1 α in acute hypoxic conditions. Two pancreatic cancer cell lines (AsPc1 and BxPc3) had low levels and two (Panc0327 and Panc1005) had higher levels of induction of both PIAS4 and HIF1 α . In contrast, with chronic hypoxia, the kinetics of PIAS4 and HIF1 α induction were divergent. For example, elevated levels of PIAS4 mRNA and protein paralleled each other in BxPc3 cells after both acute and chronic exposure to hypoxia. On the other hand, HIF1 α RNA was continuously increased during a 4- to 24-h period of hypoxia, but its protein accumulation diminished at 48 h of hypoxia in the BxPc3 cells and Panc0327 (data not shown). Phosphorylation levels of AKT and ERK also exhibited similar kinetics as HIF1 α . These data suggest that regulation of HIF1 α signalling by PIAS4 may be an acute response to hypoxia; and additional regulatory mechanisms are involved in chronic hypoxic conditions. Notably, NF κ B exhibited significant basal phosphorylation which decreased after acute and chronic hypoxia. We suspect that this represents crosstalk regulation between signalings of NF κ B and HIF1 α .

An earlier study of colon cancer demonstrated high PIAS4 expression with little angiogenesis in colon cancer tissue, which was associated with enhanced HIF1 α sumoylation and deactivation of

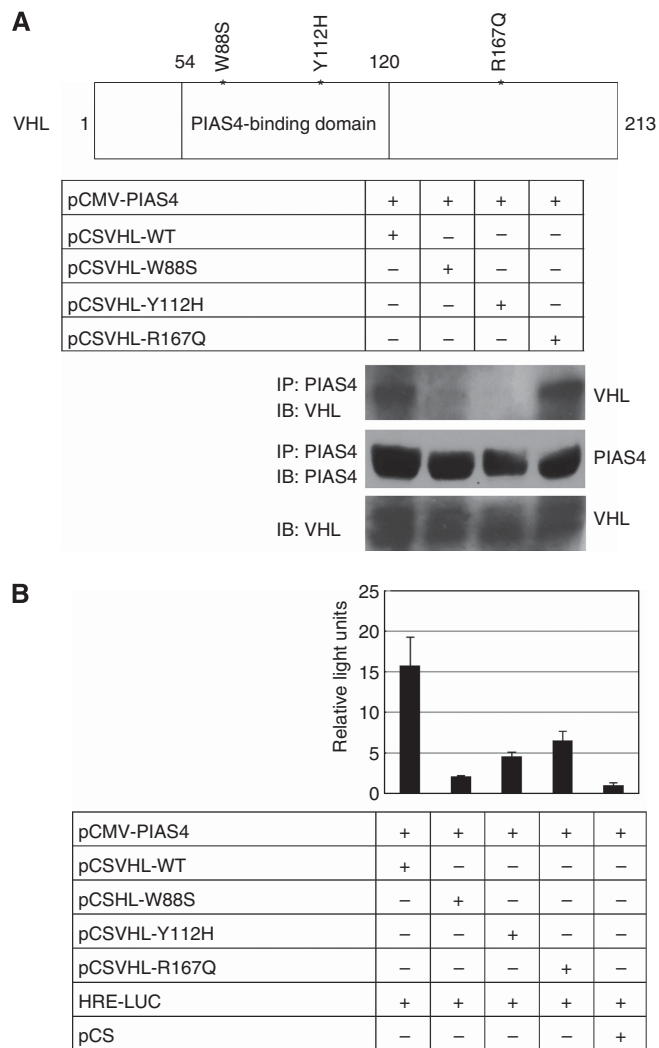


Figure 7. Regulation of HIF1 α activity by interaction between PIAS4 and VHL. Panc1 cells were co-transfected with PIAS4 and a series of VHL constructs expressing either wild-type (pCSVHL-WT) or mutant VHL (pCSVHL-W88S, pCSVHL-Y112H, pCSVHL-R167Q). W88S and Y112H are located inside and R167Q outside of the PIAS4-binding domain. **(A)** Coimmunoprecipitation assay. Immunoprecipitation (IP) with PIAS4 antibodies and blotted (IB) with either VHL or PIAS4 antibodies. Western blot analysis of total lysates was used to examine VHL expression. **(B)** Hypoxia-response element reporter assays. Cells were co-transfected with the HRE-LUC reporter construct, PIAS4, and VHL expression vectors as indicated. HRE-LUC reporter activity was normalised to Renilla activity and calculated as relative light units. HRE-LUC contains three repeats of the hypoxia-response element upstream of the thymidine kinase minimal promoter driving the firefly luciferase reporter gene. Results represent the mean of two independent experiments, each with triplicate wells.

HIF1 α activity (Kang *et al*, 2010). By contrast, our study showed that induction of PIAS4 is positively correlated with induction of HIF1 α . In the hypoxia signalling cascade, HIF1 α activates downstream target genes including VEGF and JMJD1A and promotes tumourigenesis (Tang *et al*, 2004; Krieg *et al*, 2010). Our real-time RT-PCR results showed that PIAS4 siRNA suppressed the expression of HIF1 α in pancreatic cancer cells; and when these cells were exposed to hypoxia for 48 h, the induction of the HIF1 α downstream targets, VEGF and JMJD1A, was also attenuated during PIAS4 knockdown. The histone demethylase JMJD1A is a member of the jumonji domain-containing family; and its function

is oxygen-dependent and regulated by HIF1 α (Beyer *et al*, 2008). JMJD1A has been identified as a useful biomarker for hypoxic tumour cells such as colorectal and renal cell carcinomas (Beyer *et al*, 2008; Uemura *et al*, 2010; Guo *et al* 2011). Alternatively, PIAS4 may directly regulate JMJD1A, and the study of the ability of PIAS4 to modulate epigenetic histone modifications via JMJD1A will be of interest.

Another important protein involved in hypoxic signalling is STAT3 (Gray *et al*, 2005). In cancers of diverse origins, the activities of both STAT3 and HIF1 α are upregulated in part by an autocrine loop (Lang *et al*, 2007; Anglesio *et al*, 2011). Activation of HIF1 α transcriptional activity by multiple growth stimuli and hypoxia is mediated by STAT3 (Gray *et al*, 2005; Lang *et al*, 2007). Thus, through the action of STAT3, HIF1 α upregulates genes involved in enhanced cell proliferation and survival (Lang *et al*, 2007). Our study showed that PIAS4 siRNA decreased or delayed the induction of HIF1 α expression, and suppressed phosphorylation levels of STAT3. After 48 h of hypoxia, STAT3 remained suppressed and was not reactivated suggesting an additional non-HIF1 α -dependent mechanism of STAT3 regulation by PIAS4 in these pancreatic cancer cells. In addition to STAT3, STAT5a and 5b have also been implicated in pancreatic tumourigenesis (Kataoka *et al*, 2007; Moser *et al*, 2012). Although a previous study showed weak positive staining of STAT5b in normal pancreatic ducts adjacent to the tumour and 50% of tumour samples exhibited positive staining for STAT5b (Moser *et al*, 2012), no phosphorylated STAT5a or 5b were detected in BxPc3 cells suggesting at least for the pancreatic cancer cells STAT3 has a more prominent role in the context of PIAS4. Phosphorylation of STAT3 is mediated by the Janus-activated kinase (JAK) (Zhong and Darnell, 1994); and in a KRAS-induced mouse model of PDAC, a selective JAK2 inhibitor blocked STAT3 phosphorylation and reduced the formation of pancreatic intraepithelial neoplasms and adenocarcinomas (Corcoran *et al*, 2011). Prior studies have found that the endogenous JAK2 inhibitor protein tyrosine phosphatase 1B (PTP1B) can become sumoylated and inactivated in mammalian fibroblasts (Dadke *et al*, 2007). The PIAS4 siRNA knockdown cells showed a global decrease in SUMO1 levels. Potentially, PTP1B may be a substrate of PIAS4 sumoylation resulting in increased JAK2 activity with subsequent phosphorylation of STAT3. Therefore, the JAK/STAT pathway in PDAC may be suppressed by PIAS4 siRNA.

Our observation of either decreased or delayed HIF1 α induction in PIAS4 siRNA knockdown pancreatic cancer cells was likely due to the decreased sumoylation of VHL. Von Hippel-Lindau acts as a tumour suppressor by degradation of HIF1 α (Kaelin, 2008). Von Hippel-Lindau sumoylation inactivates its tumour-suppressing activity (Cai *et al*, 2010). Coimmunoprecipitation assays demonstrated that the functional interaction between PIAS4 and VHL is essential for regulation of HIF1 α activity. We showed that loss of binding between PIAS4 and two mutant VHL constructs reduced HIF1 α transcriptional activity as demonstrated by luciferase assays. In addition, although the VHL mutant R167Q did not interfere with binding between PIAS4 and VHL, it was still able to suppress HIF1 α transcriptional activity. This amino acid is a part of the alpha helix containing domain involved in interaction between VHL and elongin BC complex that targets HIF1 α for proteasomal degradation (Iwai *et al*, 1999). How this mutation decreased the transcriptional activity of HIF1 α requires further study. On the basis of our results, we present a mechanism for the role of PIAS4 in pancreatic cancer (Figure 8). The model illustrates that in the hypoxic condition, PIAS4 sumoylates and inactivates VHL which leads to HIF1 α activation. Hypoxia also induces PIAS4 expression. In normoxic condition, PIAS4 alone is sufficient to activate STAT3, NF κ B, ERK and subsequently transcriptional activation of MYC, Cyclin D1, VEGF, and JMJD1A. As a result, pancreatic cancer cell proliferation, angiogenesis, and metastasis

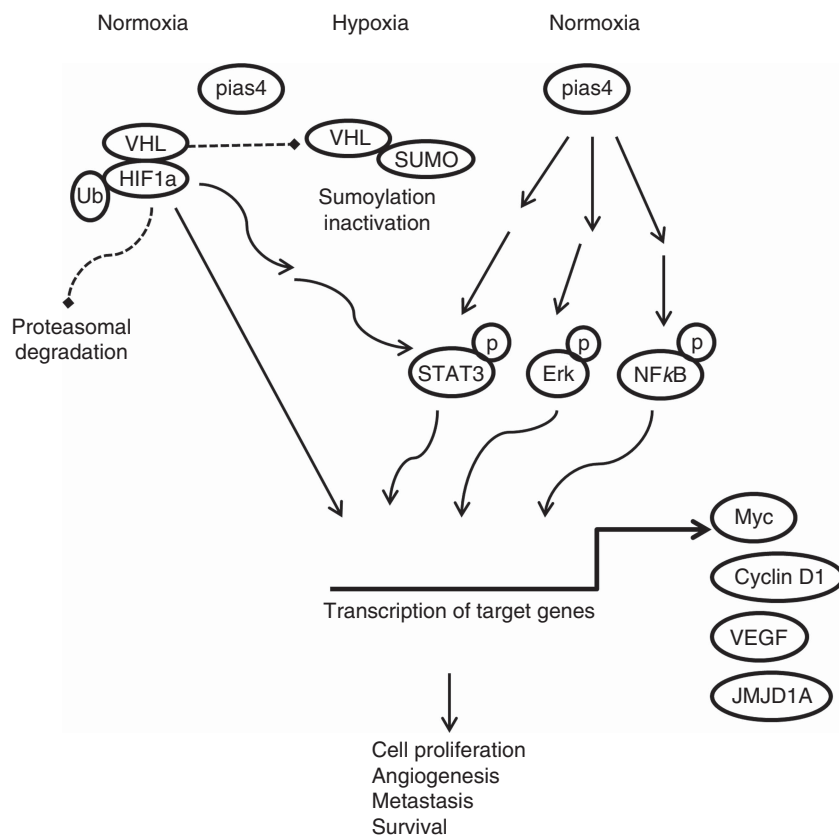


Figure 8. Mechanism of PIAS4 signalling in pancreatic cancer cells. In response to hypoxia, PIAS4 sumoylates and inactivates VHL which leads to HIF1 α activation. In normoxic condition, PIAS4 activates signalling pathways leading to cell proliferation, angiogenesis, and metastasis.

are promoted. In summary, our study demonstrated the essential role of PIAS4 in the regulation of pancreatic cancer cell growth by VHL sumoylation and enhanced HIF1 α activity. Our data strongly suggest that inhibition of PIAS4 activity can be a novel therapeutic approach in the management of pancreatic cancers.

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