# siRNA Targeting HIF-1α Induces Apoptosis of Pancreatic Cancer Cells through NF-κB-independent and -dependent Pathways under Hypoxic Conditions

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**Abstract.** *Objective: The primary objective was to explore the molecular mechanism of small interference RNA (siRNA) targeting hypoxic inducible factor-1α (HIF-1α) for inducing apoptosis of pancreatic cancer cells through a nuclear factor-kappaB (NF-κB)-independent or -dependent pathway under hypoxic conditions. Materials and Methods: A cassette encoding siRNA targeting HIF-1α mediated by recombinant adeno-associated virus (rAAV) was constructed, giving rAAV-siHIF. rAAV-siHIF or rAAV-hrGFP were transfected into exponentially growing MiaPaCa2 cells under hypoxic conditions. The expression of HIF-1α mRNA and protein and the activity of NF-κB were observed by real-time PCR, Western blot and electromobility shift assay (EMSA). The proliferation and apoptosis of MiaPaCa2 cells with or without PDTC, as an inhibitor of NF-κB, were investigated by MTT and TUNEL. Results: rAAV-siHIF inhibited the expression of HIF-1α mRNA and protein and the activity of NF-κB in MiaPaCa2 cells under hypoxic conditions. At the same time, rAAV-siHIF decreased MiaPaCa2 cell proliferation and induced apoptosis, but these effects were not abrogated by PDTC. Moreover, PDTC also inhibited MiaPaCa2 cell proliferation and induced apoptosis while rAAV-hrGFP did not have these effects. Conclusion: Under hypoxic conditions, HIF-1α plays a key role in the proliferation of MiaPaCa2 cells and inhibition of HIF-1α expression may lead to MiaPaCa2 cell apoptosis through NF-κB-independent and -dependent pathways.*

Pancreatic adenocarcinoma is a highly malignant digestive tumor with a very poor prognosis (1). Although the reason for this is still unknown, it may be related to the existence of severe hypoxia in pancreatic adenocarcinoma because hypoxic tumor cells migrate and metastasize easily and are associated with resistance to radiotherapy and chemotherapy (2, 3). Evidence has shown that hypoxia adaptation of tumor cells is regulated by hypoxia inducible factor 1 (HIF-1) (4). HIF-1 is a heterodimer which consists of 2 basic helix-loop-helix proteins, namely α subunit (120KD) and β subunit (91-94 kDa) (5), and is an essential mediator of oxygen homeostasis (6). HIF-1β subunits are constitutive proteins while the activity of the HIF-1 complex is influenced by the abundance and activity of HIF-1α subunits. Under normoxic conditions, HIF-1α protein is quickly hydroxylated by oxygen-activated HIF-1 prolyl hydroxylase (PHD) and degraded by the proteasomal pathway (7). Under hypoxic conditions, PHD is not activated so HIF-1α accumulates and associates with HIF-1β, and mature and functional HIF-1 is generated (8) which plays a key role in the growth, infiltration and metastasis of tumor cells (9-11). The over-expression of HIF-1α has been demonstrated in multiple types of human tumor as well as in their regional and distant metastases (12) and is thought to help cells resist hypoxic stress and apoptosis (13). Pancreatic adenocarcinoma, as a solid tumor, as well as MiaPaCa2 cells, a poorly differentiated human pancreatic cancer cell line, can express HIF-1α richly (11, 12). Hence, HIF-1α could play a crucial role in the pathophysiology of pancreatic cancer.

Nuclear factor-kappaB (NF-κB) is a family of related transcription factors including p50 (NF-κB1), p52 (NF-κB2), RelA (p65), RelB, and c-Rel proteins that are typically found as homo- or hetero-dimers. This family of

*Abbreviations:* hrGFP: Human recombinant green fluorescent protein; PDTC: pyrrolidine dithiocarbamate; pAAV-RC: containing *rep/cap*-gene; pAAV-hrGFP: containing human recombinant green fluorescent protein gene; CMV: cytomegalovirus; AMV: avian myeloblastosis virus; ABI: Applied Biosystems; BCA: bicinchoninic acid; NIH: National Institutes of Health; TBE: Tris-borate-EDTA; TdT: terminal deoxynucleotidyl transferase; HRP: horseradish peroxidase.

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transcription factors is unique in its capacity to coordinate transcription of a diverse array of genes, including those involved in carcinogenic processes, cell proliferation and resistance to apoptosis (14, 15). Activation of NF-κB protects cancer cells from apoptosis by the up-regulation of pro-survival mechanisms such as the anti-apoptotic Bcl-XL protein, and so on (16). Constitutive activation of NF-κB has been frequently observed in cancer cells such as pancreatic cancer cells and accordingly, inhibition of such activation has been shown to elicit cell cycle arrest and cell apoptosis (17-19). Multiple distinct signaling pathways lead to the activation of NF-κB, including proteasome independent pathways. It has been reported that HIF-1α can activate NF-κB, that NF-κB controls HIF-1α transcription and that HIF-1α activation may be concurrent with inhibition of NF-κB (20). However, it is unclear whether the resistance of hypoxic pancreatic cancer cells to apoptosis is through a NF-κB-dependent pathway activated by HIF-1α and whether inhibition of HIF-1α expression could induce hypoxic pancreatic cancer cells apoptosis through a NF-κB-dependent or -independent pathway.

RNA interference (RNAi), as a powerful tool of gene therapy, can repress the expression of target gene, thus achieving the effect of gene knock-out (21) and is also a very convenient and useful measure to study the function of genes. Studies have recently demonstrated that adeno-associated virus (AAV) is a highly efficient vector and can infect both dividing and non-dividing cells and achieve long-term gene expression (22, 23). An AAV-based vector for the delivery of small interference RNA (siRNA) targeting HIF-1α would be an efficient method of inhibiting HIF-1α expression in tumor cells, and was therefore used to investigate the role of HIF-1α in the proliferation of pancreatic cancer cells and the effect of inhibition of HIF-1α expression on the apoptosis of pancreatic cancer cells in relation to NF-κB-independent and -dependent pathways under hypoxic conditions.

## Materials and Methods

*Construction of recombinant AAV vector.* An AAV Helper-Free System including pAAV-hrGFP, pAAV-RC and pHelper was purchased from Stratagene (CA, USA) (the features of these plasmids are available from http://www.stratagene.com). The vector plasmid pAAV-hrGFP has a monoclonal site, namely Mlu I, which locates the starting point of the CMV promoter. The H1 human RNA polymerase III promoter was amplified by polymerase chain reaction (PCR) from human genomic DNA which was extracted from human blood cells. The primers for the PCR were A-Mlu I forward: 5'-ATCACGCGTCCATGGAATTCGAACGCTGA-3' and A-Mlu I-Xba I-Mun I reverse: 5'-GCTACGCGTTCTAGACAATTGGTGGTCT CATACAGAACTTATAAG-3'. The human H1 promoter was inserted into the Mlu I site of the pAAV-hrGFP, giving pAAV-H1-hrGFP. The orientation of pAAV-H1-hrGFP was confirmed by restriction and sequencing. A pair of complementary oligonucleotides with 58bp (sense: 5'-AATTGATGGAACATG ATGGTTCACTTCAA GAGAGTGAACCATCATGTTCCA TTTTTTT-3' and anti-sense: 5'-CTAGAAAAAAATGGAACATGATGGTTCACTCTCTTGAAGTG AACCATCATGTTCCATC-3') was designed according to the *HIF-1α* gene (GenBank No.U22431), annealed *in vitro* and subcloned into the pAAV-H1-hrGFP vector digested with Xba I and Mun I, giving pAAV-H1-siHIF-hrGFP. Restriction and sequencing analysis determined if the insert was correct.

Human embryonic kidney 293 cells (HEK293) (Stratagene) were cultured in Dulbecco's modified Eagle's medium (DMEM) including 10% fetal calf serum (FCS) in flasks incubated at 37˚C in 95% air and 5% $CO_2$. When the cells were 90% confluent, pAAV-H1-siHIF-hrGFP or pAAV-hrGFP, pAAV-RC and pHelper were co-transfected into the HEK293 cells using a ViraPack™ Transfection Kit (Stratagene) for packaging the rAAV-siHIF or rAAV-hrGFP. The expression of GFP in the HEK293 cells could be seen by fluorescence microscopy after 24 h and the rAAV could be purified and collected by the chloroform-PEG8000/NaCl-chloroform method (24) after 72 h. The shape of the rAAV was identified by electron microscopy and the purity and titer was assayed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and AVSachTM ELISA (Vector Gene Technology Company Ltd., Beijing, China) respectively.

*Administration in vitro*. MiaPaCa2 cells were bought from the American Type Culture Collection (Rockville, MD, USA) and cultured in DMEM containing 10% FCS. Three experimental groups were used. The Wt group consisted of non-transfected wild-type MiaPaCa2 cells, the Cv group of MiaPaCa2 cells transfected with rAAV-hrGFP ($1\times10^7$v.p/cell) as an empty control virus vector and the Si group of MiaPaCa2 cells transfected with rAAV-siHIF ($1\times10^7$v.p/cell). Over 95% of the MiaPaCa2 cells transfected by rAAV showed GFP expression by fluorescence microscopy. All the groups were cultured at 37˚C in 1% $O_2$, 94% $N_2$ and 5% $CO_2$. The expression of *HIF-1α* mRNA and protein was tested after 24 h and 48 h, the activity of NF-κB and the proliferation and apoptosis of the MiaPaCa2 cells after 24 h.

PDTC was used to inhibit the activity of NF-κB. MiaPaCa2 cells from each group were treated with 25 μmol/L PDTC (Sigma, MO, USA) and cultured for 24 h under hypoxic conditions. Once again, the proliferation and apoptosis were assayed.

*Real-time PCR to detect the expression of HIF-1α mRNA*. Total RNA was extracted from the MiaPaCa2 cells and cDNA was synthesized by AMV reverse transcriptase at 42˚C for 10 min and 95˚C for 2 min. A SYBR ExScript™ RT-PCR Kit (TaKaRa, Tokyo, Japan) and ABI (CA, USA) Prism 7900HT sequence detection system were used. The reagents were subjected to 95˚C for 30 s and were then cycled 40 times of 95˚C for 5 s and 60˚C for 15 s and 72˚C for 30 s. The primers of HIF-1α were 5'-TCATCCAAGAAG CCCTAACGTG-3' as forward primer and 5'-TTTCGCTTTCTC TGAGCATTCTG-3' as reverse primer. The primers of β-actin were 5'-TGGCACCCAGCACAATGAA-3' as forward primer and 5'-CTAAGTCATAGTCCGCCTAGAAGCA-3' as reverse primer. The relative quantity results of real-time PCR were analyzed by an ABI 7900HT software system.

*Western blot to detect the expression of HIF-1α protein*. HIF-1α monoclonal antibody was purchased from BD Biosciences (CA, USA). β-Actin polyclonal antibody was purchased from Santa Cruz (CA, USA). Total proteins were extracted from the MiaPaCa2 cells

and the concentrations were determined using a BCA protein assay kit (Pierce, Rockford, IL, USA) and by Biophotometer (Eppendorf, Hamburg, Germany). The total proteins were separated on 8% SDS-PAGE gel for HIF-1α and 12% for β-actin, transferred to polyvinylidene difluoride membranes by MINI TRANS-BLOT (BIO-RAD, CA, USA). The blot membrane was then incubated with primary and secondary antibodies and treated with enhanced chemiluminescence detection reagents (Amersham, Buckinghamshire, UK). The specific blotting band was recorded on film. The results were analyzed by ImageJ software (available from the NIH at http://rsb.info.nih.gov/ij/).

*Electromobility shift assay (EMSA) to detect the activity of NF-κB.* The nuclear proteins were extracted from the MiaPaCa2 cells and their concentrations were determined using a BCA protein assay kit (Pierce). For the EMSA (Pierce), nuclear proteins (10 mg) were mixed in 20 mL reactions with a buffer containing 10 mmol/L HEPES (pH 7.8), 50 mmol/L KCl, 0.1 mmol/L EDTA, 1 mmol/L DTT, 10% glycerol and 3 mg poly (dI-dC). Binding reactions were started by the addition of a $^{32}$P-labeled DNA probe followed by incubation at room temperature for 20 min. The oligo probe 5'-GCAGAGGGGACTTTCCGAGA-3' containing the NF-κB binding motif was annealed to the complementary oligonucleotide and end-labeled by using T4 polynucleotide kinase. Samples were electrophoresed on a native 4.5% polyacrylamide gel at 200 V in 0.5 TBE buffer. The gels were then dried and the bands visualized by exposure to film. In pancreatic cancer cells, the NF-κB band has two components: the upper component corresponds to the p50/p65 heterodimer and the lower component to the p50/p50 homodimer. In the present study, the total (combined) intensity of the NF-κB bands was analyzed by ImageJ software.

*TUNEL assay.* The MiaPaCa2 cells with or without PDTC were incubated on chamber slides under hypoxic conditions. Additionally one group of cells was cultured under normoxia and another group under hypoxia without TdT enzyme as negative controls. After 24 h, the endogenous peroxidase of the cells was inactivated with 30% $H_2O_2$ diluted 1:10 in methanol at room temperature for 10 min. The cells were incubated with the TdT equilibration buffer for 60 min at 37˚C, and then the labeling reaction mixture (Streptavidin-HRP) for 30 min at 37˚C. The cells were further stained with diaminobenzidine for 10 min at room temperature so that the nucleus of the apoptotic cells became yellow. Five fields of each slide were randomly selected and 500-1000 nuclei were counted per slide. The frequency of apoptosis was calculated as an apoptotic index (AI).

*MTT assay.* The MiaPaCa2 cells with or without PDTC were placed a 96-well culture plate ($10^4$ cells/well) under hypoxic conditions. Cell viability was measured after 24 h using the MTT assay. Routinely, 20 μL MTT (Sigma) stock solution (5 mg/mL) was added to each well with 200 μL media and incubated for 4 h. Then the media were removed and 200 μL DMSO (Sigma) was added to each well and the plate was vibrated for 10 min. Absorbance in every well was measured in a spectrophotometer at a wavelength of 570 nm.

*Statistical analysis.* The data were expressed as means±SD and statistical analysis was conducted with SPSS (Statistical Package for the Social Sciences) 11.5 using analysis of variance and Bonferroni posttest. Differences were considered statistically significant when $p<0.05$.

## Results

*Expression of HIF-1α.* Real-time PCR showed that rAAV-siHIF inhibited the expression of *HIF-1α* mRNA in the MiaPaCa2 cells after 24 h under hypoxic conditions and after 48 h the expression decreased nearly 90% compared to the unaffected Wt and Cv groups ($p<0.05$) (Figure 1A). Western blot showed that rAAV-siHIF suppressed the expression of HIF-1α protein after 24 h under hypoxic conditions and after 48 h the protein expression declined over 90% compared to the Wt and Cv groups, which were not affected ($p>0.05$) (Figure 1B, C, D).

*The activity of NF-κB.* The EMSA showed that there were two key bands, p50/p50 and p50/p65 respectively, which suggested that the EMSA test was successful. Moreover, the activity of NF-κB in the Si group decreased greatly and there was a significant difference between the Si group and the Wt or Cv groups after 24 h ($p<0.01$). However, there was no significant difference in the expression of NF-κB activity between the Wt group and the Cv group after 24 h ($p>0.05$) (Figure 2A, B).

*Apoptosis.* Under normoxic conditions, apoptotic cells were rarely seen but there were many apoptotoic cells under hypoxic conditions, indicating that hypoxia is a key factor for apoptosis of tumor cells. In the negative control group without TdT enzyme, yellow nuclei were not found although there were many apoptotic cells which indicated that our technique was satisfactory. The AI of the cells in the Si group with or without PDTC after 24 h under hypoxic conditions was significantly higher than that in the Wt group or in the Cv group ($p<0.01$). There was no effect on the AI of the Cv group cells under hypoxic conditions compared with the Wt group ($p>0.05$). However, the AI in the group with PDTC was higher than that in the corresponding group without PDTC ($p<0.01$), which suggested that PDTC may induce MiaPaCa2 cell apoptosis (Figure 3A).

*Cell proliferation.* The MTT assay showed that rAAV-siHIF inhibited proliferation of the MiaPaCa2 cells after 24 h and there was a significant difference between the Si group and the Wt and Cv groups ($p<0.01$). However, rAAV-hrGFP had no effect on the proliferation of the MiaPaCa2 cells under hypoxic conditions compared with the Wt group ($p>0.05$). At the same time, the proliferation in the groups with PDTC was lower than that in the corresponding group without PDTC ($p<0.01$), which also indicated that PDTC may inhibit MiaPaCa2 cell proliferation (Figure 3B).

## Discussion

Transfection with rAAV-siHIF inhibited the expression of HIF-1α mRNA and protein at both 24 h and 48 h while rAAV-hrGFP transfection had no effect on HIF-1α expression

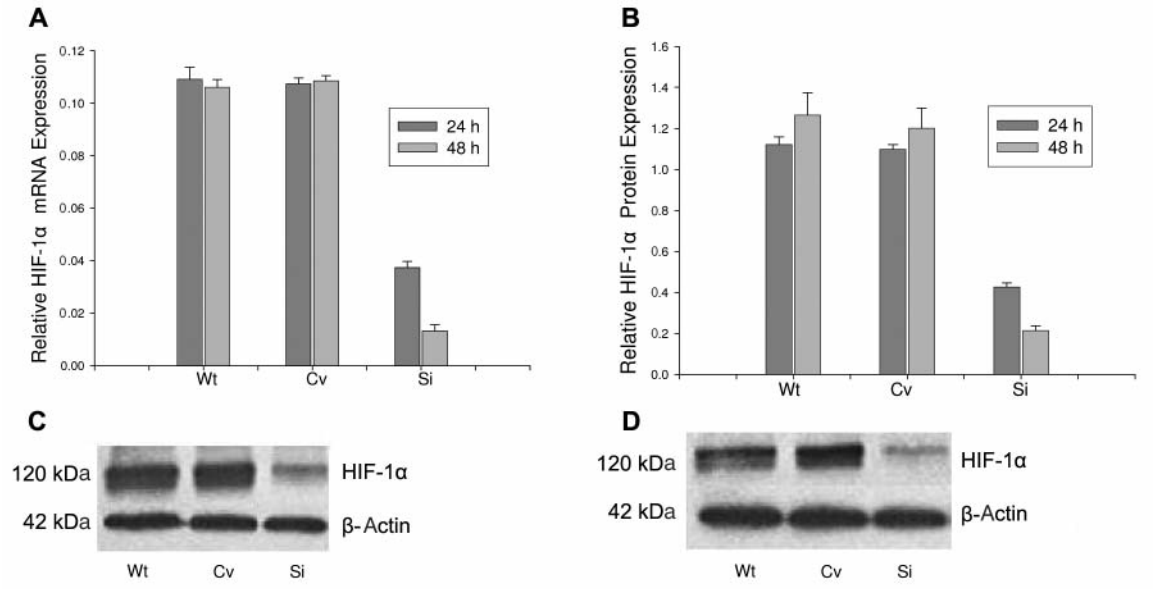


Figure 1. *Expression of HIF-1α mRNA and protein after 24 h and 48 h under hypoxic conditions. (A) Expression of HIF-1α mRNA determined by real-time PCR. (B) Expression of HIF-1α protein determined by western blot. Western blot of HIF-1α protein expression (C) after 24 h and (D) after 48 h. Wt: non-transfected wild-type MiaPaCa2 cells; Cv: MiaPaCa2 cells transfected with rAAV-hrGFP; Si: MiaPaCa2 cells transfected with rAAV-siHIF.*

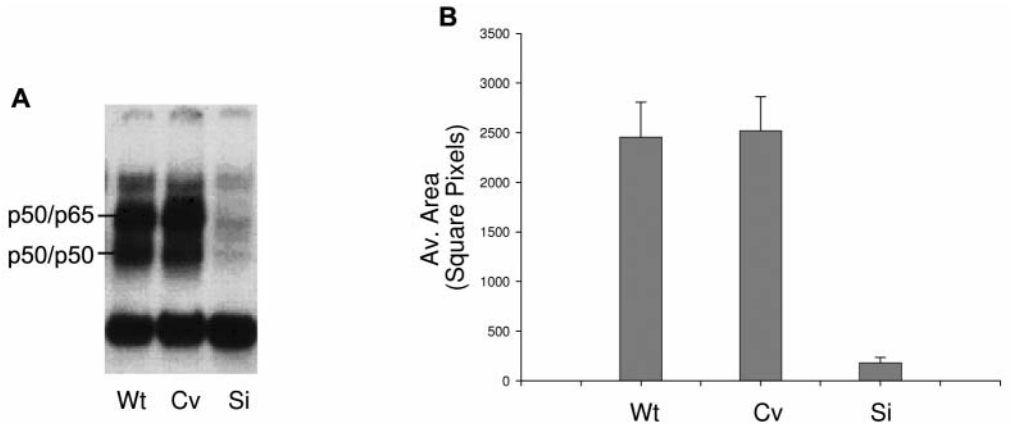


Figure 2. *The activity of NF-κB in MiaPaCa2 cells determined by EMSA under hypoxic conditions. (A) From gel electrophoresis analysis, the NF-κB complex had two bands, p50/p50 and p50/p65. (B) Relative NF-κB activity. Wt: non-transfected wild-type MiaPaCa2 cells; Cv: MiaPaCa2 cells transfected with rAAV-hrGFP; Si: MiaPaCa2 cells transfected with rAAV-siHIF.*

which indicated that the construction of the rAAV vector mediating siRNA targeting HIF-1α was successful. Because rAAV-siHIF inhibited the expression of HIF-1α within 48 h, 24 h was selected to further study the effects of rAAV-siHIF on NF-κB activity and the proliferation and apoptosis of the MiaPaCa2 cells under hypoxic conditions. The EMSA demonstrated that rAAV-siHIF inhibited the activity of NF-κB and indicated that HIF-1α is closely related to NF-κB in pancreatic cancer cells under hypoxic conditions and NF-κB may be a gene downstream of HIF-1α. Moreover, rAAV-siHIF inhibited MiaPaCa2 cell proliferation and induced apoptosis indicating that HIF-1α plays an important role in MiaPaCa2 cell proliferation and that inhibition of HIF-1α expression could induce MiaPaCa2 cell apoptosis. Interestingly, when NF-κB was abrogated by PDTC, rAAV-siHIF still induced MiaPaCa2 cell apoptosis and inhibited their proliferation. This indicated that rAAV-siHIF induced MiaPaCa2 cell apoptosis through a NF-κB-independent pathway. Moreover, the TUNEL assay indicated that PDTC led to MiaPaCa2 cells apoptosis though inhibiting the activity

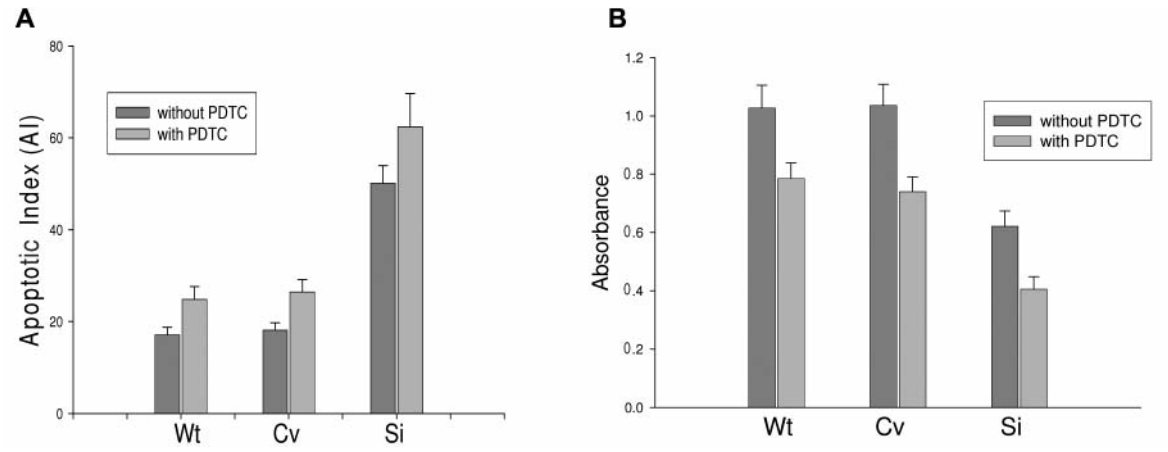


Figure 3. *(A) Apoptosis of MiaPaCa2 cells without or with PDTC under hypoxic conditions determined by TUNEL assay. (B) Proliferation of MiaPaCa2 cells without or with PDTC under hypoxic conditions determined by MTT. Wt: non-transfected wild-type MiaPaCa2 cells; Cv: MiaPaCa2 cells transfected with rAAV-hrGFP; Si: MiaPaCa2 cells transfected with rAAV-siHIF.*

of NF-κB, while rAAV-siHIF also led to MiaPaCa2 cells apoptosis and decreased NF-κB activity. Thus, rAAV-siHIF led to MiaPaCa2 cells apoptosis through a NF-κB-dependent pathway.

In conclusion, HIF-1α plays a key role in the proliferation of MiaPaCa2 cells under hypoxic conditions and inhibition of HIF-1α expression may lead to MiaPaCa2 cell apoptosis through two pathways, namely NF-κB-independent and -dependent pathways. Moreover, HIF-1α could act as an important target of pancreatic cancer genetic and pharmacological therapy.

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