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Hypoxia-inducible factor-2α (HIF-2α) mediates the effects of hypoxia on the promotion of HeLa cell viability, colony formation, and invasion capacity *in vitro*

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ABSTRACT. Hypoxia reduces the oxygen supply to tumor cells and may limit tumor cell growth. However, hypoxia promotes tumor cell metabolic adaptation, apoptosis resistance, angiogenesis, invasion, and metastasis. Hypoxia-inducible factor-2α (HIF-2α) may be responsible for these hypoxia-induced changes. In this study, we investigated the effects of hypoxia and HIF-2α knockdown in HeLa cells. HIF-2α shRNA lentivirus was used to knock down HIF-2α expression; cell viability, colony formation, invasion capacity, and gene expression were assessed. Hypoxia promoted HeLa cell growth, whereas knockdown of HIF-2α expression reduced HeLa cell viability under both normoxic and hypoxic conditions, with a greater effect observed under hypoxic conditions. Knockdown of HIF-2α expression also reduced HeLa cell colony formation and invasion capacity under both normoxic and hypoxic conditions. Expression of cyclooxygenase 2 and vascular endothelial growth factor was reduced after knockdown of HIF-2α expression, with a greater effect observed under hypoxic conditions. HIF-2α mediated the

hypoxia-induced effect on the promotion of HeLa cell viability, colony formation, and invasion capacity *in vitro*. Further studies are needed to confirm the *in vivo* relevance of hypoxia and HIF-2α.

Key words: Cell viability; Colony formation; HeLa cells; shRNA; Hypoxia-inducible factor-2α; Transwell invasion

INTRODUCTION

Cervical cancer is the second most common cancer in women worldwide, and the incidence is even higher in developing countries (Armstrong, 2010). Infection with human papillomavirus (HPV) is the most significant risk factor/cause in the development of more than 90% of cervical cancer cases (Lowy and Schiller, 2006; Armstrong, 2010). Currently, available HPV vaccines are effective against the 2 strains of HPVs predicted to cause approximately 70% of cervical cancer cases in the US, Canada, Australia, and Europe (Lowy and Schiller, 2006). The precise molecular mechanism of cervical carcinogenesis remains to be defined, but it was recently shown that oxygen levels in cervical cancer tissues (particularly in the advanced stage of disease) were much lower than in the normal cervix tissues (Höckel et al., 1991). Anoxia is a typical characteristic in the solid tumor microenvironment. During cancer progression, neoangiogenesis and vascularity of tumors consistently fall behind the demand of tumor cells because of rapid tumor cell proliferation; if blood vessel distance from tumor cells is more than 150 μm, anoxia or anaerobic conditions occur.

Anoxia can reduce the oxygen supply to tumor cells and therefore inhibit or limit tumor cell growth. However, anoxic conditions can promote tumor cell metabolic adaptation, apoptosis resistance, angiogenesis, invasion, and metastasis. Anoxia also induces the expression of a number of genes, particularly members of the hypoxia-inducible factor family (HIF). HIF-2α is an important member of this family and was identified in 1997 as one of the most important anoxia-induced factors (Zhulin et al., 1997). Previous studies showed that the HIF signaling cascade mediates the effects of hypoxia in cells. Thus, HIF may promote the formation of blood vessels, prevent cells from differentiating, and increase cell migration (Brahimi-Horn and Pouysségur, 2005; Benizri et al., 2008; Formenti et al., 2010). HIF-2α is expressed in a variety of tissues, which is affected by the surrounding environment (Warnecke et al., 2008). In cervical cancer, a recent study demonstrated the clinical significance of HIF-2α in radioresistant cervical cancer (Kim et al., 2011). Another study reported the prognostic significance of HIF-2α expression in tumor infiltrating macrophages of patients with uterine cervical cancer undergoing radiotherapy (Kawanaka et al., 2008). However, the role of HIF-2α in cervical cancer progression remains unclear. In this study, we investigated the effect of HIF-2α knockdown on the regulation of cervical cancer HeLa cell biological function.

MATERIAL AND METHODS

Cell line and culture

Cervical cancer HeLa cells were obtained from The Central Laboratory of The Second Hospital, Xiangya School of Medicine, The Central South University (Changsha, China) and cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum

in a humidified incubator containing 5% CO_2 at 37°C. In this study, HeLa cells were split into 6 groups, including i) control (CON) cells, HeLa cells were cultured under normal oxygen conditions without virus infection; ii) negative control cells, HeLa cells were cultured under normal oxygen levels and infected with a negative control virus; iii) HIF-2α knocked down cells (KD cells), HeLa cells were cultured under normal oxygen conditions and infected with HIF-2α shRNA virus; iv) anoxia control (CON+) cells, HeLa cells were cultured under the anoxia condition but without any virus infection; v) negative control + anoxia (NC+) cells, HeLa cells were cultured under anoxic conditions and infected with a negative control virus; vi) HIF-2α knocked down cells in anoxia culture (KD+), HeLa cell were cultured under anoxic conditions and infected with HIF-2α shRNA virus.

RNA interference and cell infection

To knockdown HIF-2α expression, we designed HIF-2α shRNA sequences and 4 pairs of HIF-2α shRNA were custom-synthesized by Ambion (Austin, TX, USA) according to GenBank data on HIF-2α cDNA (5'-CCAAGAGTCACCAGAACTT-3', 5'-GCTTCCTGCGA ACACACAA-3', 5'-GCGACAGCTGGAGTATGAA-3', and 5'-CAGGACATAGTATCTTTG A-3', referred to as pSC-1-4, respectively). Next, we inserted these shRNAs into a lentiviral vector and the inserted DNA sequence was confirmed. The 4 vectors, together with a negative control vector, were transfected into HEK293 cells for lentivirus production. Next, viruses were used to infect HeLa cells. Briefly, the cells were grown overnight on 12-well plates and then infected with 10 multiplicity of infection virus particles for 3 days. When 50% of the cells expressed green fluorescent protein, the growth medium was replaced and the cells were cultured for an additional 3 days. We found that pSC-4 was the most effective vector (data not shown) and we used this vector for all experiments.

RNA isolation and quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

Cells were grown to approximately 80% confluence and total cellular RNA was isolated from the cultured HeLa cells using TRIzol reagent (Sigma, St. Louis, MO, USA) and reverse-transcribed into cDNA using an AMV reverse transcription kit (Takara, Dalian, China). qPCR was performed using a SYBR Premix Ex Taq system according to manufacturer instructions (Takara). The qRT sample mix contained 15 μL SYBR Premix Ex Taq, 0.6 μL ROX Reference Dye, 3 μL cDNA, 0.6 μL of each primer, and 2 μL ddH_2O. The qPCR program was set to 95°C for 30 s and then 40 cycles of 95°C for 5 s, 60°C for 30 s, and 95°C for a 15-s loop once for melting curve analysis in a 7900HT fast RT-PCR system (Perkin Elmer, Waltham, MA, USA). β-actin mRNA was used as an internal control and the relative quantitative value was calculated using the ΔΔCt method.

Cell viability MTT assay

Cells were grown and infected with HIF-2α shRNA or negative control lentivirus for 72 h and then seeded for cell viability assay. For the methyl thiazolyl tetrazolium (MTT) assay, 20 μL MTT (5 mg/mL, Sigma) was added to each well of the 96-well plates, incubated for 4 h, and then the growth medium was removed and 100 μL dimethyl sulfoxide was added

to the wells to dissolve the MTT crystals. Next, the optical densities were measured using an automated spectrophotometric plate reader at a wavelength of 570 nm. The percentage of cell growth was calculated using the formula: % control = ODt / ODc x 100, where ODt and ODc are the optical densities for transfected and vector control cells, respectively.

Tumor cell colony formation assay

After the cells in exponential growth phase were trypsinized, seeded, and grown overnight, lentivirus carrying HIF-2α shRNA or negative control sequences was used to infect the cells for 3 days. Next, the cells were trypsinized and 200 cells were seeded into each well of 6-well plates in agar (Bio-Rad, Hercules, CA, USA) and grown for 14 days. The growth medium was replaced every 3 days. At the end of the experiment, the cells were fixed with paraformaldehyde solution and then stained with GIEMSA staining solution. Cell colonies were viewed and photographed under an inverted fluorescence microscope and a cluster of 50 cells or more was counted as 1 colony.

Tumor cell invasion assay

To assess tumor cell invasion capacity, we used the Transwell tumor cell invasion system (BD Biosciences, Franklin Lakes, NJ, USA). Briefly, HIF-2α shRNA or negative control lentivirus-infected cells were trypsinized and 100,000 cells were seeded into the upper-chamber of a Transwell in 300 μL serum-free Dulbecco's modified Eagle's medium in triplicate, and the bottom chambers were filled with 500 μL serum-containing Dulbecco's modified Eagle's medium and cultured for up to 72 h. At the end of the experiment, the cells on the upper surface were removed using a cotton swab and the cells that had invaded into the bottom surface of the Matrigel filter were stained with 1% crystal violet solution. The Matrigel filters were then gently removed from the chamber and mounted on glass slides. Six microscopic fields (100X magnification) per chamber were photographed. The cells in the photographs were then counted and the data are reported as means ± standard deviation and presented as a percentage of the control value. Next, the filters were dissolved in 33% acetic acid and the optical density values (at 570 nm) of the cell solutions were measured using a spectrophotometer.

Statistical analysis

Data are reported as means ± standard deviation (means ± SD). Comparisons between different groups of cells were analyzed for variance of multiple factors at multiple levels using different tests; for example, Student *t*-test was used to determine statistical differences between HIF-2α shRNA and negative control virus-infected HeLa cancer cells. All statistical analyses were performed using the SPSS17.0 statistics software (SPSS, Inc., Chicago, IL, USA) and $P < 0.05$ was considered to be statistically significant.

RESULTS

Effects of normoxic and hypoxic conditions on the regulation of HeLa cell growth

HeLa cells were cultured under normoxic and hypoxic conditions for up to 5 days.

The data showed that hypoxia promoted HeLa cell growth for up to 5 days of culture compared to cells in normoxic cultures (Figure 1A). However, hypoxic culture conditions induced HeLa cells to express HIF-2α mRNA (Figure 1B).

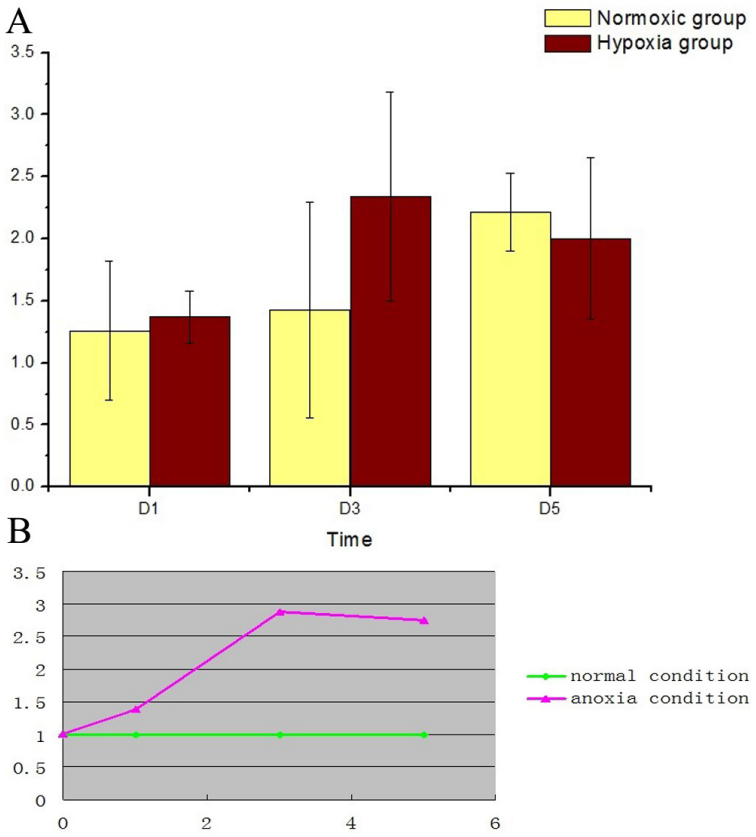

Figure 1. A. Altered HeLa cell viability under normoxic and hypoxic conditions. **B.** Expression of HIF-2α in HeLa cells.

Effects of HIF-2α knockdown in HeLa cells

Next, we performed HIF-2α knockdown experiments by culturing HeLa cells under normoxic and hypoxic conditions. We first detected the expression of HIF-2α mRNA in the 6 groups of cells and found that HIF-2α shRNA significantly knocked down HIF-2α expression under both culture conditions (Figure 2A).

Subsequently, we assessed cell viability in these 6 groups of HeLa cell cultures and found that knockdown of HIF-2α expression reduced HeLa cell viability under both normoxic and hypoxic conditions, with a greater effect observed under the hypoxic condition (Figure 2B). We then performed a tumor cell colony formation assay and found that while HIF-2α knockdown significantly inhibited HeLa cell colony formation under both normoxic and hypoxic conditions, the inhibitory effect was greater under hypoxic conditions (Figure 2C). Spe-

cifically, the CON group showed the largest number of colonies under normoxic conditions, while the lowest number of colonies was observed in the KD group under normoxia condition. There were significant differences in the number of colonies between the 3 groups ($P < 0.05$). Under anoxia conditions, the CON+ group showed the largest number of colonies, while the KD+ group showed the fewest. While there were significant differences in the number of colonies between the 3 groups ($P < 0.05$), the number of colonies in the KD+ group was lower than that in the KD group ($P < 0.05$).

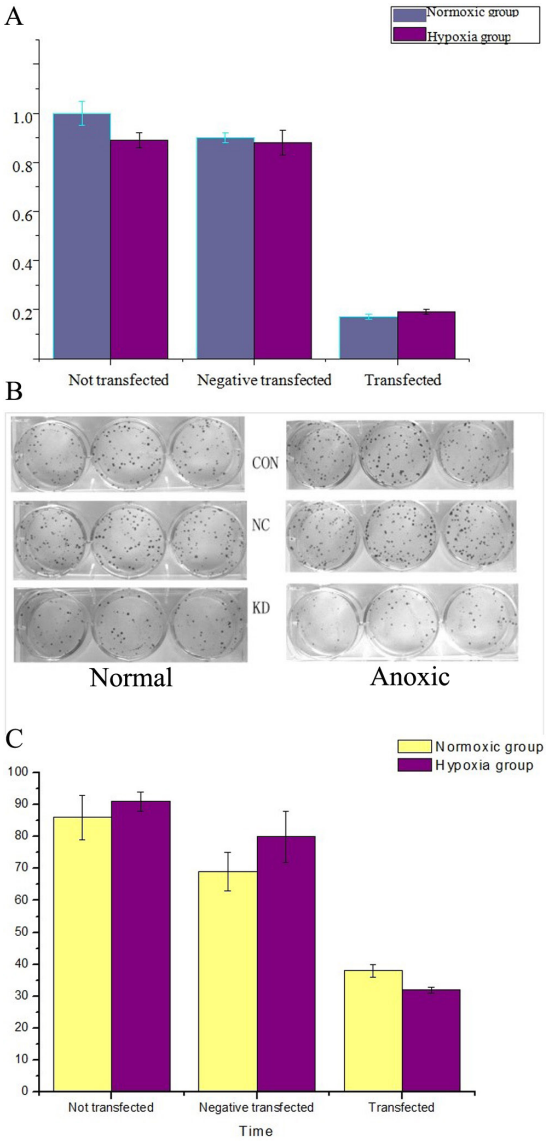

Figure 2. A. Expression of HIF-2α mRNA in HeLa cells after HIF-2α shRNA viral infection under normal or anoxia conditions. **B.** Cell viability in 6 groups of HeLa cell cultures. **C.** Colony formation assay.

We also assessed the effect of hypoxia and HIF-2α gene silencing on HeLa cell invasion capacity *in vitro* and found that hypoxia and HIF-2α knockdown promoted HeLa cell invasion (Figure 3A and B).

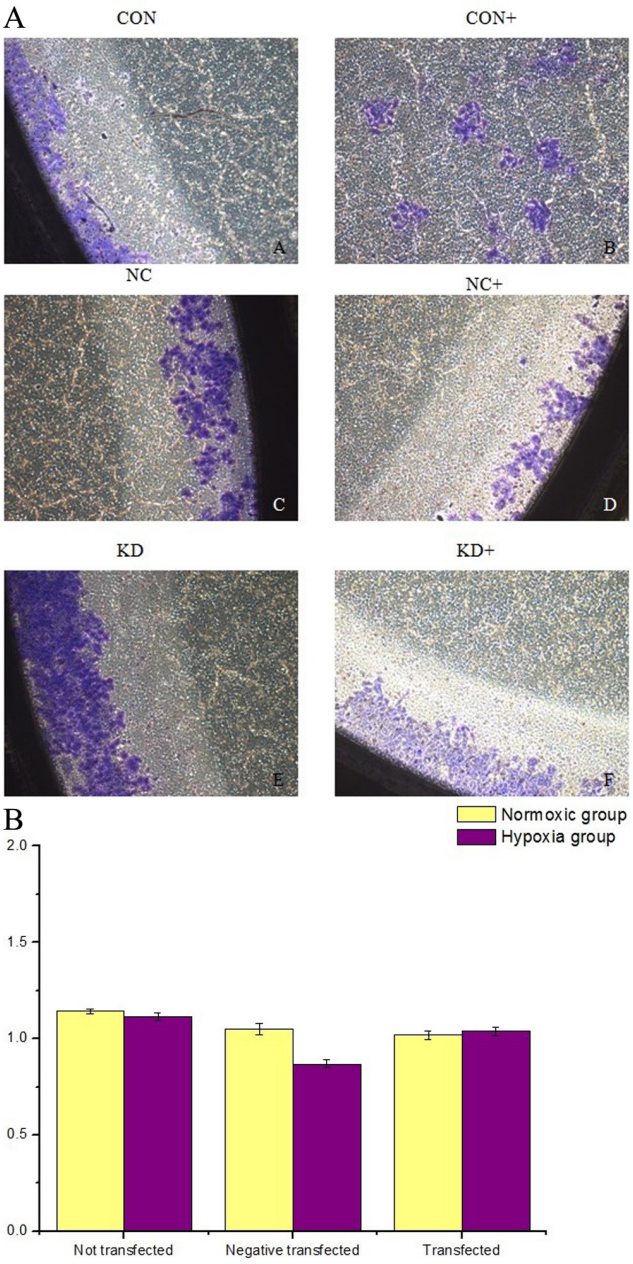

Figure 3. A. Effects of hypoxia and HIF-2α knockdown on HeLa cell invasion capacity and gene expression. **B.** Transwell tumor cell invasion assay.

Specifically, under normoxia conditions, the CON group showed the lowest invasion ability, whereas the KD group showed the highest invasion ability. There were significant differences among the 3 groups ($P < 0.05$). Under anoxia conditions, the CON+ group had the highest invasion ability, whereas the KD+ group had the lowest invasion ability. There were significant differences in the invasion capacity among the 3 groups ($P < 0.05$). In contrast to the normoxia and anoxia conditions, the invasion ability of the CON+ group was higher than that of the CON group ($P < 0.05$). The invasion ability of the NC+ group was higher than that of the NC group ($P < 0.05$) and the invasion ability of the KD+ group was not significantly different from that of the KD group ($P > 0.05$).

Effects of hypoxia and HIF-2α knockdown on gene expression in HeLa cells

We next assessed the expression of different genes following HIF-2α knockdown in HeLa cells cultured under hypoxic conditions. The data showed that under normal and hypoxia conditions, the level of cyclooxygenase-2 (COX-2) mRNA between HIF-2α knockdown and negative control HeLa cells did not significantly differ (0.90 ± 0.03 *vs* 0.84 ± 0.05 and 0.93 ± 0.05 *vs* 1.00 ± 0.04, respectively; $P > 0.05$). However, under normal culture conditions, the level of COX-2 mRNA was lower in HIF-2α knockdown HeLa cells than in the negative control lentivirus-infected cells (0.59 ± 0.03 *vs* 0.84 ± 0.05; $P < 0.05$). The same was observed for HeLa cells grown under hypoxic conditions (0.72 ± 0.02 *vs* 1.00 ± 0.04; $P < 0.05$; Figure 4A).

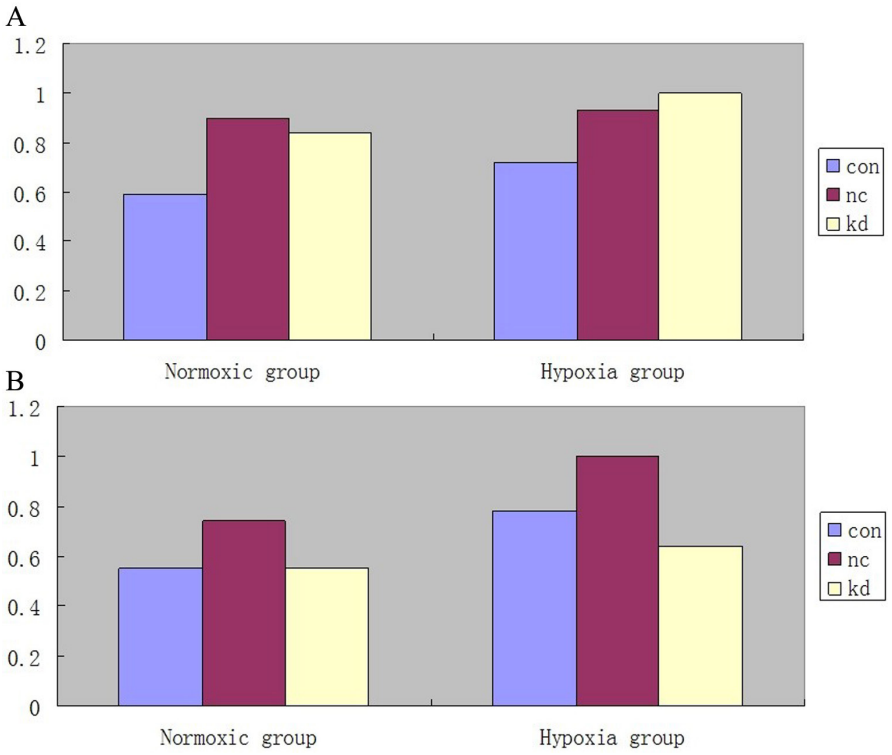

Figure 4. A. Relative expression of COX-2. **B.** Relative expression of VEGF.

Moreover, under normal and hypoxia conditions, the level of vascular endothelial growth factor (VEGF) mRNA did not differ between HIF-2α knockdown and negative control HeLa cells (0.55 ± 0.05 *vs* 0.74 ± 0.07 and 0.78 ± 0.06 *vs* 1.00 ± 0.11; $P > 0.05$). However, under hypoxia conditions, the level of VEGF mRNA was lower in HIF-2α knockdown HeLa cells than in negative control lentivirus-infected cells (0.64 ± 0.09 *vs* 1.00 ± 0.11; $P < 0.05$). HeLa cells expressed higher levels of VEGF mRNA under hypoxia condition compared to under normal conditions (0.55 ± 0.05 *vs* 0.78 ± 0.06; $P < 0.05$; Figure 4B).

DISCUSSION

Intratumor anoxia affects tumor cell migration and invasion capacity (Qing and Simon, 2009) and contributes to a poor response to radiotherapy and overall survival (Brizel et al., 1999). The HIF-2α protein is a key regulatory factor contributing to the cancer phenotype, particularly under hypoxic conditions (Loboda et al., 2010). However, interestingly, in some benign tumors, anoxic conditions exist, but HIF is not expressed. HIF becomes overexpressed and activated when cells undergo carcinogenesis. For example, in uterus liomyoma, severe anoxia is typically present, but HIF is not expressed. In contrast, HIF and its relevant genes are highly expressed in uterus leiomyosarcoma. Histologically, both liomyoma and sarcoma in the uterus showed identical microvessel density, but HIF was only expressed in the malignant tumor (Mayer et al., 2010). Furthermore, HIF-2α affects tumor growth by specifically targeting different genes (Patel and Simon, 2008). In many cancers, such as glioblastoma (Seidel et al., 2010), non-small cell lung cancer (Franovic et al., 2009), and malignant renal neoplasm (Toschi et al., 2008), HIF-2α promoted tumor growth and development, whereas in other cancers, such as colon cancer SW480 cell, HIF-2α restrained tumor growth (Imamura et al., 2009). In the current study, we demonstrated that in hypoxia culture, HeLa cell viability was increased, whereas knockdown of HIF-2α expression reduced HeLa cell viability under both normoxic and hypoxic conditions, with a reduction in cell viability more significant under the hypoxia condition. Moreover, knockdown of HIF-2α expression also reduced HeLa cell colony formation and invasion capacity under both normoxic and hypoxic conditions. Expression of COX-2 and VEGF was reduced after knockdown of HIF-2α expression. Thus, our data suggest that HIF-2α mediates the hypoxia-induced effects on the promotion of HeLa cell viability, colony formation, and invasion capacity *in vitro*. Further *in vivo* studies are required to confirm our data regarding hypoxia and HIF-2α in HeLa cells.

In the current study, we showed that HIF-2α knockdown significantly reduced colony formation of HeLa cells under both normal and anoxia conditions, indicating that this change was independent of the anoxia condition. The data confirmed the results of previous studies by Gordan et al. (2007) and Tsao et al. (2008). In a previous study, HIF-2α was able to regulate cell multiplication in mouse embryo fibroblasts; in a later study, HIF-2α induced cell multiplication in kidney cancer (Tsao et al., 2008). The effect of HIF-2α on HeLa cell invasion ability is complex. Under normal culture conditions, the expression of HIF-2α protein can enhance the invasion ability of HeLa cells, whereas HIF-2α knockdown reduced the invasion ability of HeLa cells, even under anoxia conditions. In contrast, the invasion ability of HeLa cells was high under anoxia conditions. However, following HIF-2α knockdown, the invasion ability was not significantly different between anoxia and normal conditions. This suggests that HIF-2α plays an important role in invasion ability of HeLa cells.

Molecularly, under normoxic conditions, oxygen-dependent degradation of the structural domain of HIF-2α occurs when pr0530 becomes hydroxylated by a praline hydroxylase, resulting in the association of HIF-2α protein with the VHL ubiquitin-protease complex, ultimately leading to degradation. When the cells are under anoxic conditions, praline hydroxylase loses its function and the Pro530 in the oxygen-dependent degradation structural domain cannot be hydroxylated. This leads to intact HIF-2α protein accumulation in cells, which can then form the HIF-2 protein complex with HIF-β that translocates into the nucleus. Concurrently, asparagus cochinchinensis tyrosine hydroxylation at the C-terminus of the HIF-2 protein complex with HIF-β is restrained, leading to exposure of the binding site with CREB-binding protein, CBP/p300, to form a transcription initiation complex; next, HIF-2α recognizes hypoxia response element to start transcription of targeting genes (Qing and Simon, 2009).

In our current study, we showed that HIF-2α knockdown also suppressed expression of COX-2 and VEGF under both normal and anoxic conditions. COX-2 is the rate-limiting enzyme involved in the synthesis of prostaglandins, is involved in the development of different human cancers, and promotes tumor cell proliferation and growth, tumor angiogenesis, invasion, and resistance to apoptosis (Shafie et al., 2013; Cheng and Fan, 2013; Jhang et al., 2013; Nagoya et al., 2014). In cervical cancer, COX-2 induced tumor cell invasion and metastasis (Warnecke et al., 2008). In the current study, we found that HIF-2α knockdown suppressed COX-2 expression under both normal and anoxia conditions in HeLa cells. VEGF (also known as vascular permeability factor), plays an important role in angiogenesis, cell proliferation, and tumor metastasis. In the current study, we showed that knockdown of HIF-2α expression suppressed VEGF expression under both normoxic and anoxia culture conditions in HeLa cells, which is consistent with the results of previous studies in other cancers (Ema et al., 1997; Koukourakis et al., 2002; Turner et al., 2002; Griffiths et al., 2008; Kim et al., 2009; Zimmer et al., 2010). Zimmer et al. (2010) showed that HIF-2α knockdown led to a reduction in VEGF expression, suggesting that VEGF is a target gene of HIF-2α. We also found that VEGF expression was higher under hypoxia than under normal culture conditions, suggesting that hypoxia can induce VEGF expression, consistent with previous data (Ema et al., 1997; Koukourakis et al., 2002; Turner et al., 2002; Griffiths et al., 2008; Kim et al., 2009; Zimmer et al., 2010).

The current study indicates a requirement for further investigations into the molecular mechanisms and *in vivo* relevance of HIF-2α.

Conflicts of interest

The authors declare no conflict of interest.

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