

Original Article

Anti-angiogenic and anti-proliferative effects of inhibition of HIF-1 α by p-HIF-1 α RNAi in colorectal cancer

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Received March 27, 2015; Accepted May 20, 2015; Epub July 1, 2015; Published July 15, 2015

Abstract: The aim of this study was to construct an RNA-interference plasmid (p-HIF-1 α RNAi) targeting the human HIF-1 α gene and assess its effects on HIF-1 α expression and its anti-tumour functions *in vitro*. p-HIF-1 α RNAi was constructed and confirmed by polymerase chain reaction (PCR) and DNA sequencing. Reverse transcriptase PCR (RT-PCR) and western blot were performed to detect HIF-1 α expression in HCT116 cells following transfection of p-HIF-1 α RNAi and p-control. The anti-tumour effects and mechanism of action of p-HIF-1 α RNAi in HCT116 cells were further investigated. p-HIF-1 α RNAi significantly inhibited HIF-1 α expression in the HCT116 cell line. p-HIF-1 α RNAi inhibited cell viability and reduced VEGF but not bFGF expression in the supernatant of HCT116 cells, down-regulated β -catenin and VEGF expression, and altered β -catenin location in the HCT116 cell nucleus. The plasmid p-HIF-1 α RNAi can effectively and specifically inhibit HIF-1 α expression, inhibit cell proliferation, and alter the expression of key components in the Wnt/ β -catenin signaling pathway. Thus, p-HIF-1 α RNAi is a novel and extremely promising therapeutic inhibitor of HIF-1 α .

Keywords: Colorectal cancer, HIF-1 α , anti-angiogenic, anti-proliferative, RNAi

Introduction

Colorectal cancer (CRC) is one of the most common causes of cancer death worldwide [1]. The survival and prognosis of patients with CRC depend on the stage of the tumour at the time of detection. However, about 50% of patients are diagnosed with regional or distant metastases at the time of diagnosis [2]. Metastasis development is a multistep process, in which cell survival and angiogenesis play crucial roles in colorectal cancer.

Hypoxia-inducible factors (HIFs) accumulate in both neoplastic and inflammatory cells within the tumour microenvironment and affect the progression of a variety of diseases, including colorectal cancer. HIF inhibition represents a novel therapeutic strategy for cancer treatment.

Hypoxia-inducible factor 1 α (HIF-1 α) plays an important role in regulating cell survival and angiogenesis, which are critical for tumour

growth and metastasis. Although numerous efforts have been made to identify molecules that inhibit HIF-1 α and/or target the HIF-1 signaling pathway in tumour progression, the currently available inhibitors lack specificity [3]. Thus, we constructed an RNA-interference plasmid targeting human HIF-1 α , that is, p-HIF-1 α RNAi, and assessed its effects with respect to interfering with HIF-1 α expression. Its anti-tumour effects and its mechanism of action were also investigated.

Materials and methods

Reagents

Anti-HIF-1 α , anti- β -catenin, anti-axin, anti-cyclooxygenase 2 (COX-2), anti-VEGF, and anti-GAPDH antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). All antibodies were used at the recommended concentrations. Roswell Park Memorial Institute (RPMI)-1640 (RPMI-1640) medium and foetal bovine serum (FBS) were purchased from Invi-

trogen/Life Technologies (Carlsbad, CA, USA). Penicillin/streptomycin solution (P/S, Cat. No. 0503) was purchased from Sciencell (Carlsbad, CA, USA). All other chemicals were from Sigma-Aldrich Corp. (St. Louis, MO, USA) unless otherwise stated.

Plasmids

A plasmid containing an HIF-1 α RNAi, an artificial microRNA, and carrier vector was constructed. The HIF-1 α miRNA sequence was 5'-TGCTGTGTGAGTTCGCATCTTGATGTTTTGGCCACTGACTGACATCAAGATGCGAACTCACA-3' and the control sequence was 5'-TGCUACACCGUAGCAGACACCGTTTTGGCCACTGACTGACGGTGTCTGCTAACGGTGTA-3'. The purified PCR product was then cloned into BLOCK-iT™ Pol II miR RNAi Expression Vector with EmGFP (Life) via T4 DNA ligase (Takara Bio Inc. Dalian, China). Transfection was performed with Lipofectamine™ 2000 (Invitrogen/Life Technologies) following the manufacturer's protocol.

Cell culture

The human colorectal cancer cell line used, HCT116, was purchased from Cell Bank of Type Culture Collection of Chinese Academy of Sciences (Shanghai, China) and cultured in RPMI-1640 medium supplemented with 10% bovine serum, penicillin (100 U/mL), and streptomycin (100 g/mL; Invitrogen, Carlsbad, CA) at 37°C in a 5% CO₂ humidified atmosphere.

RNA extraction and quantification

Total RNA was isolated from cells using Trizol (Invitrogen/Life Technologies) according to the manufacturer's instructions. Reverse transcription was performed using the One Step PrimeScript miRNA cDNA Synthesis Kit (Takara Bio Inc.). Real-time PCR was performed using SYBR1 Premix Ex TaqTMII (Takara Bio Inc.) with an iCycler thermal cycler (Bio-Rad, Hercules, CA, USA). The primers used for the detection of HIF-1 α using real-time PCR were as follows: forward, 5'-TGCAACATGGAAGGTATTGC-3'; reverse, 5'-TTCACAAATCAGCACCAAGC-3'. HIF-1 α levels were normalized to GAPDH transcript levels. The relative expression levels between the samples were calculated using the comparative delta CT (threshold cycle number) method ($2^{-\Delta\Delta CT}$) with a control sample as the reference point [4].

Proliferation assay

Cells were seeded on 96-well plates in regular growth medium. Proliferation of cancer cells was measured 24 h, 48 h, or 72 h after transient transfection of the p-HIF-1 α RNAi or p-control RNAi, by using the Cell Counting Kit (CCK-8) assay.

Clone formation assay

HCT116 cells were transfected with p-control or p-HIF-1 α RNAi for 48 h, and then seeded in 24-well plates at a density of 1000 cells/well or 100 cells/well in 3 mL of fresh complete RPMI-1640 medium. After 7 days, the cells were washed twice with 1 $\times$ PBS and stained with a solution of 0.2% crystal violet, 50% methanol, and 10% acetic acid in H₂O for 30 min at room temperature. Subsequently, the cells were washed with deionized H₂O and photographed.

VEGF and bFGF assay

Cells were cultured in 6-well plates after transfection with p-control or p-HIF-1 α RNAi for 24 h, 48 h, or 72 h with serum. The media were then collected, cleared by centrifugation, and VEGF or bFGF concentrations were determined using a VEGF or bFGF ELISA kit (R&D systems, Minneapolis, MN, USA) following the manufacturer's instruction. The VEGF or bFGF concentration in the culture media was assayed in duplicate at a 1:4 dilution and corrected for total cell numbers.

Laser confocal microscopy

Cells were cultured in 6-well plates after transfection with p-control or p-HIF-1 α RNAi for 24 h, then cells were collected and cultured into 8-well μ -slides (ibidi GmbH, Am Klopferspitz 19, D-82152 Martinsried, Germany) for 48 h. Then, the cells were washed with PBS and fixed with 4% paraformaldehyde for 20 min at 4°C and washed thrice for 15 min with PBS. The cells were permeabilized for 30 min using PBS, 10% BSA, 0.5% Triton X-100, followed by the anti- β -catenin antibody staining in 5% BSA at 4°C overnight. The cells were washed with PBS and incubated for 1 h at 37°C with Alexa-488 Secondary Goat anti-Rabbit antibody. The cells were washed thrice for 15 min with PBS and DAPI was used for staining nuclei. The slides were then washed with PBS and mounted with 50% glycerol at pH 7.4. Finally, β -catenin was analyzed using a Leica confocal microscope.

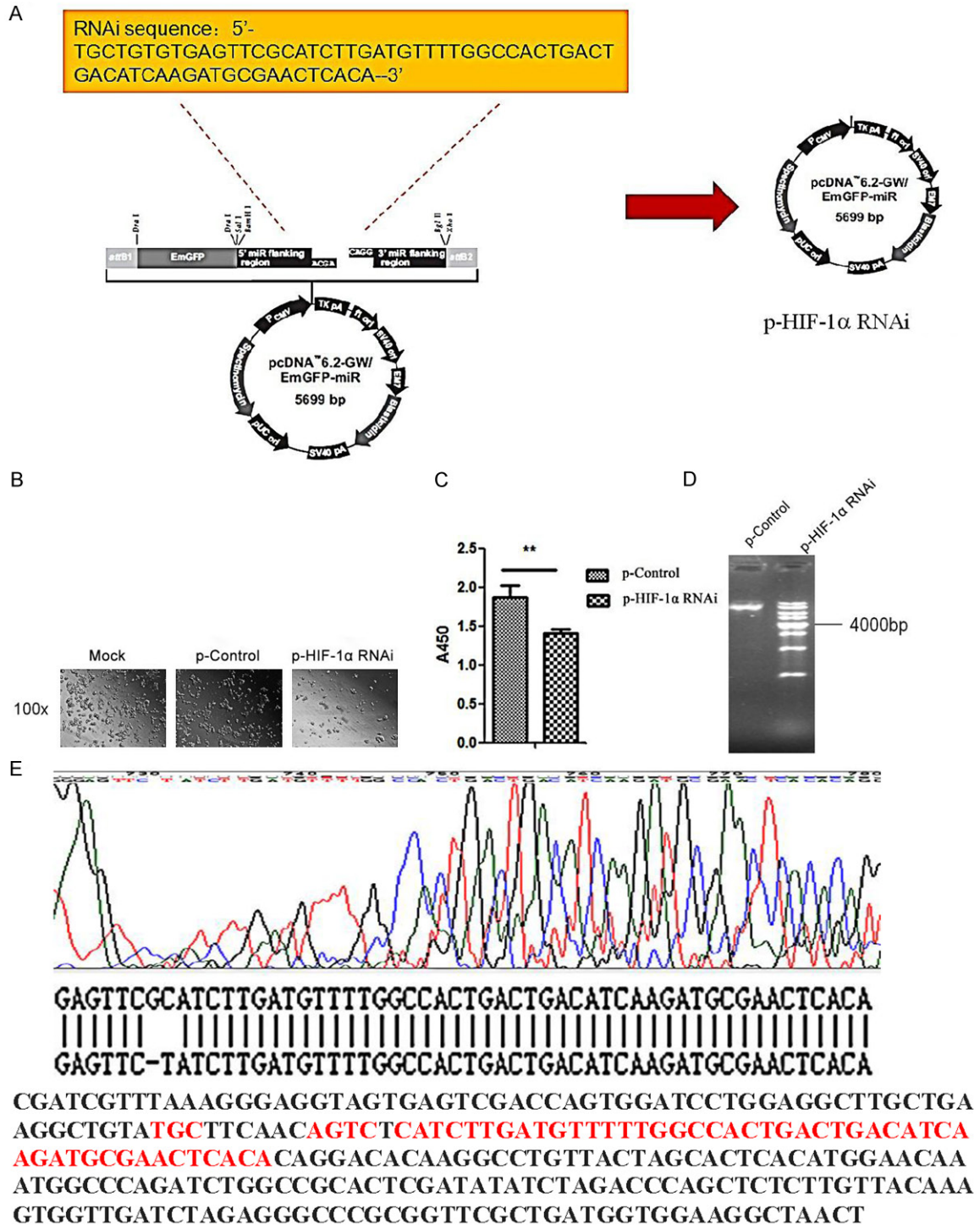


Figure 1. p-HIF-1 α RNAi construction (A) p-HIF-1 α RNAi sketch map. (B) HCT116 cells were transiently transfected with HIF-1 α RNAi or control RNAi modified by 2-MO. Photographs show HCT116 cell proliferation. (C) Results are presented as mean $\pm$ S.E.M. for the optical density (OD). Three independent experiments were performed and the results of a representative experiment are presented. (D) p-HIF-1 α RNAi plasmid visualisation on agarose gel. (E) p-HIF-1 α RNAi sequencing.

Western blot analysis

Cellular proteins were extracted and were then separated using SDS-PAGE gels. Western

blot analyses were performed according to standard procedures as previously described. GAPDH was used as the loading control.

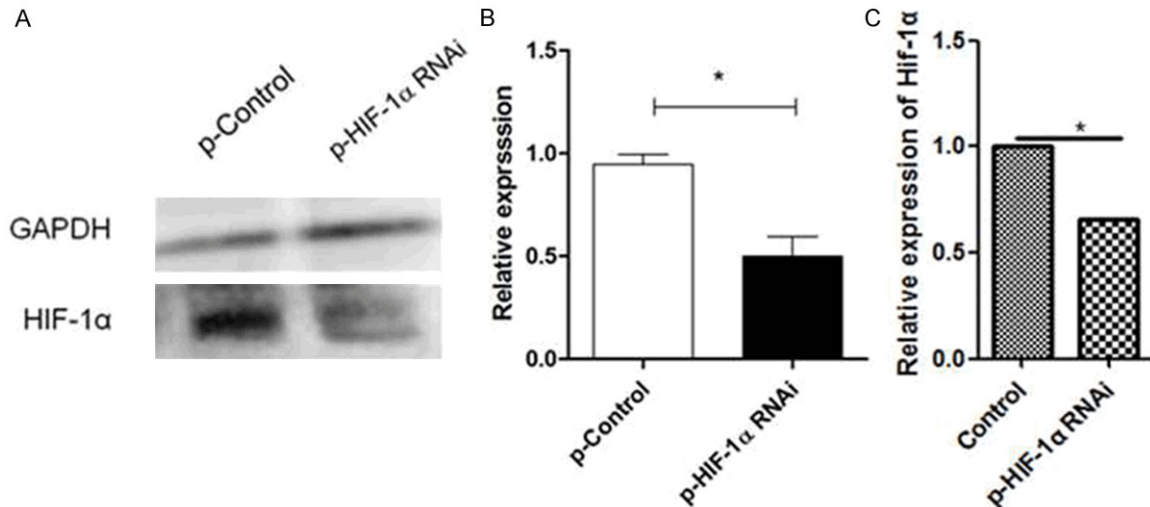


Figure 2. p-HIF-1 α RNAi down-regulated HIF-1 α expression in HCT116 cells. HCT116 cells were transfected with p-control or p-HIF-1 α RNAi. A. HIF-1 α protein expression was assessed in HCT116 cells by western blot analysis. B. The values on the vertical axis represent the relative expression of HIF-1 α in HCT116 cells. C. *HIF-1 α* mRNA expression was measured in HCT116 cells by RT-PCR.

Statistical analysis

A Student's t-test was used to analyse differences between two groups and one-way ANOVA was employed in case of data consisted of more than two groups. Data are presented as the mean $\pm$ SD from 3 independent experiments. All statistical analyses were performed using the SPSS 15.0 software. A two-tailed value of $P < 0.05$ was considered statistically significant.

Results

p-HIF-1 α RNAi construction

p-HIF-1 α RNAi sketch map is presented in **Figure 1A**. To confirm whether HIF-1 α RNAi sequence was effectively constructed, HCT116 cells were transiently transfected with HIF-1 α RNAi (5'-UGUGAGUUCGCAUCUUGAU-3') or control RNAi (5'-UACACCGUUAGCAGACACC-3') modified by 2-MO and CCK-8 assay was performed. Proliferation of HCT116 cells is shown in **Figure 1B**. Results are presented as mean $\pm$ S.E.M. for optical density (OD) ($P < 0.01$). Three independent experiments were performed and the results of one representative experiment are shown in **Figure 1C**. HIF-1 α RNAi inhibited HCT116 cell proliferation. The plasmid p-HIF-1 α RNAi construction was confirmed by agarose gel electrophoresis and DNA sequencing, presented in **Figure 1D** and **1E**.

p-HIF-1 α RNAi down-regulated HIF-1 α expression in HCT116 cells

Western blot and RT-PCR were performed to determine HIF-1 α protein and mRNA levels in CT116 cells. Cells were transfected with p-control or p-HIF-1 α RNAi for 48 h. As shown in **Figure 2A** and **1B**, p-HIF-1 α RNAi reduced HIF-1 α protein expression as well as *HIF-1 α* mRNA levels (**Figure 2C**).

p-HIF-1 α RNAi inhibited HCT116 cell viability

HIF-1 α down-regulation leads to anti-proliferative effect against CRC [5]. HCT116 cells were transiently transfected with p-HIF-1 α RNAi or p-control RNAi for 24 h, 48 h, and 72 h. Results are presented as the mean $\pm$ S.E.M. for OD. As shown in **Figure 3A** and **3B**, p-HIF-1 α RNAi significantly inhibited cell viability at 48 h ($P < 0.001$). Similar results were observed in **Figure 3C** and **3D**. Thus, we can conclude that p-HIF-1 α RNAi inhibited HCT116 cell viability.

p-HIF-1 α RNAi inhibited VEGF expression in the HCT116 cell supernatant

Angiogenesis is mediated by a number of different growth factors and is vital for tumour growth. Vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) are two potent angiogenic growth factors that stimulate vascular endothelial cell proliferation and are involved in neoplastic angio-

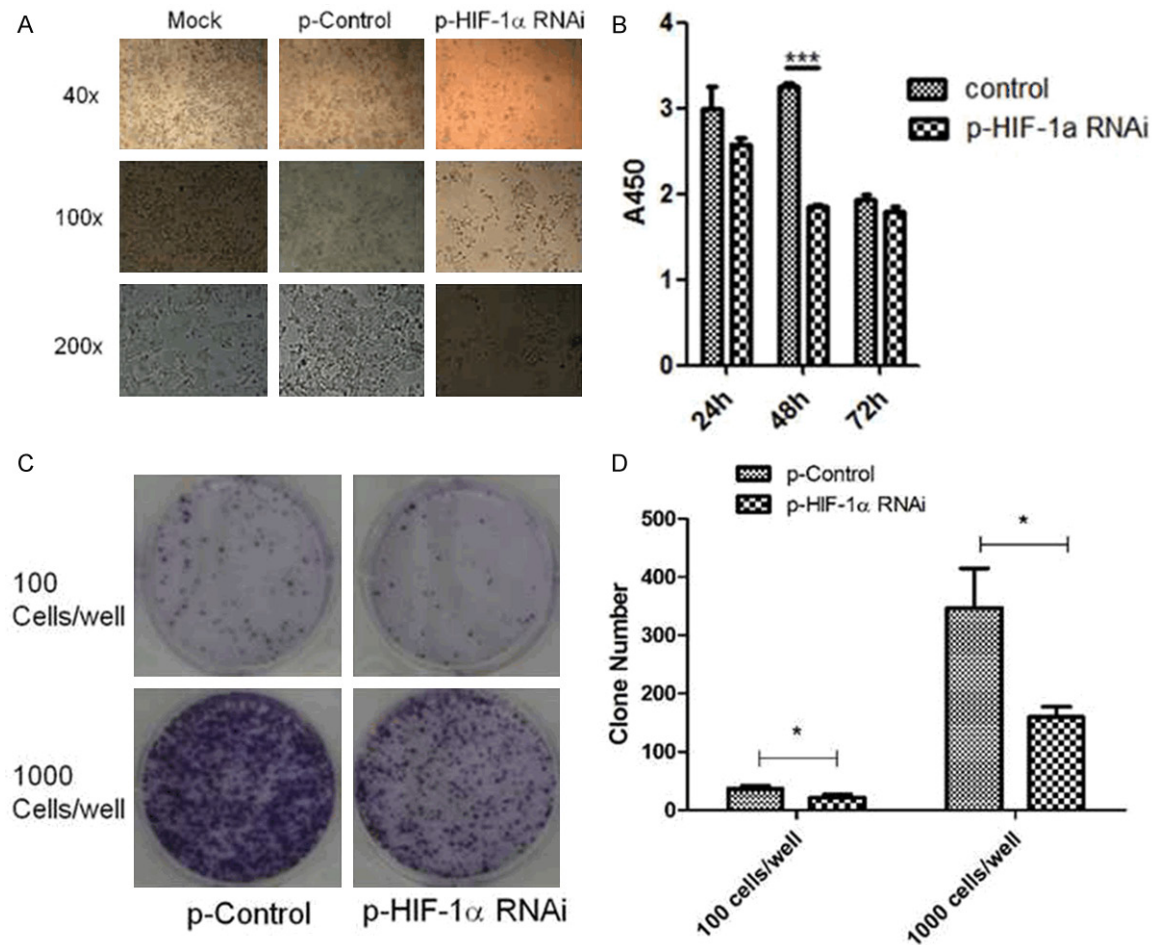


Figure 3. p-HIF-1 α RNAi inhibited HCT116 cell viability (A) HCT116 cells were transiently transfected with p-HIF-1 α RNAi or p-control RNAi for 24 h, 48 h, and 72 h. Cell proliferation was assessed 48 h later. (B) Results are presented as the mean $\pm$ S.E.M. for optical density (OD) for 24 h, 48 h, and 72 h. Three independent experiments were performed and the results of a representative experiment are shown. (C) HCT116 cells were transiently transfected with p-HIF-1 α RNAi or p-control RNAi for 48 h and were then seed in 24 wells plate at a density of 100 cells/well and 1000 cells/well. Photographs show cell proliferation after 7 days. (D) The histogram showed clone number for each group.

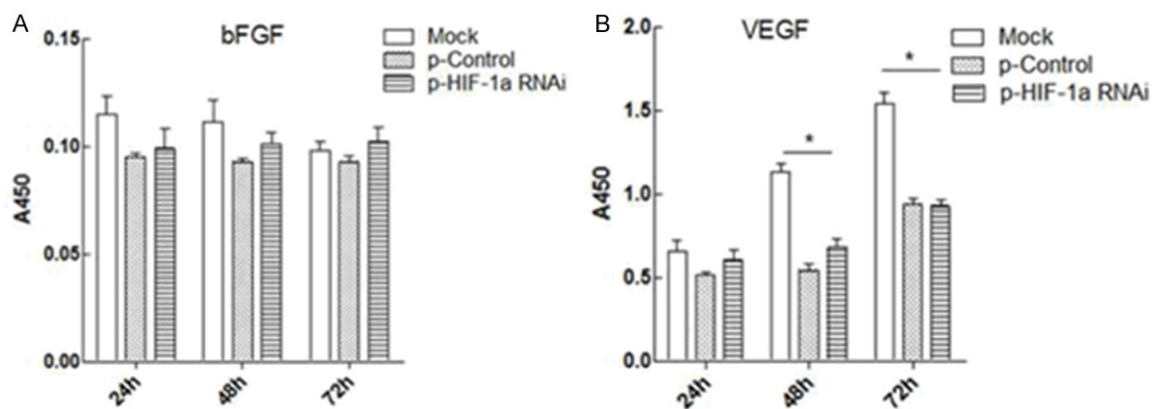


Figure 4. p-HIF-1 α RNAi inhibited VEGF expression in HCT116 cell supernatant HCT116 cells were transiently transfected with p-HIF-1 α RNAi or p-control RNAi for 24 h, 48 h, and 72 h. ELISA was performed to evaluate the expres-

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sion of bFGF and VEGF. A. bFGF expression was not affected by p-HIF-1 α RNAi expression in HCT116 cells. B. VEGF was significantly reduced in HCT116 cells transfected with p-HIF-1 α RNAi at 48 and 72 h ($P<0.05$).

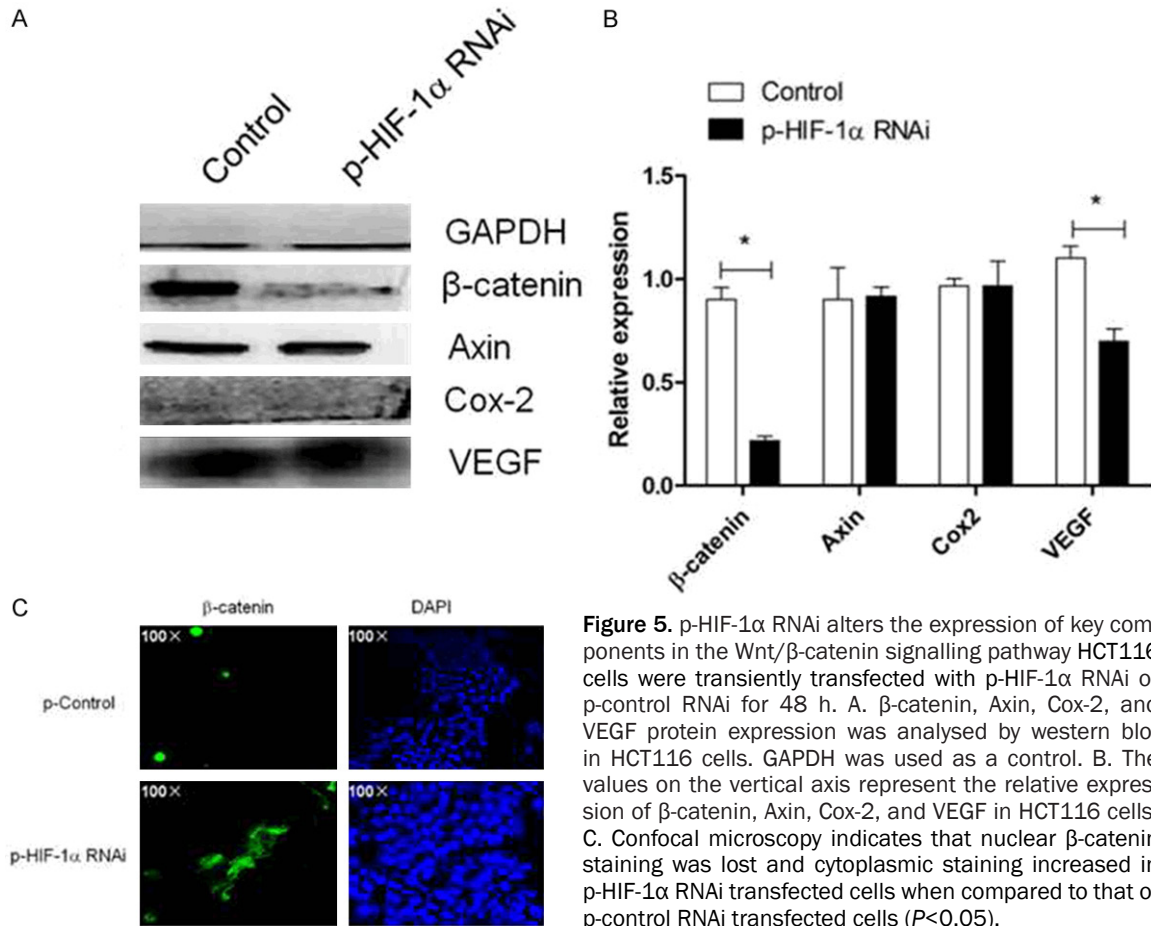


Figure 5. p-HIF-1 α RNAi alters the expression of key components in the Wnt/ β -catenin signalling pathway. HCT116 cells were transiently transfected with p-HIF-1 α RNAi or p-control RNAi for 48 h. A. β -catenin, Axin, Cox-2, and VEGF protein expression was analysed by western blot in HCT116 cells. GAPDH was used as a control. B. The values on the vertical axis represent the relative expression of β -catenin, Axin, Cox-2, and VEGF in HCT116 cells. C. Confocal microscopy indicates that nuclear β -catenin staining was lost and cytoplasmic staining increased in p-HIF-1 α RNAi transfected cells when compared to that of p-control RNAi transfected cells ($P<0.05$).

genesis of several types of tumours, including colorectal cancer [6, 7]. Several studies have reported that the enzyme linked immunosorbent assay (ELISA) is an efficient tool to evaluate tissue levels of these two angiogenic growth factors [7-9]. VEGF and bFGF expression was measured by ELISA in the supernatant of HCT116 cells in order to determine whether p-HIF-1 α RNAi affected their expression. As shown in **Figure 4A** and **4B**, p-HIF-1 α RNAi reduced VEGF expression, but did not affect that of bFGF.

p-HIF-1 α RNAi alters the expression of key components in the Wnt/ β -catenin signalling pathway

To further examine how p-HIF-1 α RNAi affects β -catenin and Wnt signalling, the expression of β -catenin, Axin, Cox-2, and VEGF, which are proteins related to the Wnt signalling pathway, was

measured by western blot in HCT116 cells. p-HIF-1 α RNAi down-regulated the expression of β -catenin and VEGF (**Figure 5A** and **5B**). Confocal microscopy indicated a rapid change in β -catenin distribution. Nuclear β -catenin staining was lost and cytoplasmic staining increased ($P<0.05$) (**Figure 5C**).

Discussion

HIF-1 α plays a key role in tumour survival in a hypoxic environment through induction of survival factors and angiogenic growth factors such as VEGF [10]. Increased expression of HIF-1 α and VEGF is correlated with a more advanced tumour stage and a poorer prognosis in patients with CRC. Additionally, previous reports suggest that HIF-1 α regulates tumour angiogenesis through VEGF induction in primary and metastatic tumours [11-13]. However, efficient drugs targeting HIF-1 α remain scarce. In this study,

we constructed an RNA-interference plasmid targeting the human *HIF-1 α* gene, p-HIF-1 α RNAi, which reduced HIF-1 α as well as VEGF.

Aberrant activation of Wnt/ β -catenin signalling is fundamental to the pathogenesis of colon cancer, and the molecular control of this pathway has become a major therapeutic focus [14, 15]. In colon cancer cells, β -catenin degradation is impaired and nuclear translocation is enhanced, leaving the Wnt-signalling pathway overactive and cells tumour-prone. Extracellular Wnt inhibitors have been investigated as potential therapeutic agents [16] and small molecular antagonists that affect β -catenin expression presented encouraging preclinical results [17, 18]. Plasmid p-HIF-1 α RNAi constructed in our study can not only effectively and specifically inhibit HIF-1 α expression and cell proliferation, but also alter the expression of key components in the Wnt/ β -catenin signalling pathway, including β -catenin and VEGF.

Thus, p-HIF-1 α RNAi is a novel and extremely promising therapeutic inhibitor of HIF-1 α .

Acknowledgements

Project supported by Shanghai Committee of Science and Technology, China (No. 114119b3100) and Putuo District Committee of Science and Technology, Shanghai China (No. 201102). This research work was also supported by Construct Program of the Key Discipline of State Administration of Traditional Chinese Medicine of People's Republic of China.

Disclosure of conflict of interest

None.

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