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Hypoxia inducible factor-1α promotes cell proliferation and migration in human pancreatic cancer cell line Patu8988

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Summary. *Aim:* Pancreatic cancer cells could survive and proliferate under severe hypoxia, with high expression of hypoxia inducible factor-1α (HIF-1α), which plays a crucial role in cell energy metabolisms, tumor angiogenesis and metastasis in cancer cells. This study investigates the role of HIF-1α on cell proliferation and migration of pancreatic cancer cells. *Materials and methods:* HIF-1α was knocked down in human pancreatic cancer cell line Patu8988 using siRNA. Cell proliferation was analyzed by MTT assay and cell cycle was analyzed by flow cytometry. Cancer cell mobility was assessed by a migration assay with Boyden chambers. *Results:* Cell proliferation analysis showed cells with decreased cell growth by MTT assay and G1/S phase cell cycle arrest using flow cytometry. Cancer cell mobility was significantly decreased when the HIF-1α was down-regulated, as assessed by a migration assay with Boyden chambers. *Conclusion:* These results suggested that down-regulation of HIF-1α could inhibit cell proliferation, and decreased tumor cell migration, which provided a new perspective in understanding the pleiotropic role of HIF-1α in pancreatic cancer.

Key words: pancreatic cancer, HIF-1α, cell cycle arrest, migration, siRNA

«Il fattore -1α inducibile da ipossia promuove la proliferazione e la migrazione cellulare nella linea di cellule pancreatiche tumorali umane Patu8988»

Riassunto. *Obiettivo*: le cellule tumorali nel pancreas possono sopravvivere e proliferare in condizioni di grave ipossia, con alta espressione del fattore -1α inducibile da ipossia (HIF-1α), che svolge un ruolo fondamentale nel metabolismo energetico delle cellule, nell'angiogenesi tumorale e nelle metastasi delle cellule tumorali. In questo studio si indaga sul ruolo di HIF-1α sulla proliferazione cellulare e la migrazione delle cellule tumorali pancreatiche. *Materiali e metodi*: HIF-1α è stato silenziato in una linea di cellule pancreatiche tumorali umane Patu8988 con siRNA. La proliferazione cellulare è stata analizzata mediante saggio MTT e il ciclo cellulare è stato analizzato mediante citometria di flusso. La mobilità delle cellule tumorali è stata valutata mediante un saggio di migrazione con camere di Boyden. *Risultati*: l'analisi della proliferazione cellulare ha mostrato una diminuzione dello sviluppo cellulare mediante il test MTT e un arresto del ciclo cellulare in fase G1/S mediante la citometria di flusso. La mobilità delle cellule tumorali era significativamente diminuita quando HIF-1α è stato silenziato come mostrato da un saggio di migrazione con camere di Boyden. *Conclusione*: questi risultati suggeriscono che il silenziamento di HIF-1α è in grado di inibire la proliferazione cellulare, e diminuire la migrazione delle cellule tumorali, il che ha fornito una nuova prospettiva nella comprensione del ruolo pleiotropico di HIF-1α nel cancro del pancreas.

Parole chiave: tumore pancreatico, HIF-1α, arresto del ciclo cellulare, migrazione, siRNA

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Introduction

Pancreatic cancer accounts for approximately 33,000 deaths in the United States every year (1, 2), and ranks as the fourth most common cause of cancer-related mortality (3, 4). The majority of patients (~85%) are diagnosed with advanced pancreatic cancer with extensive local invasion and/or metastasis, and the 1-year survival is around 20%, and the overall 5-year survival rate is less than 5% for all stages combined (5, 6). Although numerous chemotherapeutic drugs have been tested for patients with pancreatic cancer, survival rate of advanced pancreatic cancer has not improved over the past several decades. Therefore, identification of novel targets and new approaches to inhibit cell proliferation and metastasis are extremely important to improve the outcome of patients.

Hypoxia-inducible factor (HIF) is a transcription factor that is induced in hypoxic cells, and mediates the primary transcription responses to hypoxic stress. HIF is a heterodimeric complex composed of basic-helix-loop-helix Per (Period)–ARNT (aryl hydrocarbon nuclear translocator)–SIM (single minded) (bHLH-PAS) proteins including an O_2 liable subunit and a stable β subunit (7). HIF-β is a common subunit of multiple bHLH proteins and quite stable in normoxic conditions, while HIF-1α is a unique, O_2-regulated subunit that is extremely unstable and quickly degraded by the ubiquitin-proteasome system (8). Hypoxic HIF activity is primarily controlled through post-translational modification and stabilization of HIF-1α subunit, so that HIF-1α protein levels and overall HIF transcriptional activity increase as cells become more hypoxic.

HIF-1α was first described to play a central role in medicating O_2-dependent transcriptional responses. The expression of HIF-1α is inducible by oxygen concentrations less than 6% (9), and the activation of hypoxia-responsive elements by HIF-1 has been reported to correlate with decreasing oxygen concentrations in vitro (10). HIF-1 binds to the cis-acting hypoxia-response element located in the 3-flanking region of the human EPO gene (11). Elevated expression of HIF-1 has been observed in a broad array of human cancer cell types, including bladder, breast, glial, hepatocellular, ovarian, pancreatic, prostate, renal, and esophageal tumors, and associated with poor prognosis in many cases (12-14). HIF-1α plays an important role in solid tumor formation *in vivo* by promoting angiogenesis and anaerobic metabolism (15).

In our previous study, we showed that HIF-1α is important for human pancreatic cancer cell proliferation (16). Here we tested the knockdown of HIF-1α on pancreatic cancer cell proliferation and migration. The result showed that cells with decreasing HIF-1α have also reduced cell migration, demonstrating HIF-1α as important for both pancreatic cell proliferation and migration, and indicating that HIF-1α could be used as a novel target for pancreatic cancer treatment in future.

Materials and methods

Cell culture

Human pancreatic cancer cell line Patu8988 was purchased from Jiangsu Provincial Institute of Hematology. Patu8988 cells in logarithmic growth phase were maintained in RPMI1640 medium (GIBCO, Grand Island, CA, USA) containing 10% FBS (GIBCO, Grand Island, CA, USA). On the day of the experiment, cells were transferred into 6-well tissue cultured plates with density of 5×10^5/well, and a thin layer of fresh medium (0.15 mL/cm^2) with 10% FBS was added to decrease the diffusion distance of the ambient gas. Cells were cultured at low oxygen (0.5% O_2, and 5% CO_2) for 4h before the transfection.

siRNA synthesis

Twenty-one base pair siRNAs targeting HIF-1α siRNA were chemically synthesized, annealed and purified by JiKai gene Technology Co. Ltd (Shanghai, China). The custom siRNA sequences for HIF-1α were: 5'-GUGAUGAAAGAAUUACCGAAU-3' (sense), and 5'-AUUCGGUAAUUCUUUCAUC AC-3' (anti-sense). The sequences for negative control siRNA were: 5'-UUCUCCGAACGUGUCACGU UU-3' (sense) and 5'-ACGUGACACGUUCGGA GA-3' (anti-sense).

Cell transfection

Patu8988 cells in logarithmic growth phase were seeded at 5×10^5/well in 6-well plates with RPMI1640 medium with 10% FBS 24h before transfection. The medium was replaced by serum-free Opti-MEM (GIBCO, Grand Island, CA, USA) 16 h before transfection. HiPerFect Transfection Reagent and Negative control siRNA were purchased from JiKai Technology Co.Ltd (Shanghai, China). Transfection compounds were prepared in three groups as follows: siRNA group, negative control group and blank control group. Transfection compounds were incubated at room temperature for 10 minutes, and added into 6-well plates with total amount of 2200 μl in each well. The mixture was replaced by complete medium 24 h after the transfection.

Real-time PCR

Total RNA from three groups was extracted using RNAiso Reagent kit (TaKaRa, Dalian, China) at 48 h after transfection. cDNA was generated from 2 μg of total RNA by reverse transcription using random primers and PrimeScript RT Master Mix Perfect Real Time (TaKaRa, Dalian, China) according to the manufacturer's instructions. The primer sequences of HIF-1α and housekeeping gene actin, were designed using Primer5 software (Premier Biosoft International, Palo Alto, USA, and the primers were synthesized by Shanghai Daweike Biotechnology Co. Ltd (Shanghai, China). The primers for HIF-1α are: 5'-AGATTTAGACTTGAGATGTTAGC-3' and 5'-CAGTGGTGCAGTGGTAG -3', and the primers for β-actin are 5'-ACTGGAACGGTGAAGGTGAC-3' and 5'-AGAGAA GTGGGGT GGCTTTT-3'. Real-time PCR was performed using ABI PRISM 7500 Real-Time System (Life Technology, Foster City, USA). 2 μl cDNA was amplified using the SYBR Premix Ex Taq Perfect Real Time (TaKaRa, Dalian, China), and the PCR cycle conditions were 95°C for 30 s, 40 cycles of 95°C for 5 s, and 60°C for 34 s. The amplification specificity was evaluated with melting curve analysis. The mRNA level of HIF-1 in each group was normalized to β-actin.

Western blotting

At 72 h after transfection, total cell lysate were extracted with lysis buffer containing 10 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% (v/v) Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 5 mM EDTA, 1 mM PMSF, 0.28 kU/L aprotinin, 50 mg/L leupeptin, 1 mM benzamidine and 7 mg/L pepstain, protein concentration was determined using a BCA kit (Pierce, Rockford, USA). Twenty microgram of protein from each group was separated on a 10% SDS-PAGE gel, and transferred to nitrocellulose membranes (MILLIPORE, Billerica, MA, USA) in a semidry electro-transferring unit. Membranes were blocked 1h at 4°C in blocking buffer (TBST with 5% nonfat dry milk), and incubated with primary monoclonal rabbit anti-human HIF-1 antibody (Cell Signaling Technology, USA, 1:1000) in Tris-buffered saline containing 0.1% Tween-20 (TBST) and 5% nonfat dry milk overnight at 4°C. After washing, membranes were incubated with secondary HRP labeled goat anti-rabbit second antibody (Santa Cruz Biotechnology Inc.CA, USA, 1:10000) in TBST for 2 h. Membranes were washed twice and signals were detected with enhanced chemoluminescent autoradiography ECL kit (Amersham, Sunnyvale, CA, USA). Membranes were stripped by stripping buffer (gradients for stripping solution), and were reprobed with GAPDH (Cell Signaling Technology, Beverly, MA, USA, 1:1000). The signal intensity of primary antibody binding was quantitatively analyzed with Sigma Scan Pro 5 and was normalized to a loading control GAPDH.

Cell cycle analysis

For cell cycle analysis, Patu8988 cells were transected with HIF-1α siRNA or negative control RNA. After 72 h, cells were fixed in 70% ethanol for 1 h at room temperature, and then stained with 100 μL RNase A (1 mg/mL) and 400 μL propidium iodide (50 μg/mL) for 30 min at room temperature. Blank control, negative control siRNA and HIF-1α siRNA samples were run on a FACScan flow cytometer (BD Biosciences, San Jose, CA), and data were analyzed by software Light cycle (Roche Molecular Biochemi-

cals). Cells in each condition were measured in triplicate, and proliferation indexes (PI) were calculated as follows: PI = (S+G2/M)/(G0/G1+S+G2/M) ×100%.

MTT assay

Cells were seeded in 96-well plates at 1×10^4 cells per well and pre-incubated in RPMI 1640 medium containing 10% FBS. Cells were transfected by siRNAs for HIF-1α. After 72 h of transfection, cells were added with 0.5 mg/mL MTT (Sigma Chemical Co. St. Louis, MO, USA) per well for 4 h. Mixture of medium in each well was removed, and 150 μl DMSO (Sigma Chemical Co. St.Louis, MO, USA) was added. Absorbance of each group was measured at 540 nm with a microplate reader (Model 550, Bio-Rad, Hercules, USA). Each condition was repeated 8 times.

Migration assay

After transfection for 24 h, cells in all groups were treated with trypsin, and re-suspended as single cell solution. Boyden chambers with 8 μm pore polycarbonate membrane were used to study cell migration, and chambers were inserted in a transwell apparatus (Costar, Cambridge, MA), which was either coated with Matrigel (BD Biosciences, San Jose, CA) for invasive cells or no Matrigel for migration cells. A total of 2×10^5 cells in 0.5 mL of serum-free RPMI 1640 medium were seeded into the upper chamber, and 600 μL RPMI1640 medium with 20% FBS was added to the lower chamber. After the transfected cells was incubated for 36 h at 37°C, the cells on the top surface of the insert were removed by wiping with a cotton swab, and the cells that migrated to the bottom surface of the insert were fixed in 100% methanol for 2 min, stained in Giemsa for 2 min, rinsed in PBS, and were observed by microscope (OLYMPUS CX21) at 100 fold magnification. Migrated cells were counted in five fields per membrane, and cells in each condition were repeated in three independent experiments.

Statistical Analysis

Data are presented as means ± standard errors (SE). Statistical analysis was carried out by one-way ANOVA followed by Dunnett *t*-test or Student *t*-test (two-tailed comparison) using SPSS 13.0, and a *P* value of < 0.05 was considered to be statistically significant.

Results

Transfection with HIF-1α siRNA successfully down-regulates HIF-1α mRNA levels to silence HIF-1α gene

Patu8988 cells were transfected with the siRNAs, RNA was extracted at 48h and proteins were extracted 72h later. At these stages no gross morphologic changes were apparent in the cells. Real time PCR showed that a significantly decrease in mRNA level (Figure 1A) in the HIF-1α siRNA treated cells, and western blot showed a significantly decrease in protein levels in the HIF-1α siRNA treated cells compared to the control cells (Figure 1B). The bands in the western blotting were quantitatively analyzed by Sigma Scan Pro5, and we estimated that about 85% of the HIF-1α protein was depleted in these experiments (Figure 1C). These data verified the efficacy of HIF-1α siRNA, leading to decrease of HIF-1α protein in transfected cells.

Down-regulation of HIF-1 decreases cell proliferation and induced G1/S arrest

MTT assay was used to measure the cell proliferation. At 72h after transfection, HIF-1α siRNA treated cells showed a 30% decrease in absorbance compared to blank control group and negative control group, which indicated that cell proliferation of HIF-1α siRNA treated cells was inhibited (Figure 2A). Cell cycle analysis performed on cells with down-regulation of the HIF-1α suggested the reduction in DNA synthesis (Figure 2B). HIF-1α siRNA treated cells had a significant increase (24%) in G0/G1 phase of cells compared with blank control cells (34.933 ±2.593%) and negative control cells (34.600±2.552%) (Figure 2C). Correspondingly, HIF-1α down-regulation led to 13% decrease of cells in S phase and 9% decrease of cells in G2/M phase compared with blank and negative control siRNA. Moreover, proliferation indexes (PI), the percentage of cells in S and G2/M

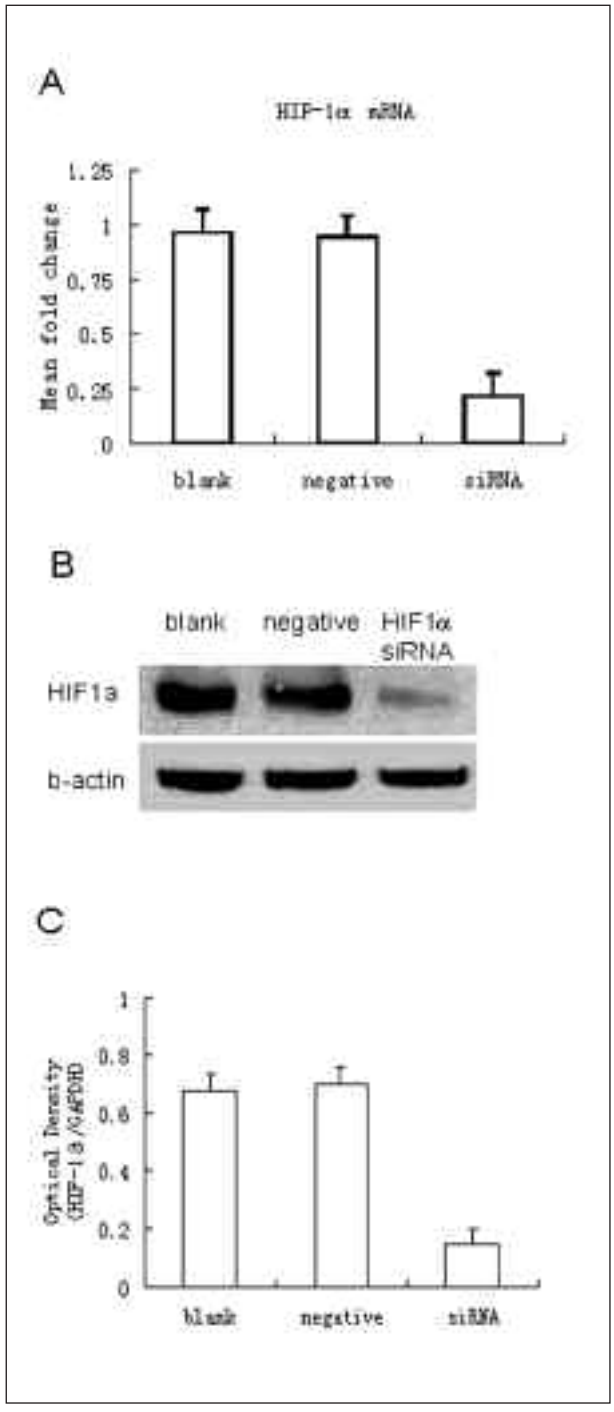

Figure 1. Transfection with siRNA successfully knocked down expression of HIF-1α. A) HIF-1α mRNA levels was decreased by specific siRNA in Patu8988 cells; B) Western blotting analysis for HIF-1α protein; C) Gray scale intensity of western blotting bands of HIF-1α protein levels (*P<0.05, compared with blank control group and negative control group respectively)

phase, suggested a significantly decrease (23%) in HIF-1α down-regulated cells (42.377±2.897%), versus blank control group (65.400±2.551%) and negative control group (64.947±2.611%) (Figure 2D). Taken together, these data indicated that down-regulation of HIF-1α results in an inhibition of G1/S phase, which leading to the inhibition of cell proliferation.

Down-regulation of HIF-1α decreases migration

Since the metastasis and migration are important characteristics for pancreatic tumor cells, we assessed cell migration in response to down-regulation of HIF-1α. Compared with the blank (306.9±6.5) and negative control siRNA treated cells (295.7±8.6), migration decreased 50% less in cells with down-regulated HIF-1α ($P < 0.05$) (Figure 3). The result suggested that the migration of pancreatic cancer cells could be inhibited by down-regulation of HIF-1α.

Discussion

Pancreatic cancer has a poor blood supply, and is able to survive and proliferate under severe hypoxia. Pancreatic cancer cells were reported to constitutively express high level of HIF-1α, which acquired the ability to survive and proliferate under severe hypoxia and nutrient deprivation (17). Accordingly, most of pancreatic cancers may require overexpression of HIF-1α to develop and progress in the context of severe hypoxia and glucose deprivation (18). Both HIF-1α and vascular endothelial growth factor are overexpressed in human pancreatic cancer, and HIF-1α is detected in the nuclei of human pancreatic carcinoma cells but only barely detected in normal pancreatic tissue (19, 20). Because angiogenesis is required for malignant cells to leave the primary tumor and proliferate at a distant site, so the expression of HIF-1α might significantly relate to metastatic status. Our study establishes that down-regulation of the HIF-1α decreases cell proliferation, and inhibits cell mobility in pancreatic cancer cell line Patu8988. These results suggests that we are able to restrain tumor growth and metastasis by silencing HIF-1α, and the HIF-1α is a

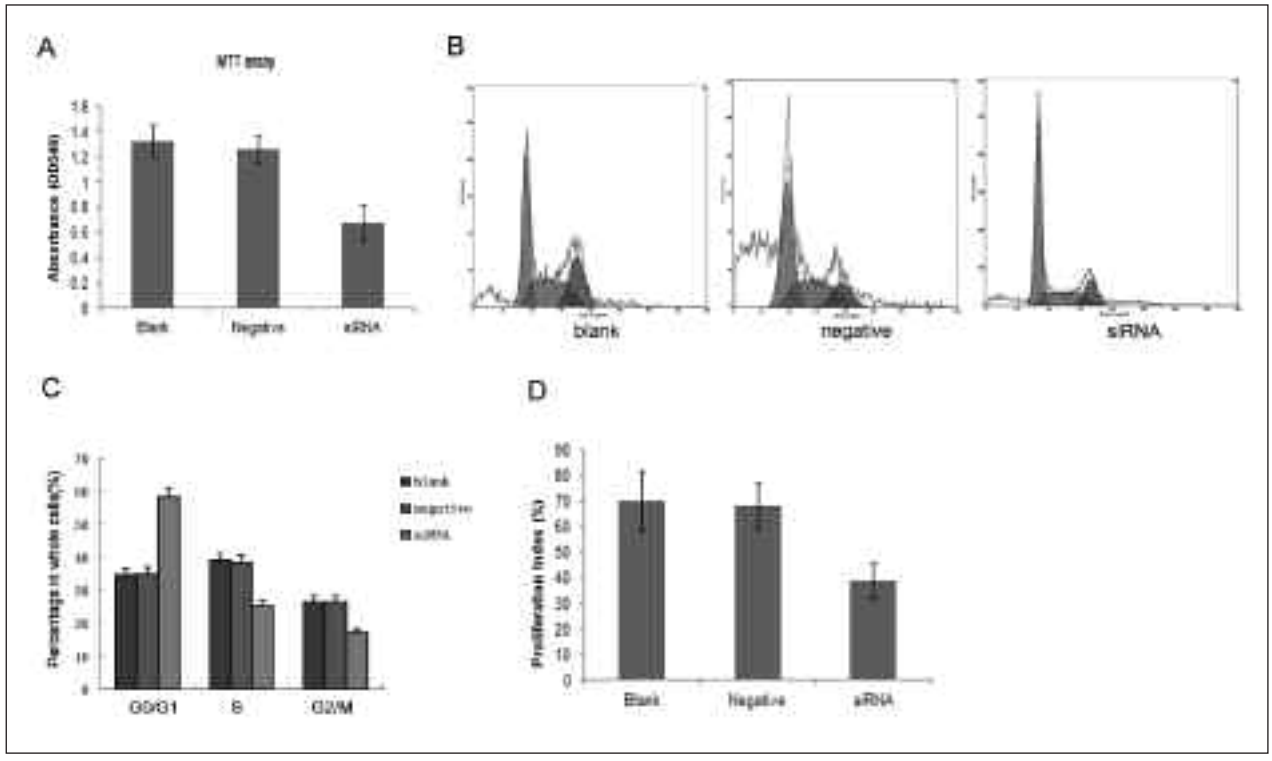

Figure 2. Knock down of HIF-1α resulted in proliferation inhibition and cell cycle arrest. A) MTT analysis for proliferation of three different groups, the mean actual absorbance of siRNA group was significantly lower than that in blank control group and negative control group respectively; B) DNA contents of Patu8988 cells treated in blank control group, negative control group and siRNA group by FACS; C) The percentages of cells at each phase in three different groups: G0/G1 phase, S phase and G2/M phase. A significant G0/G1 phase arrest after HIF-1α was knocked down; D) Analysis of the proliferation index (PI) calculated in three different groups. (* P <0.05, compared with blank control group and negative control group respectively)

potential target for developing novel strategies for the treatment of pancreatic cancer.

Although we have proved that down-regulation of HIF-1α affect cell proliferation, the pathways affected by HIF-1α signaling remains obscure. HIF-1α is involved in the transcriptional regulation of various genes, including vascular endothelial growth factor, glucose transporter 1, phosphoglycerate kinase 1, and p21 (21-24). It was reported that acute HIF-1α stabilize at 1% O_2 produces cell cycle arrest by inhibit the protoconcoprotein MYC, a transcription factor that is over-expressed in more than 40% of human cancers. MYC controls the G1/S cell cycle transition by forming heterodimers with the related protein MZAX, promoting expression of genes encoding cyclin D2 and E2F1 (25). Genes that are regulated by HIF-1α have been investigated by chromatin immunoprecipitation coupled to tile microarrays, and recent studies suggested that genes that are directly regulated by HIF-1α were largely determined by cell type-specific patterns of chromatin structure, and HIF-1α regulate the expression of multiple Jumonji-domain containing histone demethylases, which may directly contribute to changes in hypoxic target gene expression (26, 27). The pathways that affected in pancreatic cancer cells by down-regulation of HIF-1α will be investigated in future study.

To date, this study highlights that the silencing of HIF-1α would suppress cancer cell proliferation, and migration in human pancreatic cancer cell lines. The present study sheds light on the novel role of HIF-1α in pancreatic cancer. However, our results were based on a single cell line, further researches to determine the differential expression of HIF-1α in other human pancreatic cancer cell lines and the expression levels of the downstream pathway genes are required.

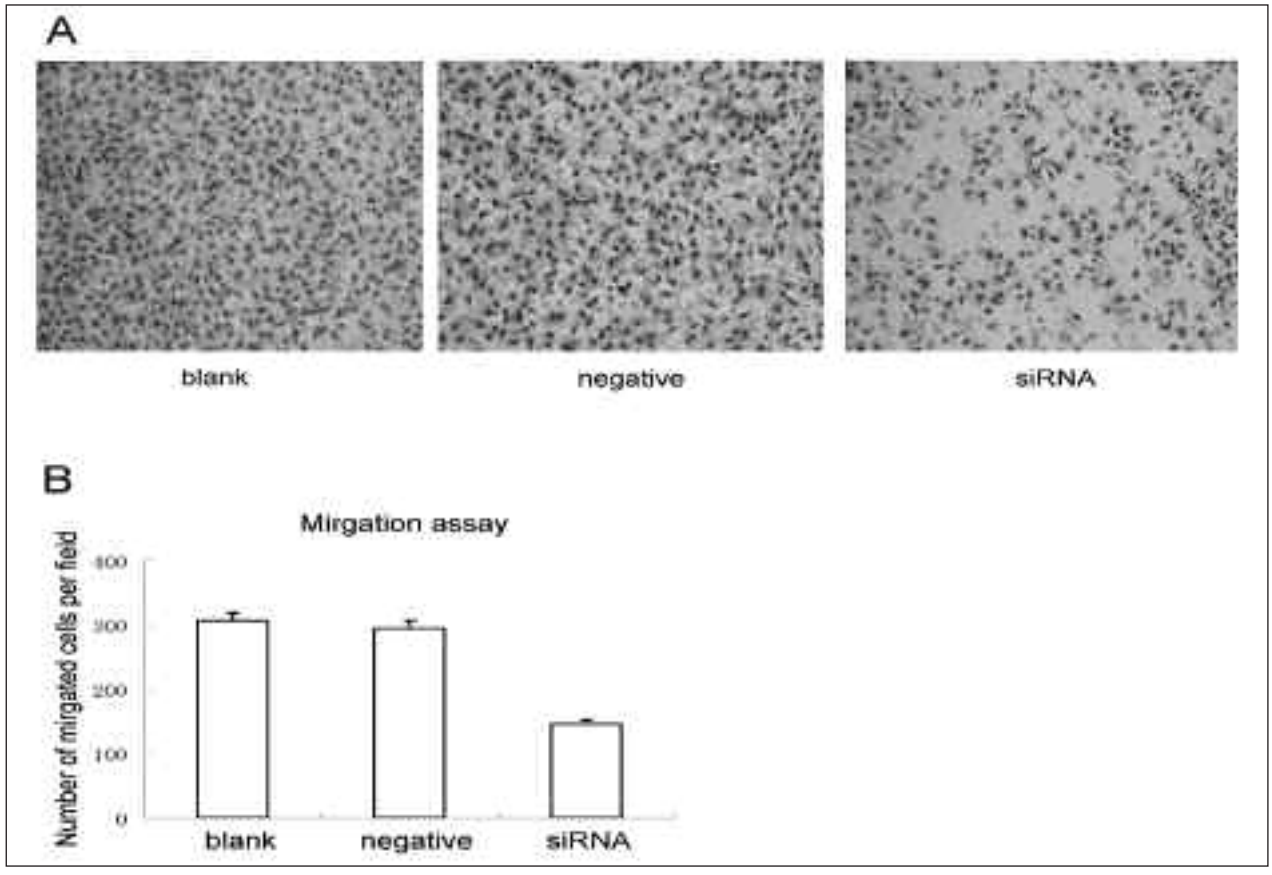

Figure 3. Knock down of HIF-1α resulted in suppression of tumor cell migration. A) The migration of cells was inhibited when HIF-1α was knocked down; B) Analysis of the number of Patu8988 cells in migration assay. The number of siRNA group was decreased compared with that of blank control group and negative control group, respectively. (* P <0.05, compared with blank control group and negative control group respectively) (Note: magnification is ×100)

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