

Activating transcription factor 4 regulates hypoxia inducible factor 1 α in chronic hypoxia in pancreatic cancer cells

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Abstract. Pancreatic ductal adenocarcinoma (PDAC) is one of the most aggressive and difficult to treat cancers with tumors typically exhibiting high levels of chronic hypoxia. Hypoxia activates hypoxia-inducible factors (HIFs) that mediate cellular responses to adapt to low oxygen environments. Hypoxia also causes endoplasmic reticulum (ER) stress, increasing activating transcription factor 4 (ATF4), a master regulator of the unfolded protein response (UPR) pathway that mediates cellular response to ER stress. ATF4 is overexpressed in PDAC and is associated with poor prognoses. While ATF4 promotes cell proliferation and tumorigenesis, most studies have been conducted under normoxia or acute hypoxia. The functions of ATF4 in chronic hypoxia remain largely unexplored. Using siRNA knockdown experiments of healthy skin fibroblast cells WS1 and PDAC cell lines PANC-1 and Mia-PaCa2 to analyze mRNA and protein expression levels, a novel ATF4 function was identified, in which it decreases HIF2 α mRNA and increases HIF1 α mRNA in chronic hypoxia while having no effect in acute hypoxia. A scratch assay was used to show that ATF4 decreases cell migration in chronic hypoxia as opposed to the increase in cell migration ATF4 imparts in acute hypoxia. Colony formation assay and cell viability assay showed that ATF4 promotes colony formation and cell

viability in both chronic and acute hypoxia. In addition to the differential response of ATF4 in chronic hypoxia compared with acute hypoxia, this is the first time ATF4 has been implicated in regulation of response to hypoxia via interaction with HIF proteins in PDAC.

Introduction

Pancreatic ductal adenocarcinoma (PDAC) is among the most aggressive cancers with a 5-year survival rate of 8-10% (1-3). Hypoxia, a stress condition in which oxygen levels are insufficient for typical cellular function, contributes to PDAC pathophysiology and treatment resistance (4). Protein synthesis in the endoplasmic reticulum (ER) demands more energy and oxygen compared with other cellular processes (5,6); as such hypoxia significantly hinders protein translation and folding (7). This results in accumulated misfolded or unfolded proteins activating ER stress sensors, thus triggering the unfolded protein response (UPR) (Fig. 1) (7,8). Within the UPR pathway, activating transcription factor 4 (ATF4) is one of the master regulators that activates transcription of genes required for amino acid metabolism, synthesis and transport (7). These genes, among others, generally alleviate hypoxia-induced ER stress to promote cell recovery and homeostasis (7,9-11).

PDAC exhibits enhanced cellular response to hypoxic stress and significantly higher ATF4 expression compared with healthy pancreatic cells (12-14). Numerous chemotherapeutic drugs are ineffective in PDAC due to ATF4 functions that promote drug resistance in hypoxia (14). ATF4 is unique in the UPR pathway because it is translated more efficiently in hypoxia compared with normoxia (7,15). This higher translation efficiency is orchestrated by upstream open reading frames (uORFs) in the mRNA transcript that promote translation of a truncated, inactive protein under normal conditions and initiate translation of functional ATF4 protein under cell stress conditions, such as hypoxia. Increased ATF4 protein expression in hypoxia is also promoted by post-translational modifications that stabilize the protein (16).

In addition to activating ATF4 in the UPR pathway, cells also respond to hypoxia by activating hypoxia-inducible factors (HIFs), a family of proteins that initiate hypoxia-response pathways (17). HIFs are well known for their roles as

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Abbreviations: ATF4, activating transcription factor 4; CA9, carbonic anhydrase IX; HAF, HIF activating factor; HIF1 α , hypoxia inducible factor 1 α ; HIF2 α , hypoxia inducible factor 2 α ; Oct4, octamer-binding transcription factor 4; PDAC pancreatic ductal adenocarcinoma; uORF, upstream open reading frame; UPR, unfolded protein response

Key words: ATF4, HIF1 α , HIF2 α , PDAC, chronic hypoxia, UPR pathway

transcription factors in hypoxia because they activate the transcription of genes that promote cell adaptation during oxygen deprivation (17). HIF1 α and HIF2 α subunits are capable of transcribing genes that play important roles in cancer progression such as carbonic anhydrase IX (CA9) and octamer-binding transcription factor 4 (Oct4), among others (18,19). Independent of its transcriptional functions, HIF2 α is also a translational factor that forms a complex with other proteins to activate an alternative cap-dependent translation mechanism to synthesize proteins from select mRNA that are necessary for response to hypoxia (17,20-22). In chronic hypoxia, the canonical translation initiation pathway is inhibited, resulting in significantly decreased global protein synthesis (20).

Previous studies on ATF4 and HIFs in pancreatic cancers have been conducted at atmospheric oxygen levels, but not in prolonged hypoxia of more than 24 h, a more pathophysiologically relevant condition in which the majority of PDAC cases exist. *In vitro* studies performed in normoxia demonstrated that ATF4 inhibition prevents cancer progression and treatment resistance. However, while ATF4 expression is higher in PDAC cells compared with healthy pancreatic cells in these studies, the cells used in normoxic experiments do not express HIFs at levels similar to those that are observed in patients, making it difficult to identify any relevant interactions between ATF4 and HIFs. Furthermore, other studies showed that more severe hypoxia of <1% oxygen levels are necessary for significant UPR activation (23,24). In the present study, ATF4 activity was explored in the hypoxia response pathway mediated by HIFs in conditions of severe, chronic hypoxia in pancreatic cancer cells.

Materials and methods

Cell culture. Pancreatic epithelial carcinoma cell lines PANC-1 (cat. no. CRL-1469) and MiaPaCa-2 [cat. no. CRL-1420; both from American Type Culture Collection (ATCC)] were cultured in Advanced DMEM media supplemented with 10% FBS, 1% GlutaMAX (cat. no. 35050061; Gibco; Thermo Fisher Scientific, Inc.), and 1% penicillin and streptomycin (cat. no. 15140-163; Gibco; Thermo Fisher Scientific, Inc.). Healthy skin fibroblasts WS1 cells (cat. no. CRL-1502; ATCC) were cultured in advanced DMEM supplemented with 15% FBS, penicillin and streptomycin, GlutaMAX, β -mercaptoethanol, and basic fibroblast growth factor. All cells were cultured under standard conditions of 37°C with 5% CO₂, 21% O₂ (normoxic) or as indicated in hypoxia. All cells were tested for mycoplasma ~every 3 months and confirmed to be free of contamination using a PCR mycoplasma detection kit (cat. no. 30-1012K; ATCC).

Hypoxia treatment. For hypoxia treatment, Ruskinn InvivO₂ 500 hypoxia workstation was used (Baker Ruskinn). Cells were seeded or plated and incubated under standard conditions in normoxia for 24 h prior to hypoxia exposure. Cells were then placed into a sterile hypoxia glove box pre-set to either 1% oxygen for healthy fibroblasts or 0.2% for cancer cell lines, 5% CO₂, a balance of N₂ and at 37°C. WS1 fibroblasts were exposed to 1% oxygen rather than 0.2% oxygen since cancer cells have a lower threshold for oxygen before hypoxia response pathways are activated, while normal fibroblasts

are less tolerant of hypoxia. Cells were handled inside of the hypoxia glove box for the duration of the experiments until they were either fixed or lysed.

Transfection. To assess the effects of gene inhibition on hypoxic PDAC, PANC-1 cells were transiently transfected using Lipofectamine RNAiMAX Transfection Reagent (Thermo Fisher Scientific, Inc.) and pre-designed Silencer Select small interfering (si)RNAs (Thermo Fisher Scientific, Inc.) at 1 pmol per well: HIF1 α (cat. no. s6539), HIF2 α (cat. no. s4698) and ATF4 (cat. no. s1702). The siRNA-lipid complexes were constructed in reduced serum Opti-MEM media (cat. no. 31985070; Thermo Fisher Scientific, Inc.) according to manufacturer protocols, incubated at room temperature for at least 5 min and were added to each well in either six-, 12- or 96-well plates. The cells were then seeded into the plates in normal growth media. Lipofectamine RNAiMAX Transfection Reagent only requires reduced serum media when siRNA-lipid complexes are made and not when treated to cells in growth media containing serum. The cells were incubated at 37°C under normoxic conditions for 24 h before they were placed in the hypoxia chamber or remained in the normoxic incubator. Knockdown efficacy was determined by reverse transcription-quantitative (RT-q) PCR.

RNA extraction, quantification and RT-q PCR. Cells incubated in normoxia were washed with PBS while hypoxic cells were washed with de-oxygenated PBS. RNA was extracted using TRIzol® (Thermo Fisher Scientific, Inc.) and purified by RNeasy column centrifugation kit (Qiagen). Purified RNA was quantified using Nanodrop spectrophotometer at a wavelength of 260 nm (Thermo Fisher Scientific, Inc.). cDNA was synthesized using qScript cDNA synthesis kit (Quantabio) according to the manufacturer's protocol and qPCR was conducted using Taqman primer-probes (Thermo Fisher Scientific, Inc.) and Taqman Gene Expression Master Mix (Applied Biosystems; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol provided for the Taqman Master Mix. The Taqman probes used were Hs00153153_m1 (HIF1 α), Hs01026149_m1 (HIF2 α), Hs00154208_m1 (CA9), Hs00999632_g1 (Oct4) and Hs00909569_g1 (ATF4). The RT-qPCR data was quantified by calculating the 2^{- $\Delta\Delta C_q$} values as defined by Livak and Schmittgen, 2001.

Protein extraction and western blot analysis. Normoxic and hypoxic cells were washed in either oxygenated PBS or de-oxygenated PBS, respectively. Mammalian Protein Extraction Reagent was supplemented with Halt Protease Inhibitor cocktail (Thermo Fisher Scientific, Inc.) and added to the cells for lysis. The cells were frozen for at least 6 h to promote lysis, and the samples were sonicated. The samples were centrifuged at 4°C for 10 min at 14,000 x g. The supernatant was transferred to new tubes and Bicinchoninic Acid (BCA) assay (Pierce; Thermo Fisher Scientific, Inc.) was conducted and measured using the EnVision plate reader (PerkinElmer, Inc.) to quantify protein.

For western blotting, the Criterion Blotter Western Blot system was used (Bio-Rad Laboratories, Inc.); 30 μ g of total protein in Laemmli sample buffer was boiled for 10 min at 95-100°C. The samples were loaded into 10% gels (Bio-Rad

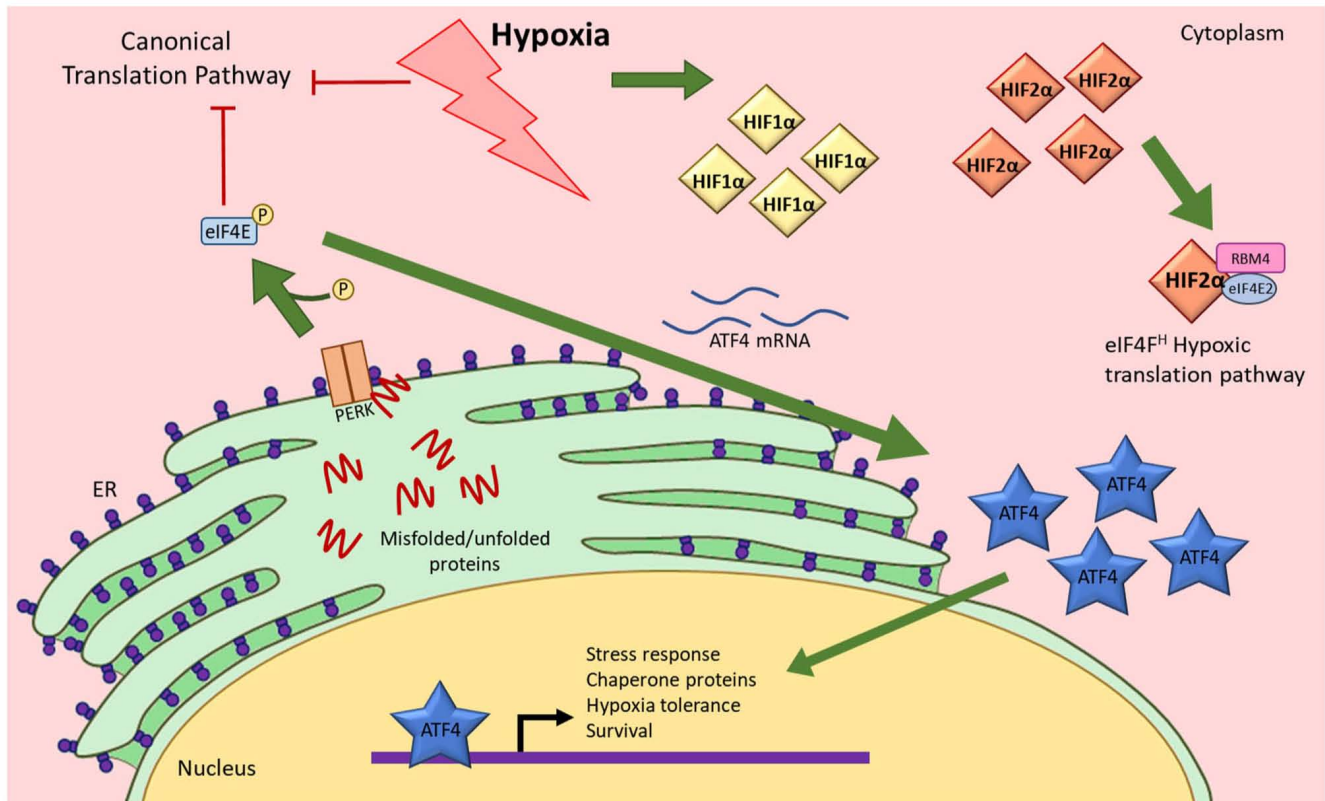


Figure 1. Hypoxia activates the UPR pathway and causes ATF4 expression to increase. Hypoxia causes inhibition of the canonical translation pathway, resulting in misfolded or unfolded proteins. These peptides activate PERK that consequently induces ATF4 mRNA translation, resulting in an increase in ATF4 protein expression. ATF4 then translocates to the nucleus to initiate gene expression changes. Hypoxia also causes increases in HIF1 α and HIF2 α . Both HIFs also translocate to the nucleus to function as transcription factors. HIF2 α also functions as a translation factor that initiates translation when cells are under hypoxic stress (17). UPR, unfolded protein response; ATF4, activating transcription factor 4; eIF4E, eukaryotic initiation factor 4E; ER, endoplasmic reticulum; HIF1 α , hypoxia-inducible factor 1 α ; HIF2 α , hypoxia-inducible factor 2 α ; PERK, protein kinase R (PKR)-like endoplasmic reticulum kinase; P, phosphate.

Laboratories, Inc.) and electrophoresis commenced at 60-100 V. The protein in the gel was transferred to PVDF membranes by wet-transfer at 100 V for 30 min. The membranes were blocked in 5% w/v fat-free powdered milk in 1X Tris buffered solution with 0.1% Tween 20 detergent (TBS-T; Sigma-Aldrich; Merck KGaA) for 1 h at room temperature, probed with primary antibodies overnight at 4°C in 5% BSA (Sigma-Aldrich; Merck KGaA) and with secondary antibodies for 1 h at room temperature in 5% BSA. The primary antibodies used were HIF1 α (1:1,000; cat. no. MA1516; Thermo Fisher Scientific, Inc.), HIF2 α (1:1,000; cat. no. ab8365; Abcam), ATF4 (1:1,000; cat. no. 11815S; Cell Signaling Technology, Inc.) and β -actin (1:1,000; cat. no. sc-47778; Santa Cruz Biotechnology, Inc.). Horseradish peroxidase-conjugated mouse IgG κ light chain binding protein (1:1,000; cat. no. sc-516102; Santa Cruz Biotechnology, Inc.) and mouse anti-rabbit secondary antibodies (1:1,000; cat. no. sc-2357; Santa Cruz Biotechnology, Inc.) were used. The blots were visualized by enhanced chemiluminescence (ECL) incubation and C-DiGit Blot Scanner (LI-COR Biosciences). The images were quantified by ImageJ software version 1.53e (National Institutes of Health).

Cell viability assay. Cells (2,000/well) were seeded into 96-well plates and incubated overnight in normoxia under standard culturing conditions. The plates were placed into hypoxia or maintained at normoxia. After hypoxic exposure,

the cells were removed from the chamber and reconstituted CellTiterGlo (Promega Corporation) reagents were added to the plate, following the manufacturer's protocol. The plate was incubated at room temperature for at least 10 min and the luminescence at 560 nm was measured on the PerkinElmer Envision plate reader.

Colony formation assay. Cells were seeded into each well (2,000 cells per well) in six-well plates. The cells were incubated at 37°C for 24 h and then placed in 0.2% oxygen or maintain in normoxia. The cells in hypoxia were incubated for 16 or 48 h and then placed in normoxia for 7-10 days of incubation for colony formation. Afterwards, the cells were washed with cold PBS, fixed with acetic acid and methanol fixing solution (1:4 mixture) at room temperature for at least 15 min until dry and stained at room temperature for at least 30 min with 0.5% w/v crystal violet (cat. no. C0775-25G; Sigma Aldrich; Merck KGaA) in 25% ethanol solution. The plates were then washed, and colonies were counted manually.

Cell migration (scratch) assay. Cells were transiently transfected following the previously outlined protocol. After siRNA-lipid complexes were added to 24-well plates, 1x10⁵ PANC-1 cells in growth media were seeded into each well and incubated at 37°C for 24 h. Each well was then 'scratched' using a P1000 pipette tip down the middle of each well. Images

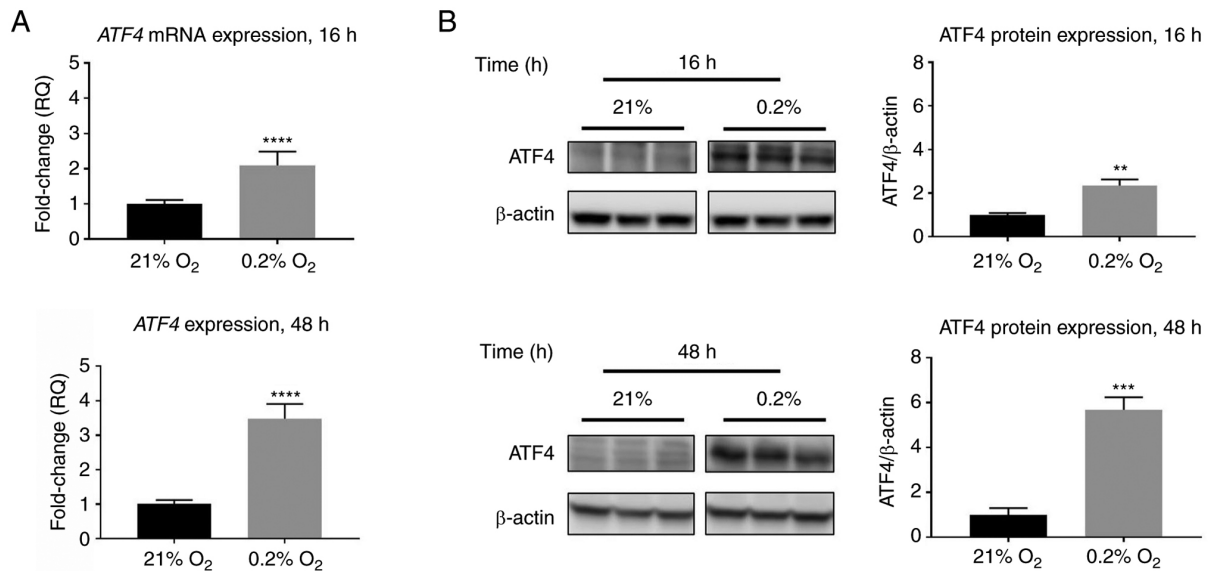


Figure 2. ATF4 mRNA and protein expression increase in response to acute and chronic hypoxia in PANC-1 cells. PANC-1 cells were treated with hypoxia (0.2% oxygen) or normoxia (21% oxygen) for 16 or 48 h. (A) RT-qPCR was conducted to determine ATF4 mRNA levels. Fold changes for RT-qPCR are shown relative to normoxic cells. Error bars represent the mean \pm SD for $n=9$. The fold changes were analyzed using unpaired Student's t-test. (B) Western blot analysis was conducted to determine ATF4 protein expression levels after 16 or 48 h of hypoxia or normoxia in PANC-1 cells. ATF4 protein levels were normalized to β -actin and are shown relative to normoxic levels. Error bars represent the mean \pm SD for $n=3$. The fold changes were analyzed using unpaired Student's t-test. ** $P<0.01$, *** $P<0.001$ and **** $P<0.0001$. ATF4, activating transcription factor 4; RT-qPCR, reverse transcription-quantitative PCR.

of all wells were obtained. The plates were then placed in either 0.2% oxygen or maintained in normoxia and incubated for either 16 or 48 h. After incubation in normoxia or hypoxia, images of each well were captured using an inverted light microscope (Invertoskop 40C; Carl Zeiss AG) and analyzed using an ImageJ/Fiji plugin tool (25).

Statistical analysis. All experiments were conducted in at least three replicates. For all experiments consisting of two groups, unpaired Student's t-test was conducted for statistical significance. For all experiments consisting of more than two groups, tests for homogeneity of variance, such as Levene's test, were performed. One-way analyses of variance (ANOVA) tests were conducted, followed by the Bonferroni multiple variance post hoc tests. Data were graphed and analyzed using GraphPad Prism software version 9.3 (GraphPad Software, Inc.). $P<0.05$ was considered to indicate a statistically significant difference.

Results

ATF4 expression increases in acute and chronic hypoxia. ATF4 is over-expressed in PDAC cells compared with healthy pancreatic cells and is associated with poor prognosis (26). To first determine ATF4 expression in PDAC cells in hypoxia, PANC-1 and Mia-PaCa2 cells were cultured in a 0.2% oxygen environment or in a 21% oxygen environment for 24 h. Hypoxia was defined as 0.2% oxygen in PDAC cells because while different tissue types activate the hypoxia-responsive pathways at varying levels of oxygen, pancreatic cancers have been shown to be severely hypoxic (<1%) with ~0.4% oxygen in tumors (27-29). It was determined that 0.2% oxygen was necessary to ensure the PDAC cells adequately activate hypoxia-response pathways by confirming HIF and ATF4 activity at 0.2% oxygen (Figs. 2 and 3). Furthermore, the UPR

pathway activates under severe hypoxic conditions, which is needed to investigate ATF4 (23,24).

PANC-1 cells were used to assess ATF4 expression in acute and chronic hypoxia. Literature values for acute hypoxia generally do not surpass 24 h, while chronic hypoxia tends to be more than 24 h. In the present study, acute was defined as 16 h and chronic as 48 h (26,30,31). RT-qPCR data revealed that ATF4 mRNA expression increased by 2.1 ± 0.1 -fold in acute hypoxia compared with cells exposed to normoxia ($P<0.0001$), while ATF4 mRNA levels in chronic hypoxia increased 3.5 ± 0.4 -fold compared with normoxia levels ($P<0.0001$; Fig. 2A). Western blot analysis revealed similar expression patterns in which ATF4 protein levels increase in hypoxia compared with normoxic levels. Under normoxic conditions, little to no ATF4 protein expression was detected, but upon hypoxic exposure, ATF4 expression increased significantly. In acute hypoxia, the PANC-1 cells exhibited a 2.3 ± 0.1 -fold increase in ATF4 protein expression ($P<0.01$), while in chronic hypoxia, ATF4 levels increased by 5.7 ± 0.3 -fold compared with normoxia ($P<0.001$; Fig. 2B).

HIFs and expression of HIF target genes vary over time in hypoxia. In addition to ATF4 expression changes, hypoxia leads to increased HIF1 α and HIF2 α expression in PDAC (17). However, HIF expression profiles differ depending on cell type and duration in hypoxia (17). While all HIF levels oscillate over time, increased HIF1 α expression is generally associated with acute hypoxic exposure whereas increased HIF2 α expression is typically associated with chronic hypoxia (17,32). HIF expression profiles can vary greatly among different cell types, and, therefore, HIF expression and activity in PANC-1 cells were determined in acute and chronic hypoxia. To confirm HIF1 α and HIF2 α expression and activity, the cells were incubated in either normoxic conditions or hypoxia over

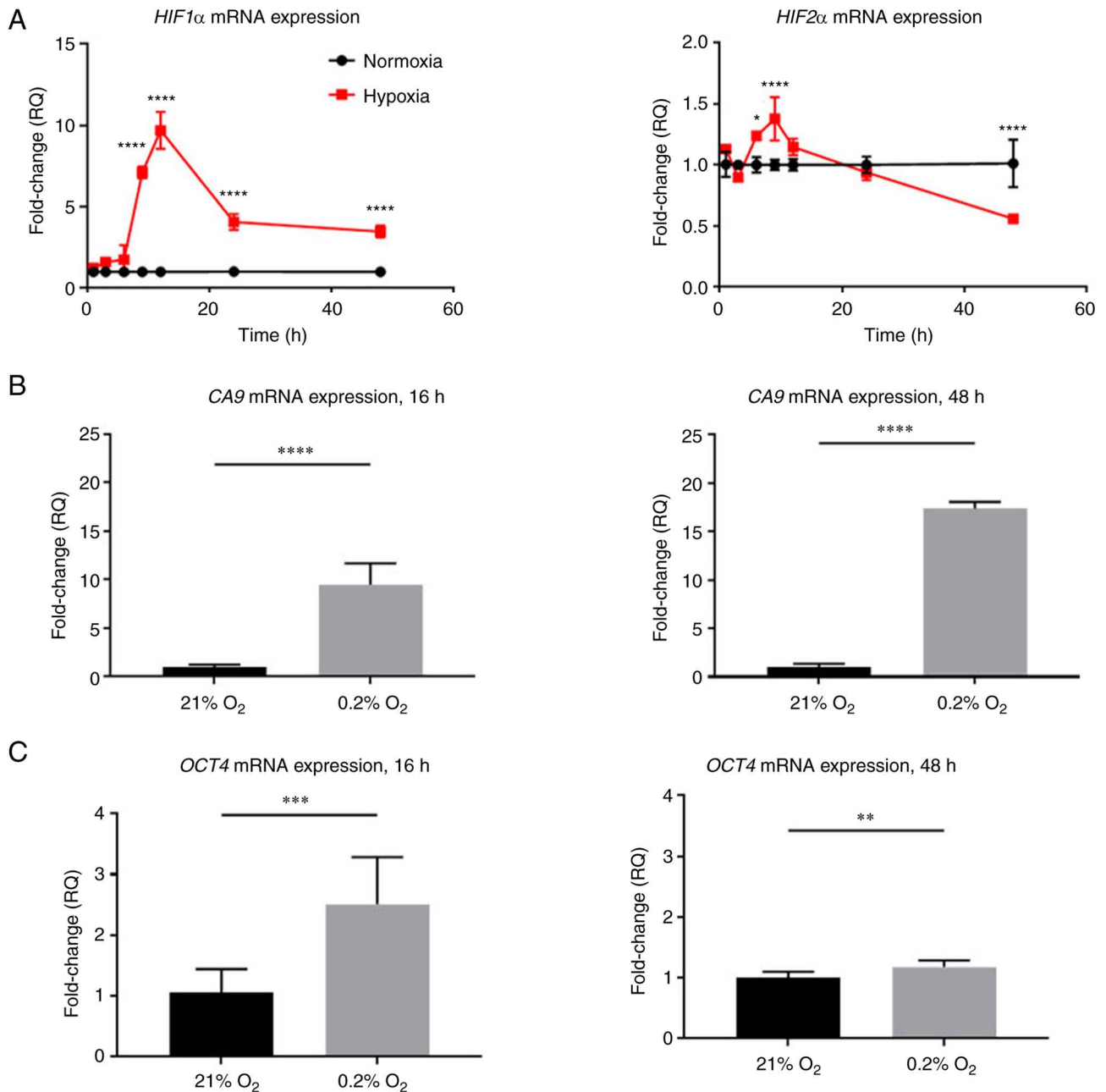


Figure 3. HIF1 α and HIF2 α mRNA levels and HIF target genes change over time in hypoxia in PANC-1 cells. PANC-1 cells were treated with hypoxia (0.2% oxygen) or normoxia (21% oxygen) for 16 or 48 h. (A-C) RT-qPCR was conducted to determine (A) HIF1 α , HIF2 α , (B) CA9 and (C) Oct4 mRNA levels. Fold changes for RT-qPCR are shown relative to normoxic cells. Error bars represent the mean \pm SD for n=9. The fold changes were analyzed using unpaired Student's t-test. *P<0.05, **P<0.01, ***P<0.001 and ****P<0.0001. HIF, hypoxia-inducible factor; RT-qPCR, reverse transcription-quantitative PCR; CA9, carbonic anhydrase IX; Oct4, octamer-binding transcription factor 4.

time. As compared with normoxic control cells, HIF1 α mRNA expression significantly increased up to 9.7 \pm 1.1-fold after 12 h of hypoxic exposure, decreased at 24 h compared with the 12-h timepoint and then ultimately plateaued at a level 3.5 \pm 0.4-fold change higher than cells in normoxia (P<0.0001). HIF2 α mRNA expression levels, while lower than HIF1 α expression levels, increased significantly up to 1.4 \pm 0.1-fold higher than normoxic levels at 9 h (P<0.0001), and then decreased to 0.6 \pm 0.02-fold lower than normoxic levels (P<0.0001; Fig. 3A).

HIFs are subject to post-translational regulation and degradation in normoxia, whereas in hypoxia, HIF proteins are stabilized and accumulate. While HIF mRNA levels may

vary over time, known HIF α target genes were also analyzed to assess HIF activities in acute and chronic hypoxia. To confirm HIF1 α activity, the mRNA levels of HIF1 α -specific transcriptional target gene CA9 were measured (Fig. 3B) (33). CA9 expression increased 9.5 \pm 2.2-fold after 16 h of hypoxia (P<0.0001) and continued to increase up to 17.8 \pm 0.7-fold after 48 h of hypoxia compared with normoxic controls (P<0.0001). To measure HIF2 α activity, the mRNA levels of HIF2 α transcriptional target gene Oct were analyzed (Fig. 3C) (19). Oct4 expression increased 2.5 \pm 0.8-fold after 16 h of hypoxia (P<0.001), and in chronic hypoxia, Oct4 mRNA levels decreased close to normoxic levels with only a 1.2 \pm 0.1-fold

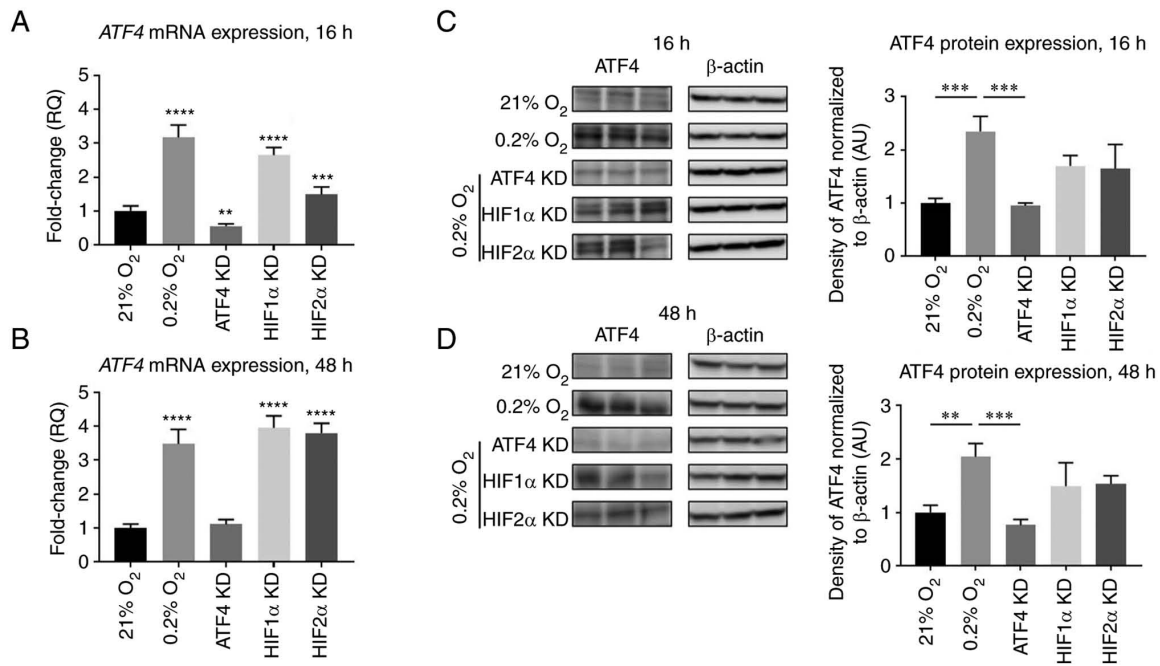


Figure 4. ATF4 mRNA and protein expression do not change upon HIF1 α or HIF2 α KD in chronic hypoxia in PANC-1 cells. PANC-1 cells were treated with RNAiMAX Lipofectamine reagent and small interfering RNA to transiently KD ATF4, HIF1 α or HIF2 α . The cells were placed in hypoxia (0.2% oxygen) or normoxia (21% oxygen) for 16 or 48 h. All cells with KD were placed in hypoxia. (A and B) RT-qPCR was conducted to determine ATF4 mRNA levels after (A) 16 and (B) 48 h of hypoxia. Fold changes for RT-qPCR are shown relative to normoxic cells. Error bars represent mean \pm SD for n=9. The fold changes were analyzed by one-way ANOVA followed by Bonferroni statistical hypothesis test. (C and D) Western blot analysis was conducted to determine the protein expression levels of ATF4 after (C) 16 or (D) 48 h of hypoxia. Fold changes are shown relative to normoxic cells. Error bars represent the mean \pm SD for n=3. Fold changes were analyzed by one-way ANOVA followed by Bonferroni statistical hypothesis test. **P<0.01, ***P<0.001 and ****P<0.0001. ATF4, activating transcription factor 4; HIF, hypoxia-inducible factor; KD, knockdown; RT-qPCR, reverse transcription-quantitative PCR.

decrease compared with normoxic levels (P<0.01). The increase in CA9 and Oct4 confirmed that HIF1 α and HIF2 α are active in acute and chronic hypoxia.

HIF1 α and HIF2 α knockdowns do not affect ATF4 mRNA or protein expression in chronic hypoxia. HIF transcriptional and translational functions make it possible that HIF1 α or HIF2 α affect ATF4 expression to mediate the UPR pathway in response to hypoxia. To understand the effects that HIF1 α and HIF2 α have on ATF4 in acute and chronic hypoxia in PDAC cells, siRNA was used to knockdown ATF4, HIF1 α or HIF2 α for loss-of-function experiments (Fig. S1). After 24 h of incubation in normal cell culture conditions, the cells were either placed in hypoxia or maintained in normoxia for 16 or 48 h. mRNA and protein expression levels of ATF4, HIF1 α and HIF2 α were determined. In acute hypoxia, knockdown of either HIF1 α or HIF2 α caused significant decreases in ATF4 mRNA levels compared with the hypoxia control (P<0.0001 and P<0.001, respectively; Fig. 4A). In chronic hypoxia, ATF4 mRNA expression significantly increased after HIF1 α knockdown compared with the hypoxia (0.2% O₂) control group (P<0.05) and was not affected after HIF2 α knockdown compared with the hypoxia (0.2% O₂) control group (Fig. 4B).

The protein expression levels of ATF4 were also determined after ATF4, HIF1 α or HIF2 α knockdowns. In normoxia, ATF4 protein expression levels were minimal, and with hypoxic exposure, ATF4 protein levels increased by 2.3 \pm 0.3-fold after 16 h (P<0.001) and 2.0 \pm 0.2-fold after 48 h compared with normoxic levels, respectively (P<0.05; Fig. 4C and D). ATF4 protein levels significantly decreased after ATF4 knockdown

(P<0.001; Fig. 4C and D). HIF1 α knockdown did not affect ATF4 protein expression levels in either acute (P>0.05) or chronic hypoxia (P>0.05). HIF2 α knockdown had no effect on ATF4 protein expression in either acute (P>0.05) or chronic hypoxia (P>0.05).

ATF4 knockdown decreases HIF1 α and increases HIF2 α expression in chronic, but not acute, hypoxia. ATF4 is a master regulator within the UPR pathway when cells are under stress, such as in hypoxia. To understand the effects that ATF4 have on HIF1 α and HIF2 α in acute and chronic hypoxia in PDAC cells, ATF4, HIF1 α or HIF2 α were knocked down using siRNA, incubated in normoxia for 24 h and then placed in either hypoxia or maintained in normoxia for 16 or 48 h. HIF1 α and HIF2 α mRNA and protein levels were then determined.

Under acute hypoxia, HIF1 α mRNA expression was 0.4 \pm 0.1-fold lower in the hypoxic vehicle-treated cells with no siRNA compared with normoxic cells (P<0.0001; Fig. 5A). There was no significant difference in HIF1 α mRNA expression between the hypoxic vehicle-treated cells and the ATF4 knockdown cells (P>0.05; Fig. 5A). HIF1 α protein expression in hypoxia alone was 2.2 \pm 0.3-fold higher than in normoxia, indicating that HIF protein levels do not appear to be solely determined by the corresponding hypoxic mRNA levels (P<0.001; Fig. 5D). Upon ATF4 knockdown in acute hypoxia, HIF1 α protein expression was 1.7 \pm 0.1-fold higher compared with the normoxia-treated cells (P<0.05) but not significantly different compared with the hypoxia treatment cells (P>0.05; Fig. 5D). HIF1 α

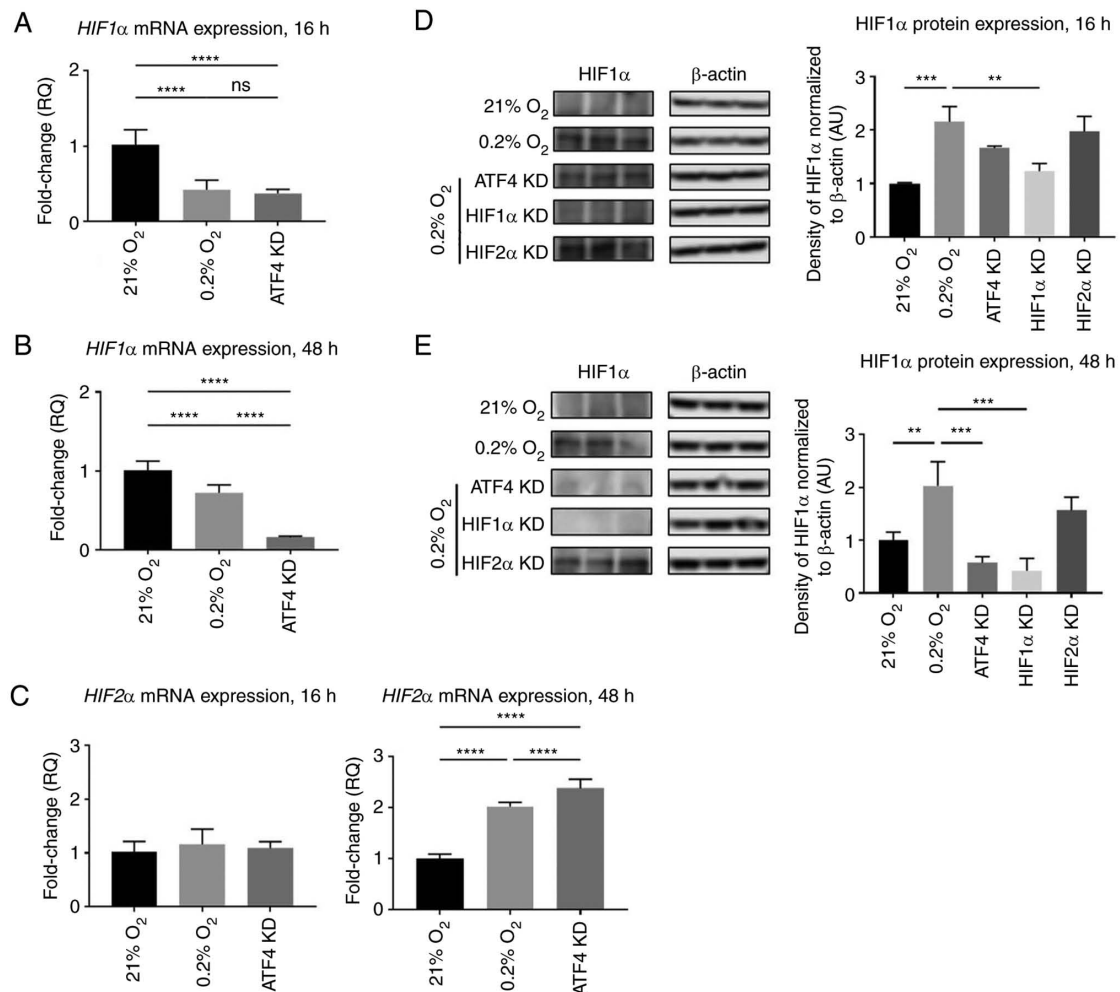


Figure 5. ATF4 KD affects HIF1 α and HIF2 α expression in acute and chronic hypoxia. PANC-1 cells were treated with RNAiMAX Lipofectamine reagent and small interfering RNA to transiently KD ATF4, HIF1 α or HIF2 α . The cells were placed in hypoxia (0.2% oxygen) or normoxia (21% oxygen) for 16 or 48 h. All cells with KD were placed in hypoxia. (A and B) RT-qPCR was conducted to determine HIF1 α after (A) 16 and (B) 48 h of hypoxia. (C) HIF2 α mRNA levels were measured using RT-qPCR after ATF4 KD in acute or chronic hypoxia. (D and E) Western blot analysis was conducted to determine the protein expression levels of HIF1 α after (D) 16 or (E) 48 h of hypoxia. Fold changes are shown relative to normoxic cells. Error bars represent the mean \pm SD for n=3. Fold changes were analyzed by one-way ANOVA followed by Bonferroni statistical hypothesis test. Fold changes for RT-qPCR are shown relative to normoxic cells. Error bars represent the mean \pm SD for n=9. Fold changes were analyzed by one-way ANOVA followed by Bonferroni statistical hypothesis test. **P<0.01, ***P<0.001 and ****P<0.0001. ATF4, activating transcription factor 4; HIF, hypoxia-inducible factor; KD, knockdown; RT-qPCR, reverse transcription-quantitative PCR.

knockdown in hypoxia significantly reduced HIF1 α protein expression compared with the hypoxia treatment group with no knockdown (P<0.01). HIF2 α knockdown did not affect HIF1 α protein expression compared with hypoxia treatment alone (P>0.05; Fig. 5D). ATF4 knockdown had no effect on HIF2 α mRNA expression in acute hypoxia with only a 1.1 \pm 0.1-fold increase compared with normoxia treated cells (P>0.05). However, in chronic hypoxia, HIF2 α mRNA expression levels increased by 2.4 \pm 0.2-fold compared with normoxia-treated cells (P<0.0001; Fig. 5C).

Furthermore, in chronic hypoxia, ATF4 knockdown significantly decreased HIF1 α mRNA levels to 0.15 \pm 0.02-fold of the normoxia-treated and hypoxia-treated cells (P<0.05; Fig. 5B). This expression pattern was also identified in protein expression where ATF4 knockdown resulted in a 0.4 \pm 0.2-fold decrease in HIF1 α protein expression levels (P<0.0001; Fig. 5E). While ATF4 did not affect HIF1 α or HIF2 α during acute hypoxia, it did affect HIF1 α and HIF2 α mRNA and protein expression levels during chronic hypoxia.

ATF4 knockdown increases cell migration in chronic but not acute hypoxia. To determine the effect of ATF4 inhibition on cell migration in acute and chronic hypoxia, a scratch assay was performed. PANC-1 cells transiently transfected with ATF4, HIF1 α or HIF2 α siRNA were seeded and scratched prior to 16 or 48 h of hypoxic exposure. After 16 h of hypoxic exposure, the negative control group, PANC-1 cells with no knockdown siRNA, exhibited the greatest gap closure among the treatment groups with 63.4 \pm 6.8% gap closure (Fig. 6A). In normoxia, there were no changes in gap closure rates among the treatment groups, with all treatments after 16 h of normoxic incubation resulted in at least 45% closure (Fig. 6C) and at least 80% closure after 48 h of incubation (Fig. 6D). Relative to the negative control group, hypoxia-treated cells without siRNA knockdowns, the ATF4 knockdown cells in acute hypoxia closed 43.7 \pm 5.7% of the gap measured at the initial scratch (P<0.05). The HIF1 α and HIF2 α knockdown cells also exhibited a significant decline in cell migration with 28.60 \pm 9.95% (P<0.001) and 39.8 \pm 13.0% (P<0.01) gap closure,

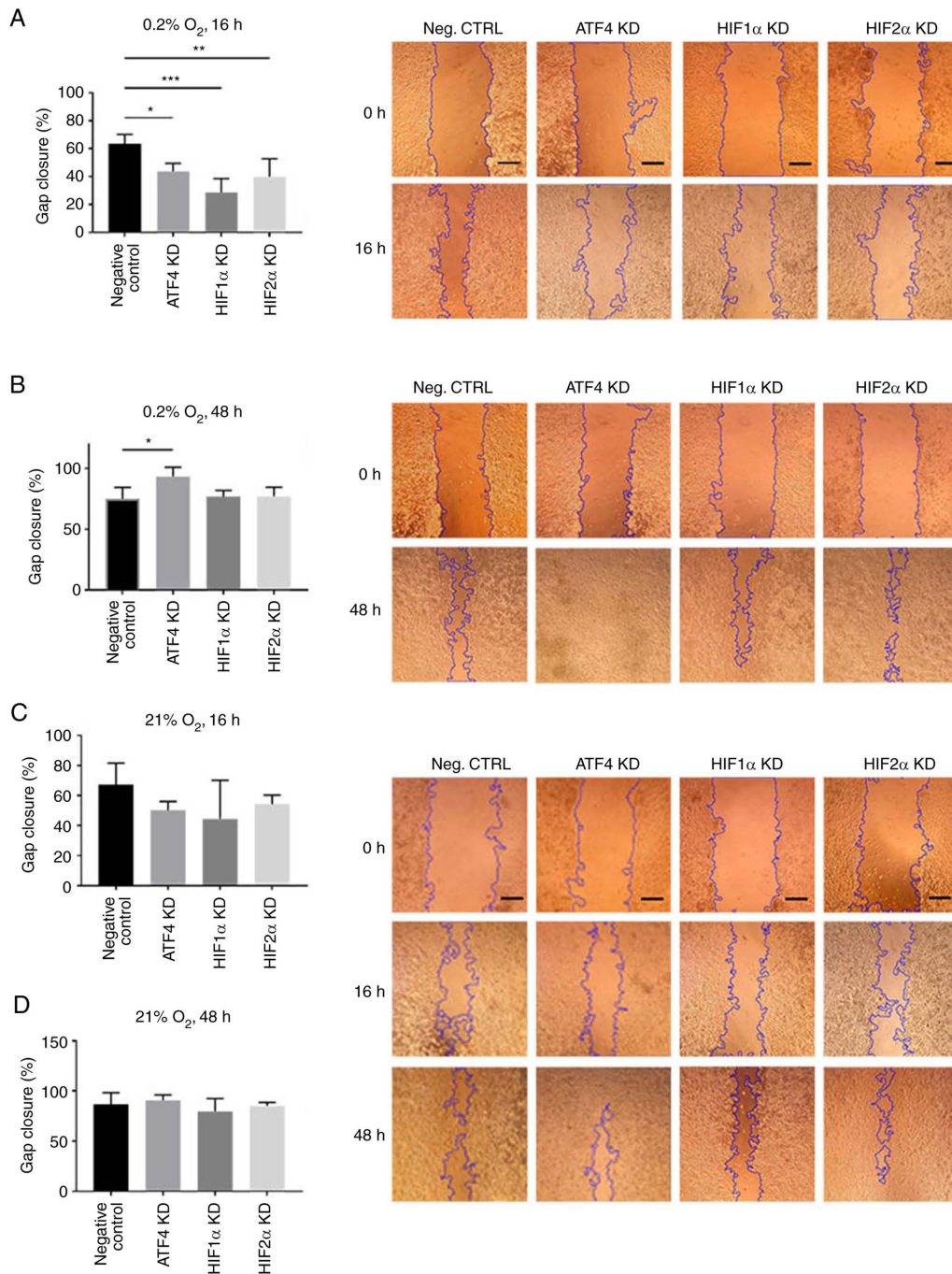


Figure 6. ATF4, HIF1 α and HIF2 α KD decrease cell migration in acute hypoxia, but ATF4 KD in chronic hypoxia increases cell migration. (A-D) PANC-1 cells were transiently transfected, scratched and incubated in hypoxia for (A) 16 or (B) 48 h or in normoxia for (C) 16 or (D) 48 h. The negative control were PANC-1 cells exposed to 0.2% oxygen with transfection reagent but no KD small interfering RNA. Images were captured by light microscope at x50 magnification. Scale bar=500 μ m. The gap closure was determined by using the ImageJ plugin tool to measure the gap area of each image and the gap closure of each treatment group was calculated after hypoxic incubation relative to images captured before hypoxic exposure. Error bars represent the mean \pm SD for n=4. The fold changes were analyzed by two-way ANOVA followed by Bonferroni post hoc statistical hypothesis test. *P<0.05, **P<0.001 and ***P<0.0005. ATF4, activating transcription factor 4; HIF, hypoxia-inducible factor; KD, knockdown.

respectively (Fig. 6A). In chronic hypoxia, the results demonstrated that HIF1 α and HIF2 α knockdown cell migration rates were comparable with those of the hypoxia-treated cells without siRNA knockdowns. The negative control cells closed $75.1 \pm 9.4\%$ of the gap after 48 h of hypoxia (Fig. 6B). HIF1 α knockdown cells showed $76.7 \pm 5.2\%$ gap closure, and HIF2 α knockdown cells exhibited $77.0 \pm 7.6\%$ gap closure. The ATF4 knockdown cells, on the other hand, showed significantly more cell migration with $93.4 \pm 7.6\%$ gap closure (P<0.05) compared

with the negative control group and HIF1 α or HIF2 α knockdown cells (Fig. 6B). The scratch assay revealed that ATF4 knockdown inhibits cell migration in acute hypoxia.

ATF4 inhibition decreases cell viability and colony formation. Considering that ATF4 knockdown promoted cell migration in chronic hypoxia, the effects of ATF4 knockdown on cell viability and clonogenicity were next determined by conducting cell viability assays and colony formation assays.

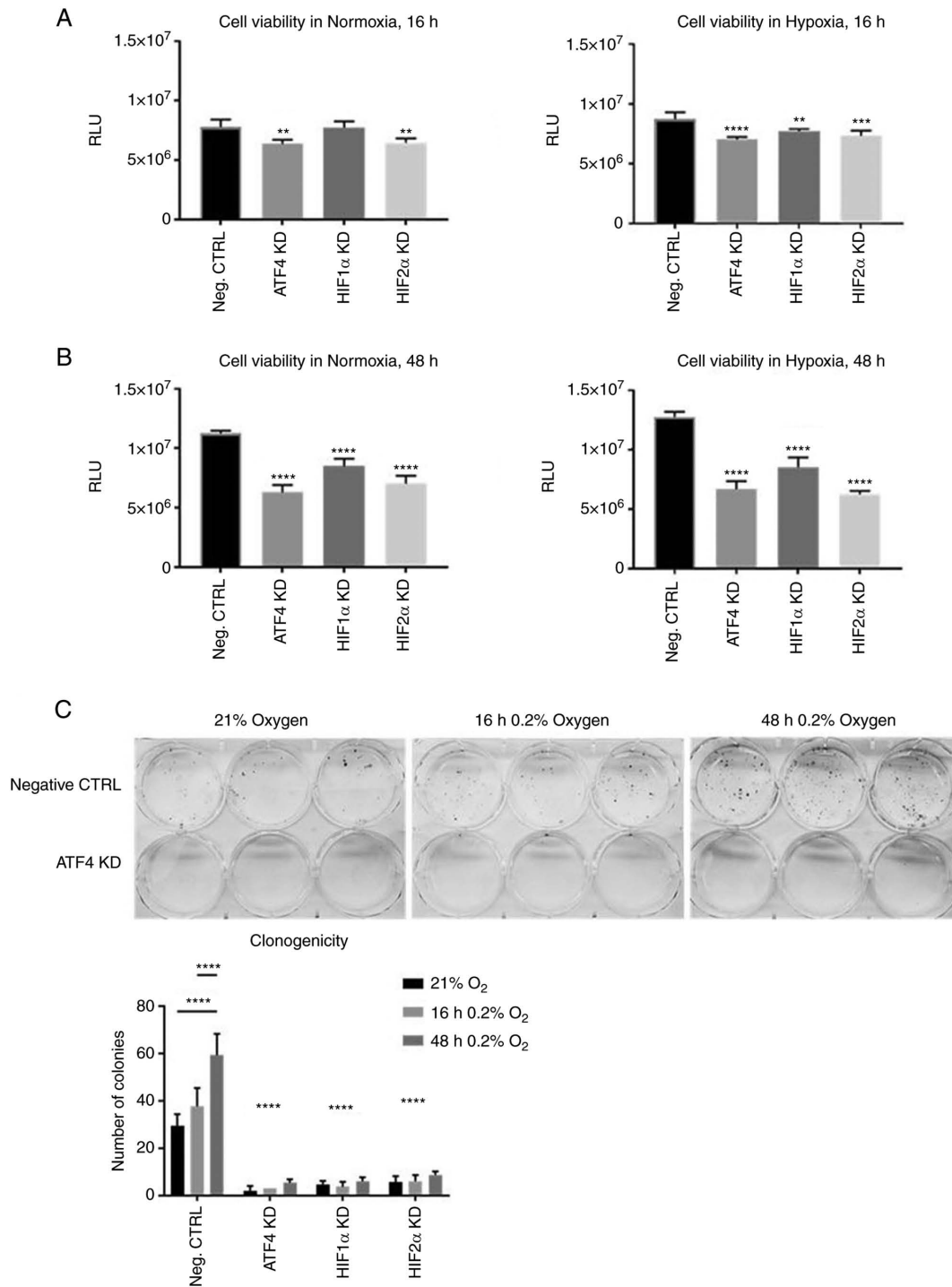


Figure 7. ATF4, HIF1 α and HIF2 α KD decrease cell viability in chronic hypoxia. (A and B) PANC-1 cells were transiently transfected and seeded into 96-well plates and incubated in hypoxia or normoxia for (A) 16 or (B) 48 h. Cell viability was measured by CellTiter Glo luminescence. The negative control were PANC-1 cells exposed to 0.2% oxygen with transfection reagent but no KD small interfering RNA. Error bars represent the mean \pm SD for n=5. The relative luminescence unit (RLU) were analyzed by one-way ANOVA followed by Bonferroni statistical hypothesis test. (C) Colony formation assays were performed by transiently transfecting PANC-1 cells and seeded into plates. The plates were placed in either normoxia or hypoxia for 16 or 48 h. The cells were fixed, stained and colonies were counted. Error bars represent the mean \pm SD for n=3. **P<0.01, ***P<0.001 and ****P<0.0001. ATF4, activating transcription factor 4; HIF, hypoxia-inducible factor; KD, knockdown.

PANC-1 cells were transiently transfected and seeded into 96-well plates. The cells were incubated in normoxia for 24 h, and then placed in either normoxia or hypoxia for 16 or 48 h. A CellTiterGlo Luminescent assay was used to measure ATP. After ATF4 knockdown, cells in normoxia and hypoxia for 16 and 48 h all showed significant decrease

in cell viability compared with the PANC-1 cells with no knockdowns (P<0.05; Fig. 7A and B). HIF1 α knockdown in normoxia did not affect cell viability but did in cells in acute hypoxia (Fig. 7A). HIF1 α and HIF2 α knockdowns significantly decreased cell viability (P<0.05) in both normoxia and chronic hypoxia (Fig. 7B) and there were no significant

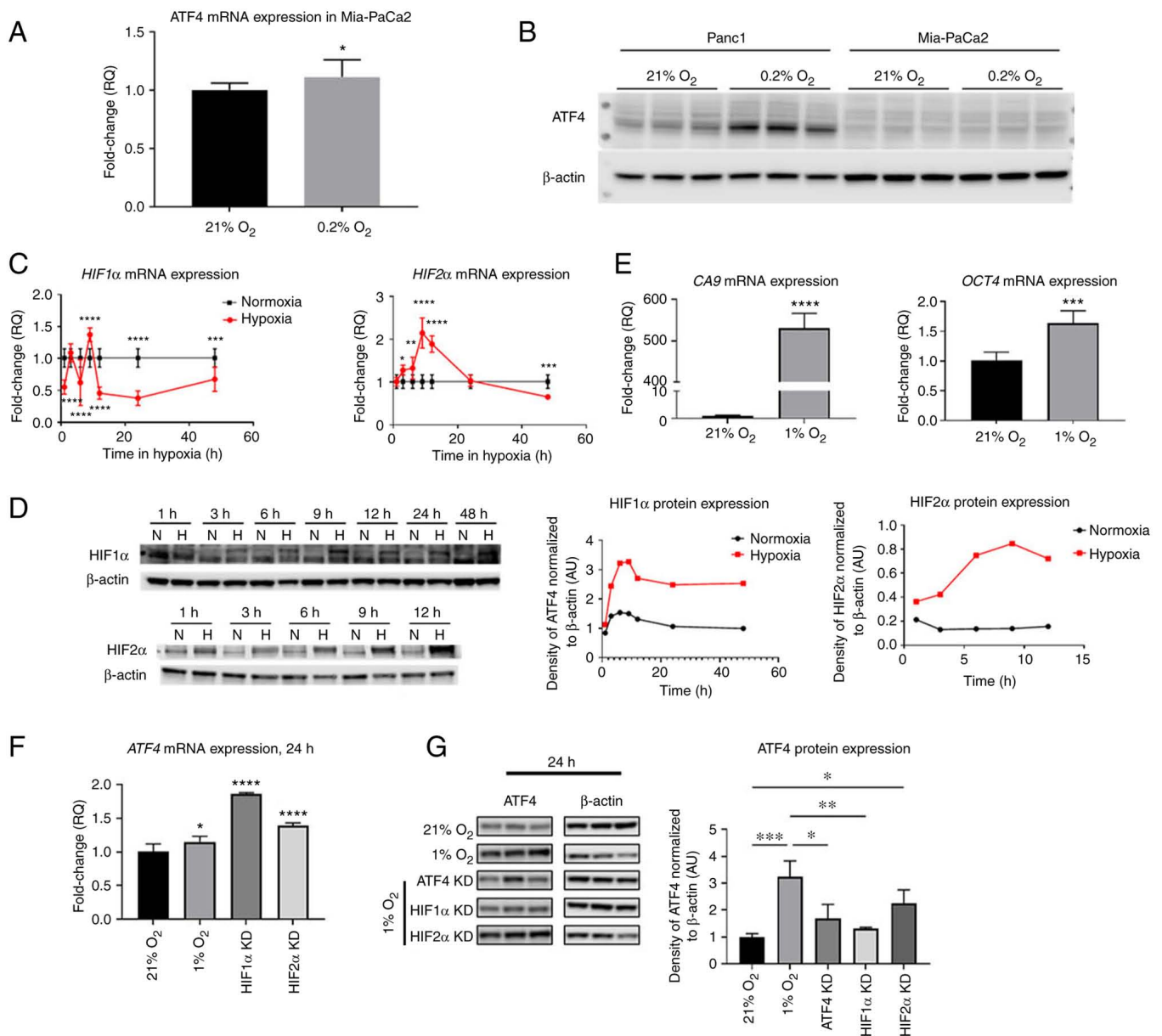


Figure 8. HIF1 α , HIF2 α and ATF4 mRNA levels and HIF target genes change over time in hypoxia in WS1 healthy cells but ATF4 was not detectable in hypoxic Mia-PaCa2 cells. PANC-1 and Mia-PaCa2 cells were placed in either normoxia (21% oxygen) or hypoxia (0.2% oxygen) for 24 h. (A) ATF4 mRNA levels in Mia-PaCa2 cells were determined by RT-qPCR, and (B) ATF4 protein levels were determined in Mia-PaCa2 cells using western blot analysis. WS1 fibroblast cells were treated with hypoxia (1% oxygen) or normoxia (21% oxygen) for durations from 1 to 48 h. (C) RT-qPCR and (D) western blot analyses were conducted to determine HIF1 α and HIF2 α mRNA and protein levels, respectively. (E) CA9 and Oct4 mRNA levels were also determined by RT-qPCR. (F and G) ATF4 mRNA and protein levels were also measured using (F) RT-qPCR and (G) western blot analysis, respectively. Fold changes for RT-qPCR are shown relative to normoxic cells. Error bars represent the mean \pm SD for n=9. The fold changes were analyzed using unpaired Student's t-test. *P<0.05, **P<0.01, ***P<0.001 and ****P<0.0001. HIF, hypoxia-inducible factor; ATF4, activating transcription factor 4; RT-qPCR, reverse transcription-quantitative PCR; CA9, carbonic anhydrase IX; Oct4, octamer-binding transcription factor 4; KD, knockdown.

differences in cell viability between normoxic and chronically hypoxic cells in each treatment group (Fig. S2). The colony formation assays showed significantly less colonies after ATF4 knockdown (P<0.0001; Fig. 7C), as well as after HIF1 α and HIF2 α knockdown (P<0.0001; Figs. 7C and S3). The negative control group cells that were exposed to chronic hypoxia had significantly more colonies compared with the acute hypoxia cells and normoxic cells (P<0.05; Fig. 7C). Overall, ATF4 inhibition significantly decreases cell viability and colony formation abilities in PANC-1 cells in both normoxia and chronic hypoxia (26).

Transforming growth factor beta (TGF- β) has been shown to be prevalent in tumors and is secreted by cancer-associated fibroblasts to supplement cancer cells and promote growth. One proposed pathway that TGF- β promotes cancer survival and growth is by inducing ATF4 expression and activity (26). However, the effect that TGF- β may have on PDAC cells in hypoxia has not yet been studied. To understand if TGF- β has the same function in hypoxic PDAC, PANC-1 wells were treated with TGF- β in acute and chronic hypoxia. Western blot analysis revealed that TGF- β did not induce ATF4 expression in normoxic cells (Fig. S4A). ATF4 was upregulated in 16 h

of hypoxic exposure, but there was little ATF4 expression detected in cells exposed to chronic hypoxia, despite TGF- β treatment (Fig. S4A). The colony formation assay used to assess clonogenicity of the cells revealed that TGF- β did improve clonogenicity in cells exposed to acute hypoxia, but not chronic hypoxia, compared with normoxic cells (Fig. S4B). There were minimal colonies formed in cells with ATF4 knockdown.

HIF1 α , HIF2 α and ATF4 expression increases in healthy WS1 skin fibroblast cells but not in Mia-PaCa2 PDAC cell line. Next, it was investigated how ATF4 and HIFs regulation in PANC-1 PDAC cells compares to another PDAC, MiaPaCa2 and to non-cancerous WS1 fibroblasts. To determine whether ATF4 is expressed in Mia-PaCa2 cells in 21 and 0.2% oxygen conditions, cells were incubated in normoxia and hypoxia for 24 h. ATF4 mRNA levels in Mia-PaCa2 cells increased in hypoxia by 1.1 \pm 0.1-fold ($P<0.1$) and protein levels in Mia-PaCa2 were not detectable in either normoxia or hypoxia (Fig. 8A and B). In PANC-1 cells, however, ATF4 protein expression increased in hypoxia compared with normoxia (Fig. 8B).

To determine HIF1 α , HIF2 α and ATF4 expression in hypoxia in healthy cells, WS1 skin fibroblast cells were incubated in normoxia (21% oxygen) or hypoxia (1% oxygen) from 1 to 48 h of treatment. After 1 h of hypoxic exposure, HIF1 α mRNA significantly decreased 0.6 \pm 0.1-fold ($P<0.0001$). At 3 h, there was no significant difference of HIF1 α mRNA levels in hypoxia compared with normoxia (1.1 \pm 0.1-fold). After 6 h of hypoxic exposure, mRNA levels decreased 0.6 \pm 0.4-fold, and they significantly increased after 9 h of exposure by 1.4 \pm 0.1-fold ($P<0.0001$). The HIF1 α mRNA levels became significantly lower than normoxic levels from 12 h of hypoxia by 0.5 \pm 0.1-fold ($P<0.0001$) to 48 h of hypoxia (0.7 \pm 0.2-fold) ($P<0.001$; Fig. 8C). HIF2 α mRNA expression steadily increased from 3 h of hypoxia and reached a peak at 9 h of hypoxia by 2.1 \pm 0.4-fold. The mRNA levels decreased continually from 12 h of exposure to 48 h, at which point, the mRNA levels were 0.7 \pm 0.1-fold lower than normoxic levels (Fig. 8C). It was confirmed that HIF1 α and HIF2 α protein levels were increased in hypoxia by western blotting, which demonstrated steady increases of HIF protein expression in all time points compared with normoxic levels (Fig. 8D).

The expression of HIF transcriptional target genes CA9 and Oct4 was also measured. CA9 is a HIF1 α -specific target gene while Oct4 is a HIF2 α -specific target gene. After 24 h of hypoxic exposure, results showed that CA9 mRNA levels significantly increased by 530.6 \pm 33.7-fold compared with normoxia while Oct4 mRNA levels were significantly higher in hypoxia by 1.6 \pm 0.2-fold compared with normoxic levels (Fig. 8E). ATF4 expression was also analyzed in normoxia, hypoxia and with HIF1 α and HIF2 α knocked down. ATF4 mRNA levels were significantly higher after 24 h in hypoxia than in normoxia by 1.1 \pm 0.08-fold ($P<0.05$; Fig. 8F). Upon HIF1 α and HIF2 α knockdowns, ATF4 mRNA expression significantly increased by 1.9 \pm 0.01 and 1.4 \pm 0.04-fold, respectively (Fig. 8F). Western blot analysis revealed that hypoxia significantly increased ATF4 protein expression in healthy cells by 3.2 \pm 0.6-fold compared with normoxia. While ATF4 mRNA increased upon HIF1 α and HIF2 α knockdown, ATF4 protein expression significantly decreased upon HIF1 α

knockdown in hypoxia (1.3 \pm 0.05-fold) compared with cells in hypoxia without knockdowns ($P<0.01$; Fig. 8G). There was no significant difference in cells with HIF2 α knockdown compared with hypoxia-treated cells without knockdowns (Fig. 8G).

Discussion

Pancreatic cancer cells exist in a hypoxic environment, and yet, most research conducted is performed under normoxic conditions or in acute hypoxia, which is less pathophysiologically relevant. Despite the chronic hypoxic condition observed in PDAC in patients, the function of ATF4 during chronic hypoxia has remained largely unstudied in pancreatic cancers. In the present study, evidence was provided that ATF4 regulates HIF1 α and HIF2 α expression and significantly affects cell migration, cell viability and colony formation in chronically hypoxic pancreatic cancer cells.

Loss-of-function experiments were conducted to ascertain the relationship between ATF4 and HIFs in PANC-1 cells in chronic hypoxia. HIF1 α and HIF2 α are regulators of cellular hypoxic response (17). The two isoforms have unique roles in mediating cell survival in hypoxia (32). Generally, HIF1 α activates genes that promote changes to cell metabolism while HIF2 α activates genes that promote processes such as stemness and extracellular signaling and remodeling (31,34,35). HIF1 α and HIF2 α are also regulated in a type of 'switch' in which when HIF1 α expression decreases or increases, HIF2 α expression tends to change inversely. This 'HIF switch' is critical to regulate the different response pathways as the time in hypoxia increases (32). The switch is largely attributed to microRNAs that control HIF1 α and HIF2 α levels by preventing HIF mRNA translation, reducing the amount of protein synthesized (32). Hypoxia-associated factors (HAFs) also downregulate HIF1 α by inducing ubiquitination and protein degradation while upregulating HIF2 α activation, including in a variety of different cancers such as pancreatic cancer and renal clear cell carcinoma (31,36). The present data revealed, for the first time to the best of our knowledge, that ATF4 functions as a HIF regulator by decreasing HIF2 α and increasing HIF1 α expression in chronic hypoxia. ATF4 regulates the HIFs inversely to microRNAs and HAFs that increase HIF2 α and decrease HIF1 α in chronic hypoxia (31,37,38). This may possibly prevent a complete shutdown of HIF1 α specific functions by counteracting the negative regulation of HIF1 α by microRNAs and HAFs. While HIF2 α is commonly associated with chronic hypoxia and HIF1 α with acute hypoxia, it was demonstrated that HIF1 α expression remains essential in chronic hypoxia for maintaining certain cell survival pathways, such as metabolic reprogramming (31,35,39). ATF4 may function to promote HIF1 α expression to maintain critical these pathways that are activated by only HIF1 α and prevent a complete inhibition of HIF1 α functions and improve hypoxic tolerance in chronic hypoxia. Whether ATF4 affects HIFs in chronic hypoxia directly or indirectly remains to be understood. Further studies are planned to analyze downstream ATF4 transcriptional target genes to determine potential mechanisms used to affect HIF expression.

Our data also showed that ATF4 promotes colony formation and cell viability in chronically hypoxic PDAC cells (26,40).

In chronic hypoxia, ATF4 knockdown significantly decreased colony formation and cell viability. Hence, ATF4 acts as a pro-survival factor for PDAC cells under conditions of chronic hypoxia and may be a viable target for PDAC therapies. ATF4 has been previously shown to promote colony formation and cell viability by regulating the Wnt/ β -catenin pathway and the PERK/eIF2 α /ATF4 axis in different cancers, including lung cancer and glioblastoma cells (41,42). It is plausible that ATF4 affects colony formation and viability of PDAC cells in chronic hypoxia the same way it does in normoxia. However, these functions of ATF4 may be more robust in chronic hypoxia compared with normoxia. Targeting ATF4 may be a therapeutic strategy for treating hypoxic PDAC which is otherwise difficult to treat.

ATF4 roles in cell migration in hypoxic PDAC cells were also determined. ATF4 stimulates cell migration and metastasis in breast cancer cells, as well as in PDAC (26,40). However, it was demonstrated that under chronic hypoxia, ATF4 inhibition increased cell migration in PDAC cells, evidence that rather than promoting cell migration as observed in normoxia, ATF4 negatively regulates cell migration in chronic hypoxia. While it has been previously revealed in breast cancer that ATF4 stimulates cell migration, these studies were conducted under normoxic conditions or in oxygen levels that are likely markedly higher than in patients (43,44). Previous studies showed that breast cancers typically exhibit oxygen levels of 0.2% oxygen or less, similar levels to that of PDAC (29,40,45,46). Furthermore, these experiments did not exceed 24 h of hypoxic exposure. There may also be differences in the physiology of the two cancers that can account for this discrepancy, which remain to be explored. Another study showed that ATF4 promotes cell migration in PDAC (26). However, this was not conducted in hypoxia, acute or chronic, and our data indicated that ATF4 functions differently in hypoxia than in normoxia. This suggested that ATF4 functions change or are different depending on the presence or absence of oxygen and the duration of hypoxia. The present results not only indicated a negative regulatory role of ATF4 on cell migration, but they also emphasized the importance of conducting pancreatic cancer research in pathophysiologically relevant conditions, particularly chronic hypoxia. The discovery of the 'HIF switch', particularly the dominance of HIF1 α in acute hypoxia and HIF2 α in chronic hypoxia, has illuminated the importance of distinguishing acute and chronic hypoxia in cancers (31,32,37,47). However, the same principles should be applied when investigating other stress response pathways in hypoxia, including ATF4 in the UPR pathway. As it was revealed, the role of ATF4 in acute and chronic hypoxia is not the same, indicative of a mechanism that facilitates this switch in function that is yet to be found.

It is unknown whether ATF4 directly interacts with HIF1 α and HIF2 α mRNA or protein to regulate expression or indirectly by way of another downstream factor that functions as a mediator between ATF4 and HIFs. It was demonstrated that HIF1 α , HIF2 α and ATF4 are all expressed at the protein level in hypoxia in healthy cells and in PANC-1 cells. Somewhat surprisingly, our data showed that hypoxia does not induce ATF4 expression in Mia-PaCa2 cells compared with PANC-1 cells which exhibit a robust increase in ATF4 protein expression. This can be due to several reasons, including the different

phenotypes exhibited by Mia-PaCa2 cells and PANC-1 cells. For instance, low expression of neural cell adhesion molecule CD56, a protein found in PANC-1 cells but not Mia-PaCa2 cells, has been revealed to result in low levels of ER stress markers, including ATF4 (48,49). The lack of CD56 in Mia-PaCa2 cells may contribute to the low levels of ATF4, despite hypoxic exposure. Another protein that may contribute to the low ATF4 levels in Mia-PaCa2 but high induction in PANC-1 cells is E-cadherin, a cell-to-cell adhesion molecule. High E-cadherin levels, as observed in Mia-PaCa2 cells but not in PANC-1 cells, are associated with low expression of ATF4 (43). These types of differing expression profiles observed in the two cell lines may contribute to the low ATF4 expression identified in Mia-PaCa2 cells and high levels in PANC-1 cells. Notably, PANC-1 have been described as more aggressive and with greater metastasizing potential (48). The ATF4 related mechanism, found only in the PANC-1 cells and which is notably quite different than healthy WS-1 cells, is a candidate for mediating such a phenotype and therefore represents a potential biomarker for metastatic potential, though further studies are required to explore this hypothesis.

One major finding from the present study was that in chronically hypoxic PANC-1 cells, ATF4 was revealed to affect HIF1 α expression while HIFs did not affect ATF4. This, however, was not observed in healthy cells. As observed in PANC-1 cells, ATF4 mRNA expression significantly increased when HIF1 α was knocked down. However, in healthy cells, HIF1 α knockdown caused ATF4 protein expression levels to be significantly lower than the hypoxic cells expressing HIF1 α . This result is in contrast to what was observed in PANC-1 cells, in which HIF1 α knockdown did not change ATF4. The present findings indicated that in healthy cells, HIF1 α may regulate ATF4 in hypoxia, while in PDAC cells, this regulation pathway is either inhibited or is negated by other mechanisms that cause ATF4 overexpression (14,26,50). One such mechanism is the protein CD56 found in PANC-1 cells that causes high ATF4 expression (48,49). Since HIF1 α has never been shown to affect ATF4 in hypoxia in healthy cells, the mechanism in which HIF1 α may affect ATF4 protein expression requires further study. It will also be important to further validate these findings with other methods in *in vitro* PDAC models, such as the use of 3D spheroids, and in *in vivo* models which replicate in an improved way the heterogeneity of the tumor microenvironment observed in patients. This shall be the focus of our future efforts.

In conclusion, it was demonstrated that ATF4 is upregulated in chronically hypoxic PDAC cells and that HIF1 α expression is dependent on ATF4 while ATF4 negatively regulates HIF2 α in PDAC cells in chronic hypoxia. It was also demonstrated that while ATF4 may decrease cell migration in chronic hypoxia, its inhibition is detrimental to clonogenicity and cell viability in hypoxia, and therefore, is an attractive target for chronically hypoxic PDAC. The present study has emphasized the importance of conducting research in pathophysiologically relevant systems related to hypoxia. These findings elucidated a novel understanding of PDAC in which hypoxia causes the UPR pathway to regulate the hypoxia response pathways, possibly contributing to the aggressive qualities of PDAC.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

NTC and SPB conceptualized the idea and designed experiments. NTC conducted experiments, procured and analyzed data and led manuscript writing and organization. ZM and CHC provided feedback on data interpretation and experimental design. SW was involved in planning and executing experiments. NTC and SPB confirm the authenticity of all raw data. All authors contributed to the analysis of results and writing of the manuscript. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

- Siegel RL, Miller KD and Jemal A: Cancer statistics, 2018. *CA Cancer J Clin* 68: 7-30, 2018.
- Hessmann E, Buchholz SM, Demir IE, Singh SK, Gress TM, Ellenrieder V and Neeße A: Microenvironmental determinants of pancreatic cancer. *Physiol Rev* 100: 1707-1751, 2020.
- Orth M, Metzger P, Gerum S, Mayerle J, Schneider G, Belka C, Schnurr M and Lauber K: Pancreatic ductal adenocarcinoma: Biological hallmarks, current status, and future perspectives of combined modality treatment approaches. *Radiat Oncol* 14: 141, 2019.
- Yuen A and Díaz B: The impact of hypoxia in pancreatic cancer invasion and metastasis. *Hypoxia (Auckl)* 2: 91-106, 2014.
- Buttgereit F and Brand MD: A hierarchy of ATP-consuming processes in mammalian cells. *Biochem J* 312: 163-167, 1995.
- Fähling M: Surviving hypoxia by modulation of mRNA translation rate. *J Cell Mol Med* 13: 2770-2779, 2009.
- Wortel IMN, van der Meer LT, Kilberg MS and van Leeuwen FN: Surviving stress: Modulation of ATF4-mediated stress responses in normal and malignant cells. *Trends Endocrinol Metab* 28: 794-806, 2017.
- B'chir W, Maurin AC, Carraro V, Averous J, Jousse C, Muranishi Y, Parry L, Stepien G, Fafournoux P and Bruhat A: The eIF2 α /ATF4 pathway is essential for stress-induced autophagy gene expression. *Nucleic Acids Res* 41: 7683-7699, 2013.
- Guo X, Aviles G, Liu Y, Tian R, Unger BA, Lin YT, Wiita AP, Xu K, Correia MA and Kampmann M: Mitochondrial stress is relayed to the cytosol by an OMA1-DELE1-HRI pathway. *Nature* 579: 427-432, 2020.
- Pathria G, Scott DA, Feng Y, Sang Lee J, Fujita Y, Zhang G, Sahu AD, Ruppin E, Herlyn M, Osterman AL and Ronai ZA: Targeting the Warburg effect via LDHA inhibition engages ATF4 signaling for cancer cell survival. *EMBO J* 37: e99735, 2018.
- Fernandez MR and Cleveland JL: ATF4-amino acid circuits: A recipe for resistance in melanoma. *EMBO J* 37: e100600, 2018.
- Chiou SH, Risca VI, Wang GX, Yang D, Grüner BM, Kathiria AS, Ma RK, Vaka D, Chu P, Kozak M, *et al*: BLIMP1 induces transient metastatic heterogeneity in pancreatic cancer. *Cancer Discov* 7: 1184-1199, 2017.
- Mesclon F, Lambert-Langlais S, Carraro V, Parry L, Hainault I, Jousse C, Maurin AC, Bruhat A, Fafournoux P and Averous J: Decreased ATF4 expression as a mechanism of acquired resistance to long-term amino acid limitation in cancer cells. *Oncotarget* 8: 27440-27453, 2017.
- Palam LR, Gore J, Craven KE, Wilson JL and Kore M: Integrated stress response is critical for gemcitabine resistance in pancreatic ductal adenocarcinoma. *Cell Death Dis* 6: e1913, 2015.
- Ait Ghezala H, Jolles B, Salhi S, Castrillo K, Carpentier W, Cagnard N, Bruhat A, Fafournoux P and Jean-Jean O: Translation termination efficiency modulates ATF4 response by regulating ATF4 mRNA translation at 5' short ORFs. *Nucleic Acids Res* 40: 9557-9570, 2012.
- Blais JD, Filipenko V, Bi M, Harding HP, Ron D, Koumenis C, Wouters BG and Bell JC: Activating transcription factor 4 is translationally regulated by hypoxic stress. *Mol Cell Biol* 24: 7469-7482, 2004.
- Chee NT, Lohse I and Brothers SP: mRNA-to-protein translation in hypoxia. *Mol Cancer* 18: 49, 2019.
- Logsdon DP, Shah F, Carta F, Supuran CT, Kamocka M, Jacobsen MH, Sandusky GE, Kelley MR and Fishel ML: Blocking HIF signaling via novel inhibitors of CA9 and APE1/Ref-1 dramatically affects pancreatic cancer cell survival. *Sci Rep* 8: 13759, 2018.
- Zhang S, Zhao L, Wang J, Chen N, Yan J and Pan X: HIF-2 α and Oct4 have synergistic effects on survival and myocardial repair of very small embryonic-like mesenchymal stem cells in infarcted hearts. *Cell Death Dis* 8: e2548, 2017.
- Uniacke J, Holterman CE, Lachance G, Franovic A, Jacob MD, Fabian MR, Payette J, Holcik M, Pause A and Lee S: An oxygen-regulated switch in the protein synthesis machinery. *Nature* 486: 126-129, 2012.
- Ho JJD, Wang M, Audas TE, Kwon D, Carlsson SK, Timpano S, Evagelou SL, Brothers S, Gonzalgo ML, Krieger JR, *et al*: Systemic reprogramming of translation efficiencies on oxygen stimulus. *Cell Rep* 14: 1293-1300, 2016.
- Uniacke J, Perera JK, Lachance G, Francisco CB and Lee S: Cancer cells exploit eIF4E2-directed synthesis of hypoxia response proteins to drive tumor progression. *Cancer Res* 74: 1379-1389, 2014.
- Rzyski T, Milani M, Pike L, Buffa F, Mellor HR, Winchester L, Pires I, Hammond E, Ragoussis I and Harris AL: Regulation of autophagy by ATF4 in response to severe hypoxia. *Oncogene* 29: 4424-4435, 2010.
- Koumenis C and Wouters BG: 'Translating' tumor hypoxia: Unfolded protein response (UPR)-dependent and UPR-independent pathways. *Mol Cancer Res* 4: 423-436, 2006.
- Suarez-Arnedo A, Torres Figueroa F, Clavijo C, Arbeláez P, Cruz JC and Muñoz-Camargo C: An image J plugin for the high throughput image analysis of in vitro scratch wound healing assays. *PLoS One* 15: e0232565, 2020.
- Wei L, Lin Q, Lu Y, Li G, Huang L, Fu Z, Chen R and Zhou Q: Cancer-associated fibroblasts-mediated ATF4 expression promotes malignancy and gemcitabine resistance in pancreatic cancer via the TGF- β 1/SMAD2/3 pathway and ABCC1 transactivation. *Cell Death Dis* 12: 334, 2021.
- Koong AC, Mehta VK, Le QT, Fisher GA, Terris DJ, Brown JM, Bastidas AJ and Vierra M: Pancreatic tumors show high levels of hypoxia. *Int J Radiat Oncol Biol Phys* 48: 919-922, 2000.

28. Graffman S, Björk P, Ederoth P and Ihse I: Polarographic pO₂ measurements of intra-abdominal adenocarcinoma in connection with intraoperative radiotherapy before and after change of oxygen concentration of anaesthetic gases. *Acta Oncol* 40: 105-107, 2001.
29. McKeown SR: Defining normoxia, physoxia and hypoxia in tumours-implications for treatment response. *Br J Radiol* 87: 20130676, 2014.
30. Saxena K and Jolly MK: Acute vs chronic vs cyclic hypoxia: Their differential dynamics, molecular mechanisms, and effects on tumor progression. *Biomolecules* 9: 339, 2019.
31. Koh MY, Lemos R Jr, Liu X and Powis G: The hypoxia-associated factor switches cells from HIF-1 α - to HIF-2 α -dependent signaling promoting stem cell characteristics, aggressive tumor growth and invasion. *Cancer Res* 71: 4015-4027, 2011.
32. Koh MY and Powis G: Passing the baton: The HIF switch. *Trends Biochem Sci* 37: 364-372, 2012.
33. Onnis B, Rapisarda A and Melillo G: Development of HIF-1 inhibitors for cancer therapy. *J Cell Mol Med* 13: 2780-2786, 2009.
34. Downes NL, Laham-Karam N, Kaikkonen MU and Ylä-Herttua S: Differential but complementary HIF1 α and HIF2 α transcriptional regulation. *Mol Ther* 26: 1735-1745, 2018.
35. Holmquist-Mengelbier L, Fredlund E, Löfstedt T, Noguera R, Navarro S, Nilsson H, Pietras A, Vallon-Christersson J, Borg A, Gradin K, *et al*: Recruitment of HIF-1 α and HIF-2 α to common target genes is differentially regulated in neuroblastoma: HIF-2 α promotes an aggressive phenotype. *Cancer Cell* 10: 413-423, 2006.
36. Koh MY, Darnay BG and Powis G: Hypoxia-associated factor, a novel E3-ubiquitin ligase, binds and ubiquitinates hypoxia-inducible factor 1 α , leading to its oxygen-independent degradation. *Mol Cell Biol* 28: 7081-7095, 2008.
37. Serocki M, Bartoszewska S, Janaszak-Jasiecka A, Ochocka RJ, Collawn JF and Bartoszewski R: miRNAs regulate the HIF switch during hypoxia: A novel therapeutic target. *Angiogenesis* 21: 183-202, 2018.
38. Lu ZJ, Lu LG, Tao KZ, Chen DF, Xia Q, Weng JJ, Zhu F, Wang XP and Zheng P: MicroRNA-185 suppresses growth and invasion of colon cancer cells through inhibition of the hypoxia-inducible factor-2 α pathway *in vitro* and *in vivo*. *Mol Med Rep* 10: 2401-2408, 2014.
39. Uchida T, Rossignol F, Matthay MA, Mounier R, Couette S, Clottes E and Clerici C: Prolonged hypoxia differentially regulates hypoxia-inducible factor (HIF)-1 α and HIF-2 α expression in lung epithelial cells: Implication of natural antisense HIF-1 α . *J Biol Chem* 279: 14871-14878, 2004.
40. Nagelkerke A, Bussink J, Mujcic H, Wouters BG, Lehmann S, Sweep FC and Span PN: Hypoxia stimulates migration of breast cancer cells via the PERK/ATF4/LAMP3-arm of the unfolded protein response. *Breast Cancer Res* 15: R2, 2013.
41. Du J, Liu H, Mao X, Qin Y and Fan C: ATF4 promotes lung cancer cell proliferation and invasion partially through regulating Wnt/ β -catenin signaling. *Int J Med Sci* 18: 1442-1448, 2021.
42. Dadey DYA, Kapoor V, Khudanyan A, Thotala D and Hallahan DE: PERK regulates glioblastoma sensitivity to ER stress although promoting radiation resistance. *Mol Cancer Res* 16: 1447-1453, 2018.
43. Zeng P, Sun S, Li R, Xiao ZX and Chen H: HER2 upregulates ATF4 to promote cell migration via activation of ZEB1 and downregulation of E-cadherin. *Int J Mol Sci* 20: 2223, 2019.
44. González-González A, Muñoz-Muela E, Marchal JA, Cara FE, Molina MP, Cruz-Lozano M, Jiménez G, Verma A, Ramírez A, Qian W, *et al*: Activating transcription factor 4 modulates TGF β -induced aggressiveness in triple-negative breast cancer via SMAD2/3/4 and mTORC2 signaling. *Clin Cancer Res* 24: 5697-5709, 2018.
45. Rundqvist H and Johnson RS: Tumour oxygenation: Implications for breast cancer prognosis. *J Intern Med* 274: 105-112, 2013.
46. Vaupel P, Schlenger K, Knoop C and Höckel M: Oxygenation of human tumors: Evaluation of tissue oxygen distribution in breast cancers by computerized O₂ tension measurements. *Cancer Res* 51: 3316-3322, 1991.
47. Lando D, Peet DJ, Whelan DA, Gorman JJ and Whitelaw ML: Asparagine hydroxylation of the HIF transactivation domain a hypoxic switch. *Science* 295: 858-861, 2002.
48. Gradiz R, Silva HC, Carvalho L, Botelho MF and Mota-Pinto A: MIA PaCa-2 and PANC-1-pancreas ductal adenocarcinoma cell lines with neuroendocrine differentiation and somatostatin receptors. *Sci Rep* 6: 21648, 2016.
49. Yoshida T, Ri M, Kinoshita S, Narita T, Totani H, Ashour R, Ito A, Kusumoto S, Ishida T, Komatsu H and Iida S: Low expression of neural cell adhesion molecule, CD56, is associated with low efficacy of bortezomib plus dexamethasone therapy in multiple myeloma. *PLoS One* 13: e0196780, 2018.
50. Garcia-Carbonero N, Li W, Cabeza-Morales M, Martinez-Useros J and Garcia-Foncillas J: New hope for pancreatic ductal adenocarcinoma treatment targeting endoplasmic reticulum stress response: A systematic review. *Int J Mol Sci* 19: 2468, 2018.



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