

HIF-1 α promotes the stemness of oesophageal squamous cell carcinoma by activating the Wnt/ β -catenin pathway

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Abstract. Hypoxia-inducible-factor 1 α (HIF-1 α) is a marker for poor prognosis in the majority of the cancer types, and it has been revealed to be essential for maintaining cancer stem cells (CSCs). In the present study, it was determined that the expression of HIF-1 α and CSC-related genes under hypoxic conditions was upregulated. Stable knockdown of HIF-1 α significantly inhibited cell proliferation, migration and tumour growth *in vivo* in oesophageal squamous cell carcinoma (ESCC). A previous study revealed that the Wnt/ β -catenin pathway may play a key role in maintenance and progression of CSCs. Therefore, it was also revealed that stable knockdown of HIF-1 α reduced the formation of spheroid body cells, the expression of CSC-related genes and Wnt/ β -catenin pathway-related target genes, as well as the activity of the Wnt/ β -catenin pathway. Collectively, the present results indicated that HIF-1 α may regulate the stemness of ESCC by activating the Wnt/ β -catenin pathway.

Introduction

Oesophageal squamous cell carcinoma (ESCC) is one of the most highly malignant neoplasms of the digestive system. In China, the dominant histological subtype of oesophageal

cancer (EC) is ESCC, and it has a low 5-year survival rate after surgery, chemotherapy, radiotherapy and target therapy (1,2). Cancer stem cells (CSCs) have been defined as a small population of tumour cells that may contribute to tumour self-renewal, tumour maintenance, differentiation into heterogeneous lineages of cancer cells, tumour progression and resistance to therapies (3,4). Previous research on ESCC stem-like cells were related to the detection of side population (SP) cells in ESCC cell lines. Further studies identified that p75NTR, CD133, CD44, KLF4, ALDH1A1, OCT4, SOX2 and NANOG were oesophageal stem-like cancer cell markers that can maintain the self-renewal and progression ability of cancer cells. Concurrently, spheroid body cells could be enriched and maintain the stem-like cell characteristics of ESCC (5-10). In a previous study, our group demonstrated that ESCC KYSE450 spheroid body cells overexpressed the stemness genes SOX2, ALDH1A1 and NANOG, and were highly tumorigenic in xenograft models. In addition, it was revealed that Lgr5-positive spheroid body cells may represent a type of ESCC cancer stem cell, and Lgr5 may be an essential regulatory factor modulating stemness in ESCC (11).

Numerous studies have discovered that hypoxia promoted the self-renewal of embryonic stem cells, haematopoietic stem cells, neurospheres and maintained the proliferation of CSCs (12). Hypoxia-inducible factor 1 α (HIF-1 α) is an important transcription factor that regulates cell responses to hypoxia. Increased expression of HIF-1 α is associated with cancer cell metabolism, proliferation, invasiveness, angiogenesis and metastasis, and HIF-1 α is a marker for poor prognosis in the majority of cancer types (13,14). In addition, accumulating evidence supports the hypothesis that HIF-1 α is essential for maintaining CSCs and that the ability of CSCs and tumour-initiating cells exists in a hypoxic niche micro-environment (12,15). Mazumdar *et al* reported that HIF-1 α regulates the Wnt/ β -catenin signalling pathway in hypoxic embryonic stem cells by promoting β -catenin activation and the expression of downstream effectors (16). Furthermore, certain researchers revealed that the depletion of HIF-1 α decreased the expression of colon cancer stem cell markers and the Wnt/ β -catenin signalling transcriptional activity (17). The Wnt/ β -catenin signalling pathway has also been identified

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to play a critical role in the regulation, formation and maintenance of stemness in cancer stem cells (18). Previously, we revealed that *Lgr5* activated the Wnt/ β -catenin signalling pathway, leading to the progression of ESCC, and the related stem cell gene *Lgr5* also played a key role in maintaining the functions of ESCC stem-like cells through the Wnt/ β -catenin signalling pathway (11).

The present study examined the effects of stable HIF-1 α knockdown on the proliferation, migration and tumour growth *in vivo* of ESCC. Moreover, the role of stable HIF-1 α knockdown in the expression of CSC-related genes and spheroid body formation was investigated in serum-free medium at low adherence in ESCC cells. Subsequently, the activity of the Wnt/ β -catenin signalling pathway and the expression of Wnt/ β -catenin pathway-related target genes were detected in ESCC cells. The present results indicated that HIF-1 α promotes the stemness of ESCC by activating the Wnt/ β -catenin pathway.

Materials and methods

Cell lines and culture. The human ESCC cell lines KYSE450, KYSE70, Eca9706 and Eca109 were obtained from the Institute of the Chinese Academy of Medical Sciences. For normoxic conditions, all of the cell lines were cultured in RPMI-1640 supplemented with 10% foetal bovine serum (both from HyClone; GE Healthcare Life Sciences) at 37°C in a 5% CO₂ humidified incubator (Thermo Fisher Scientific, Inc.). For hypoxic conditions, the chemical anoxic agent CoCl₂ (Sigma-Aldrich; Merck KGaA) was used to induce hypoxia in ESCC using standard methods.

Lentivirus transfection assay. Lentivirus vectors encoding shRNA targeting HIF-1 α were constructed using the sequence 5'-TTCTCCGAACGTGTCACGT-3', and designed by GenePharma. Lentiviruses were packaged in ESCC by co-transfection with the plasmid pTOPFlash and the void plasmid pSuper. The supernatant was collected and concentrated 48 h after co-transfection. The lentivirus was transfected using an enhanced solution with 8 μ g/ml polybrene (Sigma-Aldrich; Merck KGaA), and then the packed lentivirus was added to cells with a multiplicity of infection (MOI) of 10. Following incubation for 12 h, the lentivirus solution was replaced with normal culture medium supplemented with 10% FBS. The infected cells were subcultured every 5-7 days.

Quantitative real-time polymerase chain reaction (qRT-PCR). Total RNA was isolated with TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.), and cDNA was synthesized with the PrimeScript RT Reagent kit (Takara Bio, Inc.). qRT-PCR was performed using a SYBR Green I kit (Roche Diagnostics) and was assessed in triplicate using Agilent Mx3005P. The following primers were used for HIF-1 α : forward, 5'-GAACGTCGAAAAGAAAAGTCTCG-3' and reverse, 5'-CCTTATCAAGATGCGAACTCACA-3'; β -actin: Forward, 5'-AGGCACCAGGGCGTGAT-3' and reverse, 5'-GCCACATAGGAATCCTTCTGAC-3'. Other primer sequences and additional PCR conditions are available upon request. PCR was conducted at 95°C for 15 min, followed by 40 cycles at 94°C for 15 sec, 55°C for 30 sec, and 64°C for

30 sec. Gene expression values were normalized to the house-keeping gene, and relative expression values were calculated based on the 2^{- $\Delta\Delta$ C_q} method (19).

Tissue patient sample collection. The ESCC tissues and normal esophageal squamous epithelial tissues were collected from patients who underwent surgical resection with curative intent at the First Affiliated Hospital of Henan University of Traditional Chinese Medicine. The study included 20 human tissues (9 females and 11 males, aged 46-75 years) collected from February 2018 to November 2018. The Institutional Ethics Review Board of The First Affiliated Hospital of Henan University of Traditional Chinese Medicine (Zhengzhou, China) approved the use of human samples and patients provided written informed consent.

Western blotting. ESCC cells, ESCC tissues and normal esophageal squamous epithelial tissues were lysed, and the total protein was extracted using lysis buffer (Beyotime Institute of Biotechnology, Haimen, China). A Nuclear and Cytoplasmic Protein Extraction kit (Sigma-Aldrich; Merck KGaA) was used to separate nuclear and cytoplasmic proteins. Total protein (20-40 mg) and nuclear protein extracts were separated by 10% SDS-PAGE and transferred onto PVDF membranes. After blocking with 5% skim milk at room temperature for 1 h, the primary antibodies were incubated overnight at 4°C. Then, the membranes were washed three times in TBST and incubated with a secondary antibody at room temperature for 1 h. Protein expression was quantified by densitometry (Quantity One software; Bio-Rad Laboratories, Hercules, CA, USA). The following primary antibodies were used: Anti-HIF-1 α (cat. no. 66730; dilution 1:10,000), anti-*Lgr5* (cat. no. 29025; dilution 1:1,000), anti-NANOG (cat. no. 14295; dilution 1:1,000), anti-p75NTR (cat. no. 55014; dilution 1:1,000), anti-ALDH1A1 (cat. no. 15910; dilution 1:1,000), anti-SOX2 (cat. no. 11064; dilution 1:1,000), anti- β -catenin (cat. no. 17565; dilution 1:2,000), anti-c-Myc (cat. no. 10828; dilution 1:2,000), anti-cyclin D1 (cat. no. 60186; dilution 1:2,000) and anti- β -actin (cat. no. 20536; dilution 1:2,000) (all antibodies from Proteintech Group Inc., Wuhan, China). Subsequently, the membranes were incubated with specific horseradish peroxidase-conjugated antibodies (cat. no. bs-2188R; dilution 1:5,000; from Beijing Biosynthesis Biotechnology Co., Ltd., Beijing, China) The immunoreactive proteins were detected using an enhanced chemiluminescence (ECL) detection system (Tanon Science and Technology Co., Ltd., Shanghai, China).

Culture of spheroid body cells. The ESCC cells were plated in 6-well ultra-low attachment plates (Corning, Inc.), and were incubated in serum-free DMEM/F12 medium (Invitrogen; Thermo Fisher Scientific, Inc.) supplemented with 20 ng/ml EGF (PeproTech, Inc.), B27 (1:50; Gibco; Thermo Fisher Scientific, Inc.), and 20 ng/ml bFGF (PeproTech, Inc.). Then the number and the formation rate of spheroid body cells were examined under light microscopy in ESCC cells after 7 days.

Cell proliferation and migration assay. Isolated cells were seeded into 96-well culture plates at 2,000 cells/well in three replicates for cell proliferation assays and then were incubated

for 24 h. Viable cells were counted by Cell Counting Kit-8 (CCK-8) (Dojindo Molecular Technologies, Inc.) at 24, 36, 48, 60 and 72 h. Subsequently, the cells were incubated with CCK-8 reagent for 1 h at 37°C. Colour intensity was assessed using a microplate reader set at 450 nm to obtain cell growth curves. For the migration assay, cells were seeded in the upper chamber of Transwell plates (BD Biosciences) with serum-free DMEM/F12. The lower chambers were filled with chemoattractant medium (DMEM/F12 plus 10% FBS). After 20 h of incubation at 37°C, the cells that did not migrate to the lower chamber were removed with a cotton swab, while the migrating cells were stained with 0.1% crystal violet after 36-48 h at room temperature and counted under a light microscope in five different fields.

Luciferase reporter assay. The 3'untranslated regions (UTRs) of HIF-1 α were amplified using the following primers: HIF-1 α -F, 5'-GATCTCGAGGCTTTTTCTTAATTTTCATTCCT-3' and HIF-1 α -R, 5'-GATGCGGCCGCGCCTGGTCCACAGAAGATGTTTA-3'. The ESCC cells (2x10⁵)/well were cultured in 24-well plates and co-transfected with 0.5 μ g of the TOP-flash or FOP-flash plasmid (Promega, Madison, WI, USA) using 2 μ l Lipofectamine 2000 transfection reagent (Invitrogen; Thermo Fisher Scientific, Inc.) in serum-free DMEM/F12 medium. After 6 h of transfection, the DMEM/F12 medium was replaced with DMEM/F12 medium containing 10% serum. Subsequently, the cells were lysed and luciferase activity was detected using the Dual-Luciferase reporter assay (Promega) system kit after 24 h of incubation. For each experiment, the assay was performed three times. *Renilla* luciferase values were then divided by the firefly luciferase activity values to normalize the difference in transfection efficiency.

In vivo xenograft tumourigenicity assay. Sixty 5-week-old male BALB/c nude mice with a body weight of ~15.5 g were housed under specific pathogen-free conditions and were cared at a temperature of 25°C with a humidity level of 40-60% with food and water provided *ad libitum* at the Laboratory Animal Centre of The First Affiliated Hospital of Henan Medical University of Traditional Chinese Medicine. The protocol of raising the animals was approved by the Institutional Ethics Review Board of the First Affiliated Hospital of Henan University of Traditional Chinese Medicine. A total of 1x10⁶ cancer cells were injected subcutaneously into 5-week-old female BALB/c nude mice (5 mice per group), and tumour size was determined with callipers by measuring the long and short diameters every 3 days. Animals were sacrificed by cervical dislocation after 4 weeks, then tumours were photographed, collected and measured by bodyweight.

Statistical analysis. The data represent the mean \pm SD from three independent experiments. The statistical analysis was performed using GraphPad Prism (GraphPad Software, Inc., La Jolla, CA, USA). The data were then analyzed using Student's t-test when only two groups were present. In addition, one-way analysis of variance (ANOVA) was used to assess comparison in three groups, and multiple comparison between the groups was performed by analyzing data with the Student-Newman-Keuls (SNK) method. All comparisons were performed relative to untreated groups, and significant differences are indicated as P<0.05 and P<0.01.

Results

HIF-1 α and CSC-related genes are upregulated in ESCC in hypoxic conditions. To detect the levels of HIF-1 α expression in ESCC cells, western blotting and qRT-PCR techniques were used to detect HIF-1 α expression in four ESCC cell lines (KYSE70, KYSE450, Eca109 and Eca9706). The results demonstrated that HIF-1 α expression was markedly higher in KYSE450 cells than in the other ESCC cell lines (Fig. 1A). Furthermore, it was revealed that the protein expression of HIF-1 α in hypoxic conditions (CoCl₂ treatment) was higher than in normoxic conditions in ESCC cell line KYSE450 (Fig. 1B). In addition, to assess the clinical importance of the expression of HIF-1 α , the protein level of HIF-1 α in ESCC tissues and normal esophageal squamous epithelial tissues was examined by western blotting. The results revealed that the protein level of HIF-1 α was increased in ESCC tissues, compared with normal esophageal squamous epithelial tissues (Fig. 1C). Subsequently, the expression levels of the CSC-related genes Lgr5, ALDH1A1, NANOG, SOX2 and p75NTR were evaluated in KYSE450 cells under normoxic and hypoxic conditions. Compared with expression levels in the normoxic environment, the mRNA and protein expression of CSC-related genes in hypoxic conditions were significantly enhanced in the ESCC cell line KYSE450 (Fig. 1D).

Stable knockdown of HIF-1 α decreases ESCC cell proliferation. Among the four ESCC cell lines, KYSE450 cells were used for further experiments. The proliferation of ESCC cells was detected by the CCK-8 assay. After transfection with the lentivirus, the results from the CCK-8 assay demonstrated that the proliferation rate of the HIF-1 α -knockdown cell group was inhibited compared to the control cell (CON) group and the negative control cell (NC) group on the third day (P<0.05) (Fig. 2).

Stable knockdown of HIF-1 α diminishes ESCC cell migration. To study whether HIF-1 α knockdown affects tumour migration, Transwell assays were performed to investigate the effects of HIF-1 α in ESCC cells. Cells from the HIF-1 α knockdown group, CON group and NC group were seeded in Transwell chambers and cultured for 24 h. A significant decrease in migration was observed in the HIF-1 α -knockdown cells compared to the migration of the cells in the CON group and the NC group (P<0.05). The migration rate of HIF-1 α knockdown cells was 65% lower than that of the cells in the CON group. The data indicated that HIF-1 α was involved in ESCC progression (Fig. 3).

Stable knockdown of HIF-1 α significantly reduces the stemness of ESCC cells. It has been revealed that stem cell properties in ESCC cells contribute to ESCC recurrence and metastasis. To investigate whether HIF-1 α plays a major role in ESCC stem cells, it was first determined that hypoxia facilitates the maintenance of ESCC stem cells. Mounting evidence demonstrates that CSCs can be enriched and maintained using spheroid body formation assay. Thus, ultra-low attachment surface plates and serum-free culture conditions were used to culture the spheroid bodies in the HIF-1 α -knockdown group, CON group and NC group. The results revealed that the number of

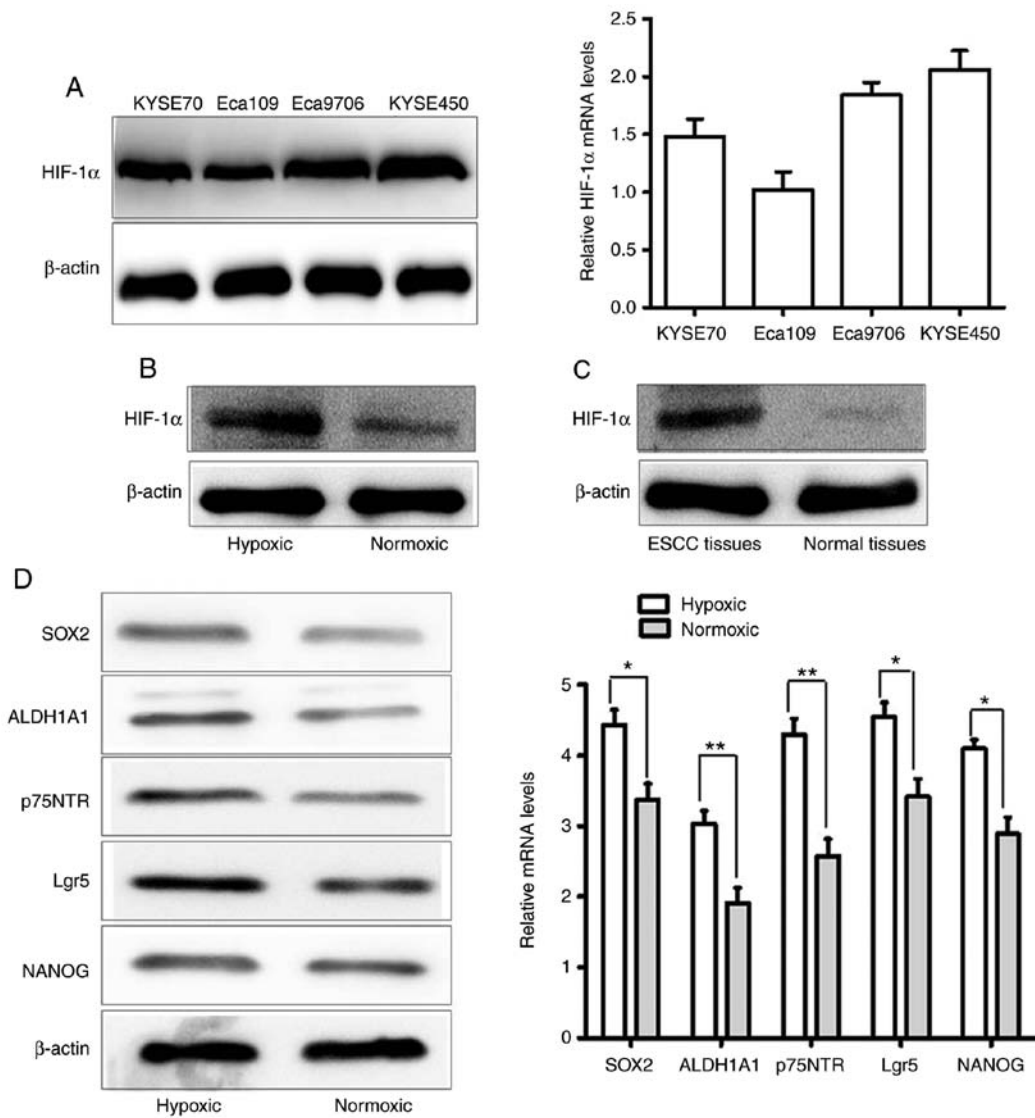


Figure 1. HIF-1 α and CSC-related genes are upregulated in ESCC in hypoxic conditions. (A) mRNA and protein levels of HIF-1 α in four ESCC cell lines (KYSE70, KYSE450, Eca109 and Eca9706). (B) The protein expression of HIF-1 α in hypoxic conditions (CoCl₂ treatment) was higher than in normoxic conditions in ESCC cell line KYSE450. (C) The protein level of HIF-1 α was elevated in ESCC tissues, compared with normal esophageal squamous epithelial tissues. (D) qRT-PCR and western blot analysis revealed that the mRNA and protein expression levels of the CSC-related genes Lgr5, ALDH1A1, NANOG, SOX2 and p75NTR in KYSE450 cells was increased under hypoxic conditions, compared with in normoxic conditions (* P <0.05 and ** P <0.01). HIF-1 α , hypoxia-inducible factor 1; CSC, cancer stem cell; ESCC, oesophageal squamous cell carcinoma.

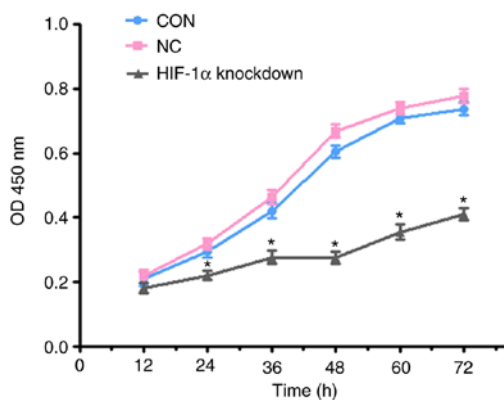


Figure 2. Stable knockdown of HIF-1 α decreases ESCC cell proliferation. A cell proliferation assay revealed that the proliferation rate of the HIF-1 α -knockdown cell group was inhibited compared to the CON group and the NC group on the third day (P <0.05). HIF-1 α , hypoxia-inducible factor 1; ESCC, oesophageal squamous cell carcinoma; CON, control cells; NC, negative control cells.

the spheroid body cells in the HIF-1 α knockdown group was markedly reduced, compared with CON group and NC group (Fig. 4A). Subsequently, the effect of HIF-1 α knockdown on the expression of CSC-related markers was examined by western blotting and qRT-PCR. The results revealed that knockdown of HIF-1 α decreased the protein and mRNA expression of the CSC-related genes Lgr5, ALDH1A1, NANOG, SOX2 and p75NTR compared with the CON group and the NC group. These data revealed that HIF-1 α may have an important function in promoting the stemness of ESCC (Fig. 4B).

Downregulated HIF-1 α directly suppresses the activity of the Wnt/ β -catenin pathway in ESCC cells. Accumulating evidence supports that the Wnt/ β -catenin pathway plays a major role in cancer cell proliferation, activation, and progression and the renewal of cancer stem cells. The Wnt/ β -catenin pathway could enter into the nucleus through β -catenin and combine

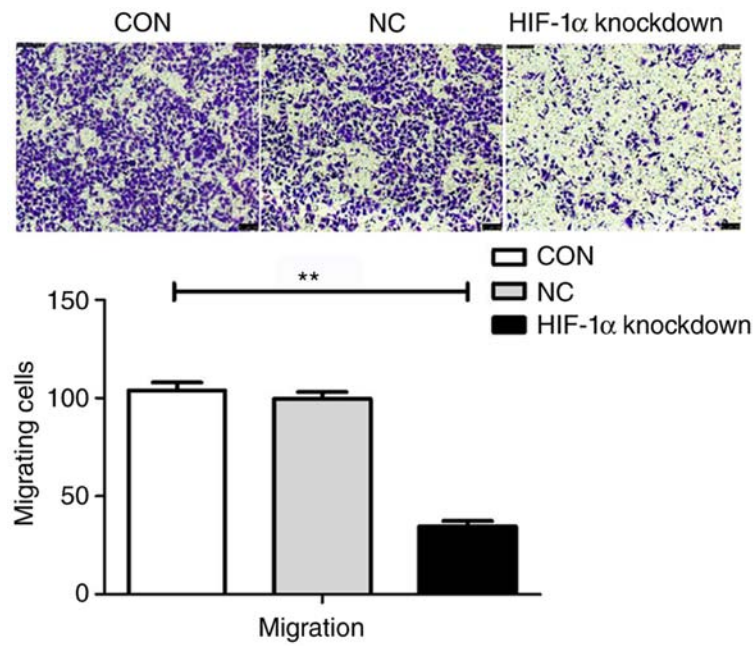


Figure 3. Stable knockdown of HIF-1 α diminishes ESCC cell migration. Transwell migration assays revealed that the migration rate of HIF-1 α -knockdown cells was 65% lower than the CON group (**P<0.01). HIF-1 α , hypoxia-inducible factor 1; ESCC, oesophageal squamous cell carcinoma; CON, control cells; NC, negative control cells.

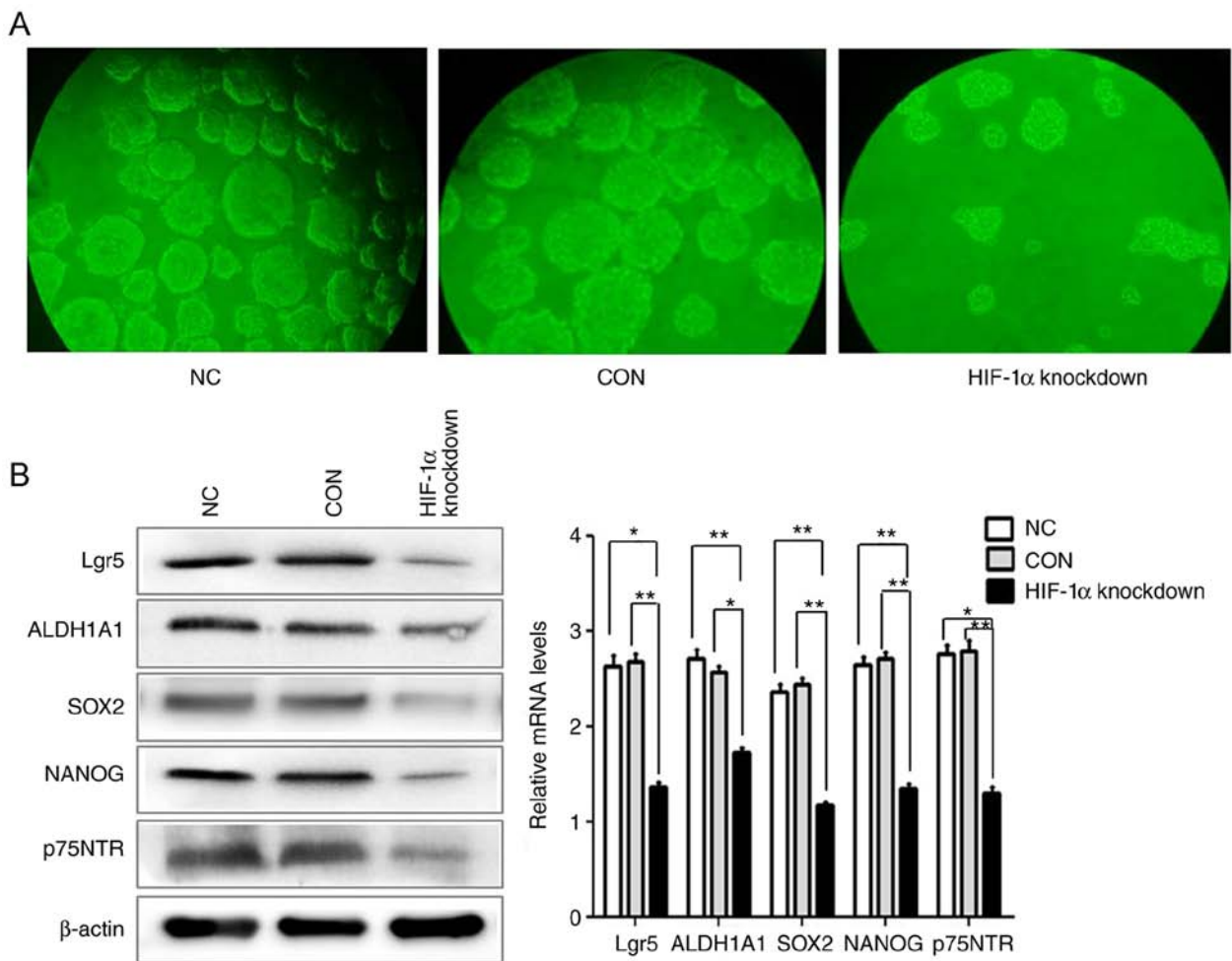


Figure 4. Stable knockdown of HIF-1 α significantly reduces the stemness of ESCC cells. (A) The number of spheroid body cells in the HIF-1 α -knockdown group was reduced significantly, compared with CON group and NC group. (B) Protein and mRNA levels of the CSC-related genes Lgr5, ALDH1A1, NANOG, SOX2 and p75NTR were reduced in the HIF-1 α -knockdown group compared with the expression levels in the CON and NC group (*P<0.05 and **P<0.01). HIF-1 α , hypoxia-inducible factor 1; ESCC, oesophageal squamous cell carcinoma; CON, control cells; NC, negative control cells.

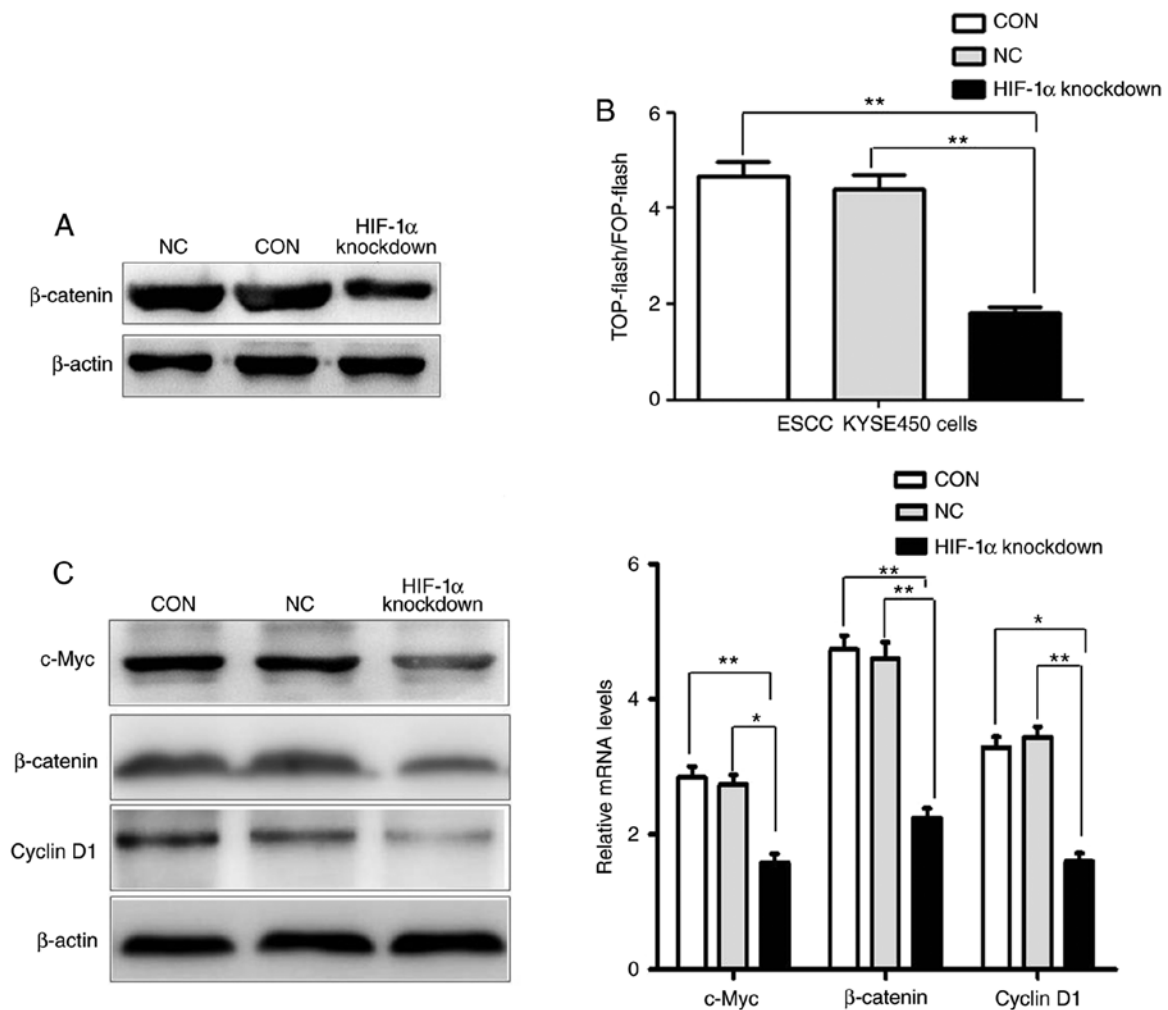


Figure 5. Downregulated HIF-1 α directly suppresses the activity of the Wnt/ β -catenin pathway in ESCC cells. (A) Knockdown of HIF-1 α in ESCC cells decreased the protein expression of nuclear accumulation of β -catenin as revealed by subcellular fractionation assays. (B) A TOP-flash and FOP-flash dual luciferase reporter assay revealed that transcriptional activity of TCF/LEF transcription factors in the HIF-1 α -knockdown group of cells was significantly reduced, compared with the CON group and NC group. (C) The total protein and mRNA expression of β -catenin and important target genes cyclin D1 and c-Myc in the Wnt/ β -catenin pathway were decreased in the HIF-1 α -knockdown group of cells, compared with the expression in the CON group and the NC group ($P < 0.05$ and $^{**}P < 0.01$). HIF-1 α , hypoxia-inducible factor 1; ESCC, oesophageal squamous cell carcinoma; CON, control cells; NC, negative control cells.

with TCF/LEF transcription factors, thereby activating transcription of important target genes cyclin D1 and c-Myc. Thus, subcellular fractionation assays were used to confirm that the knockdown of HIF-1 α in ESCC cells decreased the protein expression of nuclear accumulation of β -catenin (Fig. 5A). Furthermore, the TOP-flash and FOP-flash dual luciferase reporter assay revealed that transcriptional activity of TCF/LEF transcription factors in the HIF-1 α -knockdown group of cells was significantly reduced, compared with the CON group and NC group (Fig. 5B). Moreover, the total protein and mRNA expression of β -catenin and important target genes cyclin D1 and c-Myc in the Wnt/ β -catenin pathway were decreased in the HIF-1 α -knockdown group of cells compared with the expression in the CON group and the NC group (Fig. 5C). Collectively, our data indicated that knockdown of HIF-1 α could suppress the activity of the Wnt/ β -catenin pathway in ESCC cells.

Stable knockdown of HIF-1 α inhibits tumour growth in vivo. To further evaluate the role of HIF-1 α in the progression of

ESCC, tumour models were established. The HIF-1 α knockdown cells and CON cells were injected subcutaneously into the dorsum of the BALB/c male nude mice. Tumour growth in the mice was measured weekly after implantation. After four weeks, the mice were euthanized, and the tumours were removed and weighed. As revealed in Fig. 6, compared with the CON cells, the mean volumes and the growth rate of the tumour treated with HIF-1 α -knockdown cells exhibited a decrease in the BALB/c male nude mice (Fig. 6).

Discussion

Hypoxia-inducible factors (HIFs) are a crucial pathological feature of all solid tumours and play a key role in cancer proliferation, migration, progression, metabolic reprogramming, angiogenesis, poor clinical prognosis and CSC maintenance (20–22). HIF-1 α is a key transcriptional factor that responds to hypoxia, and accumulating data indicate that HIF activation is stimulated by increased transcriptional and translational activity of HIF-1 α (23). Consistent with these

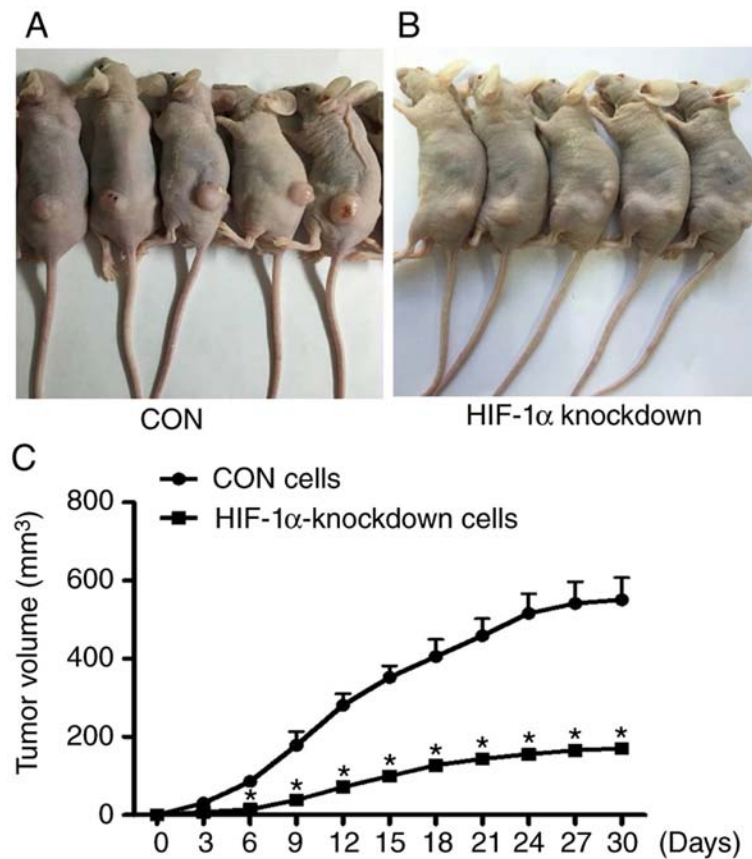


Figure 6. Stable knockdown of HIF-1 α inhibits tumour growth *in vivo*. (A and B) HIF-1 α -knockdown cells and CON cells were injected subcutaneously into the dorsum of BALB/c male nude mice. Tumours developed four weeks post-cell injection. (C) The average tumour volume was plotted every 3 days (*P<0.05). HIF-1 α , hypoxia-inducible factor 1; CON, control cells.

functions, increased HIF-1 α expression has been observed in various human cancer cell types and has been associated with cancer proliferation, migration, progression and poor prognosis in many cases. Conley *et al* revealed that the expression of HIF-1 α increased under hypoxic conditions in breast cancer (24). In this respect, it was determined in the present study that HIF-1 α expression was markedly higher in KYSE450 cells than in other ESCC cell lines, and compared with a normoxic environment, the protein expression level of HIF-1 α under hypoxic conditions was upregulated significantly in the ESCC cell line KYSE450. In addition, it was demonstrated that the protein expression of HIF-1 α was markedly higher in ESCC tissues than normal esophageal squamous epithelial tissues.

Furthermore, previous studies revealed that knockdown of HIF-1 α resulted in lower rates of proliferation, migration, tumour metabolism and tumorigenic activity in colon cancer cell lines. Researchers have revealed that HIF-1 α plays an important role in the progression of colon cancer (17,25). In the present study, it was demonstrated that the proliferation rate of the HIF-1 α -knockdown group of ESCC cells was inhibited compared with the CON group cells and NC group cells. It was also revealed that the migration rate of HIF-1 α -knockdown ESCC cells was 65% lower than that of the CON group and NC group cells. These data indicated that HIF-1 α was involved in the modulation of ESCC proliferation and progression.

Numerous studies have indicated that hypoxia may result in poor clinical outcomes in cancers through enhanced

survival, self-renewal, and tumorigenesis abilities of the CSC subpopulation. Previous studies have revealed that hypoxia could induce the CSC phenotype in breast and colon cancer, as well as glioma and pancreatic cancer through the activity of HIFs (12,26-28). Xiang *et al* demonstrated that HIF-1 α could stimulate both the expression and activity of TAZ, a transcriptional co-activator that is required for maintenance of breast cancer stem cells. They also observed that suppression of HIF-1 α decreased the expression of CSC-related genes, tumour angiogenesis, tumour proliferation and tumorigenicity (29). Qiang *et al* revealed that HIF-1 α enhanced the self-renewal activity of CD133-positive glioblastoma cells and inhibited the induction of glioblastoma stem cell differentiation. They determined that HIF-1 α may play an important role in the hypoxia-mediated maintenance of glioblastoma stem cells partly due to its interaction with NICD (30). A previous study revealed that SOX2, ALDH1A1, NANOG, p75NTR and Lgr5 may be CSC-related genes in ESCC, and spheroid body cell culture is used to enrich and identify potential CSCs (8-11). Emerging evidence has indicated that CSCs contribute to tumour maintenance, tumour progression, migration and tumorigenicity (4,31,32). To investigate the biological role of HIF-1 α in ESCC CSCs, it was observed that knockdown of HIF-1 α decreased the number of spheroid body cells, and suppressed the protein and mRNA expression of the CSCs-related genes Lgr5, ALDH1A1, NANOG, p75NTR and SOX2, compared with the CON group and NC group cells. In addition, it was also

observed that HIF-1 α -knockdown cells exhibited a decreased growth rate of tumours *in vivo*. Collectively, these data revealed that HIF-1 α plays an important role in regulating the stemness of ESCC.

Accumulating evidence supports the hypothesis that the Wnt/ β -catenin signalling pathway plays a critical role in the regulation, formation, renewal and maintenance of stem cells and cancer stem cells (18,33). Similar to the results from our previous study, the Wnt/ β -catenin signalling pathway may play a key role in the maintenance and progression of ESCC stem cells. Certain researchers have revealed that HIF-1 α depletion not only decreases β -catenin protein levels and nuclear β -catenin levels, but also decreases β -catenin transcriptional activity in colon cancer cells. Finally, researchers have revealed that HIF-1 α is essential for the maintenance of the stemness and malignancy of colon cancer cells by activating the Wnt/ β -catenin signalling pathway (17). β -catenin is a crucial signalling molecule in the Wnt/ β -catenin signalling pathway that functions as a transcriptional factor to activate the expression of cell proliferation, migration, and important target genes, such as cyclin D1 and c-Myc (34-36). In the present study, it was observed that the knockdown of HIF-1 α in ESCC cells decreased the expression of nuclear accumulation of β -catenin. In addition, the TOP-flash and FOP-flash dual luciferase reporter assay revealed that transcriptional activity of TCF/LEF transcription factors in HIF-1 α -knockdown group of cells was significantly reduced, compared with the CON group and NC group. Furthermore, the total protein and mRNA expression of β -catenin and the Wnt/ β -catenin pathway-related target genes cyclinD1 and c-Myc were decreased in the HIF-1 α -knockdown group of cells compared with the CON group and NC group of cells. This indicated that knockdown of HIF-1 α could reduce the activity of Wnt/ β -catenin pathway in ESCC cells.

Collectively, the present results demonstrated that HIF-1 α -induced activation of the Wnt/ β -catenin pathway is essential for self-renewal, tumourigenesis and progression of ESCC stem cells. These results could serve as a foundation for the study of new targets for the development of potential targeted therapeutic strategies by inhibiting the expression of HIF-1 α in ESCC. In addition, the further exploration of the impact of HIF-1 α in ESCC stem cells, and the relationship of ESCC stem cell-related genes and HIF-1 α is required.

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Availability of data and materials

The datasets used during the present study are available from the corresponding authors upon reasonable request.

Authors' contributions

Collection, analysis and drafting the manuscript was performed by ZL, XQC and RDL. Analysis and interpretation of figures and data was performed by XQR, BW and LFL. Statistical analysis was conducted by XQC, YSG and XJC. Revision of manuscript for important intellectual content was performed by XQR and XQC. All authors read and approved the final manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

The human and animal protocols were approved by the Institutional Ethics Review Board of The First Affiliated Hospital of Henan University of Chinese Medicine, Zhengzhou, Henan, China. Written informed consent was obtained from all patients and consent for the publication of the human tissues from all patients who were involved in the present study.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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