Inhibition of hypoxia inducible factor 1α expression suppresses the progression of esophageal squamous cell carcinoma

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Key words: hypoxia inducible factor 1α, esophageal squamous cell carcinoma, gene silencing, proliferation, apoptosis, YC-1

Hypoxia inducible factor 1α (HIF-1α) is highly expressed and is implicated in the progression of esophageal squamous cell carcinoma. To investigate the potential mechanism by which HIF-1α contributes to the progression of esophageal squamous cell carcinoma, here we established stable esophageal carcinoma cell lines Eca-109 and TE-13 in which HIF-1α was depleted by shRNA mediated gene silencing. In addition, we used specific inhibitor YC-1 to inhibit HIF-1α expression. Our in vitro studies demonstrated that shRNA or chemical mediated inhibition of HIF-1α led to reduced proliferation and increased apoptosis of esophageal carcinoma cells, as well as the downregulation of VEGF, MMP2 and BCL2. Furthermore, we employed xenograft nude mice model to validate the in vitro findings and proved that depletion of HIF-1α suppressed the tumorigenicity of esophageal carcinoma cells in vivo. In conclusion, our results provide new insight into the potential role of HIF-1α in esophageal squamous cell carcinoma and open up the possibility of inhibiting HIF-1α for targeted therapy of esophageal squamous cell carcinoma.

Introduction

Hypoxia is a typical aspect of locally advanced solid tumors due to the imbalance between oxygen supply and consumption. Tumor blood vessel formation often lags behind the growth of tumor tissue, leading to the formation of hypoxic microenvironment. Accumulating evidence has shown that hypoxic microenvironment can promote tumor progression because the tumor cells under this condition show malignant phenotypes such as higher rate of proliferation, improved metastasis potential and stronger antiapoptosis ability. Hypoxia inducible factor 1 (HIF-1) plays an important role in mediating the adaptation and tolerance of tumor cells to hypoxic microenvironment. HIF-1 is composed of HIF-1α and HIF-1β subunits. Under hypoxia, HIF-1α subunit is accumulated and translocated to the nucleus where it forms HIF-1 heterodimer with HIF-1β and binds hypoxia response elements to activate the transcription of downstream genes. Among the HIF-1α target genes, vascular endothelial growth factor (VEGF) is important to increase the immediate availability of oxygen from capillaries through increased vascular permeability and induce the formation of new vessels. BCL2 contributes to the resistance of hypoxic cells to apoptosis. Matrix metalloproteinase 2 (MMP2) is a member of the matrix metalloproteinase family that plays crucial role in tumor invasion and metastasis. Recent study reported that hypoxia promotes the invasiveness of human pancreatic cancer cells by upregulation of MMP2. Consequently, HIF-1α contributes to tumor metastasis and angiogenesis and the overexpression of HIF-1α has been documented in a wide range of cancers with a negative impact on their therapy and prognosis. Among gastrointestinal cancer, esophageal squamous cell carcinoma has a high incidence in East Asia. The patients are often diagnosed at later stages and the tumor is hard to be eradicated by surgery, is resistant to radiation therapy and has a poor prognosis. Several studies have demonstrated that the expression of HIF-1α in esophageal squamous cell carcinoma tissues is higher than surrounding normal tissues, and that high HIF-1α expression is closely related to the angiogenesis, lymph node metastasis and resistance to radiotherapy and chemotherapy of esophageal squamous cell carcinoma.

Given the crucial role of HIF-1α in tumorigenesis, great efforts have been made to screen potential inhibitors of HIF-1α. 2,3-(5’-hydroxymethyl-2’-furyl)-1-benzylindazole (YC-1) is able to suppress both protein synthesis and the stability of HIF-1α and demonstrate antitumor effect. Notably, YC-1 can inhibit the transcription of HIF-1α and the expression of hypoxia-related downstream targets, significantly reducing the tolerance of tumor cell to hypoxia. In this study, we aim to investigate the potential mechanism by which HIF-1α contributes to the progression of esophageal squamous cell carcinoma and evaluate
the therapeutic effects achieved by inhibition of HIF-1α. We employed both shRNA and YC-1 to inhibit the expression of HIF-1α in esophageal carcinoma cell lines Eca109 and TE13, and observed reduced proliferation and increased apoptosis of esophageal carcinoma cells. Moreover, inhibition of HIF-1α led to the downregulation of HIF-1α targets VEGF, MMP2 and BCL2, which may contribute to the cell phenotypes we observed. Finally, we employed xenograft nude mice model to validate the in vitro findings and proved that the depletion of HIF-1α suppressed the tumorigenicity of esophageal carcinoma cells in vivo.

Results

Silencing of HIF-1α in esophageal carcinoma cells. To evaluate whether HIF-1α shRNA vectors could lead to HIF-1α silencing in esophageal carcinoma cells, we performed protein gel blot analysis to detect the HIF-1α protein level in esophageal carcinoma cells Eca109 and TE13 transfected with vectors harboring different targeting sequences against HIF-1α. The results demonstrated that compared to untransfected cells or cells transfected with HIF-1α Neo control shRNA vector, HIF-1α level was significantly decreased in pGCsi-HIF-1α 3 shRNA transfected cells both in normoxia and hypoxia conditions (Fig. 1A). Since pGCsi-HIF-1α 1 and 2 shRNA vectors did not show strong inhibitory effect on HIF-1α expression as pGCsi-HIF-1α 3 shRNA vector (data not shown), we employed Eca109 and TE13 cells stably transfected with pGCsi-HIF-1α 3 shRNA vector for the following experiments and named them as Eca109 HIF-1α shRNA and TE13 HIF-1α shRNA. The cells stably transfected with pGCsi negative control shRNA vector were named as Eca109 HIF-1α Neo and TE13 HIF-1α Neo, which served as negative controls for the following experiments.

To verify that shRNA-mediated silencing of HIF-1α has biological effects in esophageal carcinoma cells, we detected the expression of HIF-1α targets VEGF and MMP2 by protein gel blot. As expected, hypoxia induced the upregulation of VEGF, MMP2 and p53 expression (Fig. 1B). However, HIF-1α knockdown significantly inhibited both basic level expression and hypoxia-induced expression of VEGF and MMP2. In contrast, p53 levels at both normoxia and hypoxia conditions were upregulated upon HIF-1α knockdown (Fig. 1B). Collectively, these data prove that HIF-1α shRNA could inhibit the expression of HIF-1α and its targets in esophageal carcinoma cells.

HIF-1α knockdown inhibits the proliferation of esophageal carcinoma cells. MTT assay demonstrated that Eca109 HIF-1α shRNA and TE13 HIF-1α shRNA cells proliferated much slower than normal Eca109 and TE13 cells or Eca109 HIF-1α Neo and TE13 HIF-1α Neo cells (Fig. 2). On the 7th day, HIF-1α
To provide the apoptosis rate (Fig. 4C) α 11,12). These results suggest that HIF-1α could promote the proliferation of esophageal carcinoma cells (p < 0.005).

HIF-1α knockdown promotes the apoptosis of esophageal carcinoma cells. The apoptosis rate in different groups of esophageal carcinoma cells was determined by Annexin V-FITC as follows: in normoxia condition, 8.8 ± 1.21% (Eca109), 14.1 ± 2.35% (Eca109 HIF-1α Neo), 25.8 ± 4.32% (Eca109 HIF-1α shRNA), 1.5 ± 0.11% (TE13), 1.7 ± 0.09% (TE13 HIF-1α Neo) and 44.2 ± 5.21% (TE13 HIF-1α shRNA); in hypoxia condition, 10.8 ± 1.32% (Eca109), 13.5 ± 2.74% (Eca109 HIF-1α Neo), 27.7 ± 4.48% (Eca109 HIF-1α shRNA), 1.3 ± 0.09% (TE13), 1.5 ± 0.08% (TE13 HIF-1α Neo) and 54.7 ± 6.35% (TE13 HIF-1α shRNA) (Fig. 3). Compared to control groups, the apoptosis rate in HIF-1α knockdown cells was significantly increased in both normoxia and hypoxia conditions. These data indicate that HIF-1α has inhibitory effect on apoptosis of esophageal carcinoma cells (p < 0.05).

HIF-1α inhibitor YC-1 inhibits the proliferation and promotes the apoptosis of esophageal carcinoma cells. To provide further evidence that HIF-1α modulates the proliferation and apoptosis of esophageal carcinoma cells, we employed a chemical inhibitor YC-1 to inhibit HIF-1α expression in Eca109 cells and examined the consequent biological effects. The results demonstrated that YC-1 inhibited the proliferation of Eca109 in a dose and time dependent manner (Fig. 4A). In addition, treatment of Eca109 cells in normoxia condition with 20 μmol/L YC-1 for 24 h significantly increased the apoptosis rate to 16.2 ± 3.89% compared to 7.6 ± 1.78% in the absence of treatment. In hypoxia condition, the apoptosis rate was further increased to 32.9 ± 5.82% after YC-1 treatment compared to 10.3 ± 2.46% in the absence of treatment (Fig. 4B). Lastly, we examined the expression of HIF-1α, VEGF, MMP2 and BCL2 in Eca109 cells treated by YC-1 and found that YC-1 inhibited both basic level expression and hypoxia-induced expression of HIF-1α, VEGF, MMP2 and BCL2 in Eca109 cells (Fig. 4C). Taken together, these results suggest that YC-1 inhibits the proliferation and promotes the apoptosis of esophageal carcinoma cells by downregulating the expression of HIF-1α, VEGF, MMP2 and BCL2 (p < 0.05).

HIF-1α knockdown inhibits the tumorigenicity of Eca109 cells in vivo. To complement the above in vitro studies with in vivo studies, we established xenograft tumors by subcutaneous injection of HIF-1α knockdown Eca109 cells or the corresponding control cells into the flanks of BALB/c nude mice. The time for tumor formation was 7 ± 1.2 days for Eca109 cells, 8 ± 1.6 days for Eca109 HIF-1α Neo cells, and 14 ± 3.1 days for Eca109 HIF-1α shRNA cells. Furthermore, the size of tumor derived from Eca109 HIF-1α shRNA cells was significantly smaller (0.737 ± 0.138 cm3, 4 weeks after tumor formed) than those derived from Eca109 cells (1.479 ± 0.288 cm3, 4 weeks after tumor formed) and Eca109 HIF-1α Neo cells (1.322 ± 0.122 cm3, 4 weeks after tumor formed) (Fig. 5A) (p < 0.01).

We also examined the effects of HIF-1α knockdown on the expression of HIF-1α, VEGF, MMP2 and BCL2 in vivo. Protein gel blot analysis demonstrated that the expression levels of HIF-1α, VEGF, MMP2 and BCL2 were significantly lower in tumor tissues derived from Eca109 HIF-1α shRNA cells than those derived from Eca109 and Eca109 HIF-1α Neo control cells (Fig. 5B). These in vivo studies support the in vitro findings and prove that HIF-1α knockdown inhibits the tumorigenicity of Eca109 cells in vivo.

Discussion

The present study demonstrated that shRNA or chemical mediated inhibition of HIF-1α expression led to reduced proliferation and increased apoptosis of esophageal carcinoma cells, as well as the downregulation of VEGF, MMP2 and BCL2 expression. Furthermore, we employed xenograft nude mice model to validate the in vitro findings and proved that shRNA mediated HIF-1α knockdown suppressed the tumorigenicity of esophageal carcinoma cells in vivo.

Hypoxia is an important factor in the pathogenesis of solid tumors and HIF-1 functions as the most critical factor for hypoxia tolerance in various malignant diseases. HIF-1α expression is low in normoxia but is rapidly upregulated upon hypoxia. In addition, various factors such as heavy metals, nitric oxide, reactive oxygen species promote the expression of HIF-1α and increase its stability.11,12 Stabilized HIF-1α would translocate into the nucleus to bind to hypoxia response element (HRE) in target genes to drive their transcription. VEGF, MMP-2 and BCL2 are known HIF-1α target genes and are crucially involved in cell proliferation and apoptosis, angiogenesis, invasion and metastasis, thus mediating the function of HIF-1α in tumor progression.13

Recent studies have demonstrated that the inhibition of HIF-1α expression could inhibit the development of liver,
In the present study we found that HIF-1α shRNA or YC-1 inhibited the expression of VEGF in vitro. Furthermore, HIF-1α shRNA inhibited the expression of VEGF in xenografted tumors. These data suggest that HIF-1α shRNA mediated inhibition of esophageal squamous cell carcinoma is at least partially due to the downregulation of VEGF expression.

Tumor invasion and metastasis crucially depend on MMPs. Under hypoxic conditions, the invasive ability of tumor cells is greatly enhanced due to hypoxia-induced upregulation of MMPs. MMP2 and MMP9 have been reported to be highly expressed in esophageal tumor tissues. In this study, both in vitro and in vivo results showed that MMP2 expression was decreased following the inhibition of HIF-1α, suggesting that downregulation of MMP2 contributes to the antitumor effect.

Angiogenesis is a very important feature of malignant tumors. HIF-1α mediated VEGF expression has been shown to be important for the angiogenesis of esophageal squamous cell carcinoma. In the present study we found that HIF-1α shRNA or YC-1 inhibited the expression of VEGF in vitro. Furthermore, HIF-1α shRNA inhibited the expression of VEGF in xenografted tumors. These data suggest that HIF-1α shRNA mediated inhibition of esophageal squamous cell carcinoma is at least partially due to the downregulation of VEGF expression.

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![Figure 3. shRNA mediated HIF-1α knockdown promotes the apoptosis of esophageal carcinoma cells. Eca109, TE13 and their derived stable cells as indicated were cultured under normoxia or hypoxia condition and the apoptosis was detected by Annexin V staining.](image)

stomach, lung and other tumors, indicating that targeting HIF-1α appears to be effective for cancer treatment. While HIF-1α expression has been reported to be correlated with the tumor stage and lymph node metastasis of esophageal squamous cell carcinoma, few studies have investigated the biological effects of inhibiting HIF-1α in esophageal squamous cell carcinoma. In this study we employed both shRNA and chemical inhibitor to inhibit HIF-1α expression in esophageal squamous cell carcinoma cells and both in vitro and in vivo results demonstrate that inhibition of HIF-1α expression could inhibit the development of esophageal squamous cell carcinoma.
of HIF-1α shRNA and YC-1 in esophageal squamous cell carcinoma.

Our data also suggest that HIF-1α shRNA and YC-1 may inhibit the proliferation of esophageal carcinoma cells through promoting apoptosis. HIF-1α is known to upregulate the expression of anti-apoptotic proteins such as BCL2 and HIF-1α in hypoxia can promote tumor progression by preventing apoptosis and thus facilitating cell proliferation.20,21 In the present study we found that both HIF-1α shRNA interference or YC-1 treatment of esophageal squamous cell carcinoma cells inhibited the expression of BCL2, which may result in the increased apoptosis we observed. It is noteworthy that esophageal squamous cell carcinoma cells treated by YC-1 demonstrated much higher apoptosis rate under hypoxia than under normoxia. This may be due to the cytotoxicity of YC-1.22,23

In conclusion, our results demonstrate that HIF-1α is highly expressed in esophageal squamous cell carcinoma cells and shRNA mediated HIF-1α knockdown or inhibition of HIF-1α by YC-1 reduced the proliferation, increased the apoptosis and decreased the in vivo tumorigenicity of esophageal carcinoma cells at least partially by downregulating the expression of VEGF, MMP2 and BCL2. Therefore, our study provides new insight into the potential role of HIF-1α in esophageal squamous cell carcinoma and opens up the possibility of inhibiting HIF-1α for targeted therapy of esophageal squamous cell carcinoma.

**Materials and Methods**

**Cell culture.** Eca109 and TE13 esophageal squamous cell carcinoma cell lines were purchased from Shanghai Institute of Cell Biology and cultured in DMEM high glucose medium and 1640 high glucose medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 μg/mL streptomycin, respectively. The cells were normally cultured at 37°C with 5% CO₂ and 95% air incubator. Hypoxia treatment was performed by culturing the cells of the logarithmic growth phase in the anaerobic incubator (5% CO₂ and 95% N₂, the oxygen concentration less than 0.1%) for 24 h.

**HIF-1α shRNA constructs.** Based on the sequence of human HIF-1α (Genbank No. NM001530), three target sequences and one negative control (Neo) sequence were synthesized and cloned into pGCsi vector to make pGCsi-HIF-1α 1, pGCsi-HIF-1α 2 and pGCsi-HIF-1α 3 shRNA constructs or HIF-1α Neo construct. The detailed sequences were as follows: (1) 5’-GAT CCC GAG GAA GAA CTA TGA ACA TTA TTC AAG AGA TTA TGT TCA TAG TTC TTC CTC TTT TTG GAT-3’ and 5’-AGC TAT CCA AAA AGA GGA AGA ACT ATG AAC ATA ATC TCT TGA ATT GTG GAT-3’ and 5’-AGC TAT CCA AAA AGA GGA AGA ACT ATG AAC ATA ATC TCT TGA ATT GTG GAT-3’ and 5’-AGC TAT CCA AAA AGA GGA AGA ACT ATG AAC ATA ATC TCT TGA ATT GTG GAT-3’ and 5’-AGC TAT CCA AAA AGA GGA AGA ACT ATG AAC ATA ATC TCT TGA ATT GTG GAT-3’ and 5’-AGC TAT CCA AAA AGA GGA AGA ACT ATG AAC ATA ATC TCT TGA ATT GTG GAT-3’ and 5’-AGC TAT CCA AAA AGA GGA AGA ACT ATG AAC ATA ATC TCT TGA ATT GTG GAT-3’ and 5’-AGC TAT CCA AAA AGA GGA AGA ACT ATG AAC ATA ATC TCT TGA ATT GTG GAT-3’ and 5’-AGC TAT CCA AAA AGA GGA AGA ACT ATG AAC ATA ATC TCT TGA ATT GTG GAT-3’ and 5’-AGC TAT CCA AAA AGA GGA AGA ACT ATG AAC ATA ATC TCT TGA ATT GTG GAT-3’ and 5’-AGC TAT CCA AAA AGA GGA AGA ACT ATG AAC ATA ATC TCT TGA ATT GTG GAT-3’ and 5’-AGC TAT CCA AAA AGA GGA AGA ACT ATG AAC ATA ATC TCT TGA ATT GTG GAT-3’ and 5’-AGC TAT CCA AAA AGA GGA AGA ACT ATG AAC ATA ATC TCT TGA ATT GTG GAT-3’ and 5’-AGC TAT CCA AAA AGA GGA AGA ACT ATG AAC ATA ATC TCT TGA ATT GTG GAT-3’ and 5’-AGC TAT CCA AAA AGA GGA AGA ACT ATG AAC ATA ATC TCT TGA ATT GTG GAT-3’ and 5’-AGC TAT CCA AAA AGA GGA AGA ACT ATG AAC ATA ATC TCT TGA ATT GTG GAT-3’ and 5’-AGC TAT CCA AAA AGA GGA AGA ACT ATG AAC ATA ATC TCT TGA ATT GTG GAT-3’ and 5’-AGC TAT CCA AAA AGA GGA AGA ACT ATG AAC ATA ATC TCT TGA ATT GTG GAT-3’ and 5’-AGC TAT CCA AAA AGA GGA AGA ACT ATG AAC ATA ATC TCT TGA ATT GTG GAT-3’ and 5’-AGC TAT CCA AAA AGA GGA AGA ACT ATG AAC ATA ATC TCT TGA ATT GTG GAT-3’ and 5’-AGC TAT CCA AAA AGA GGA AGA ACT ATG AAC ATA ATC TCT TGA ATT GTG GAT-3’ and 5’-AGC TAT CCA AAA AGA GGA AGA ACT ATG AAC ATA ATC TCT TGA ATT GTG GAT-3’ and 5’-AGC TAT CCA AAA AGA GGA AGA ACT ATG AAC ATA ATC TCT TGA ATT GTG GAT-3’ and 5’-AGC TAT CCA AAA AGA GGA AGA ACT ATG AAC ATA ATC TCT TGA ATT GTG GAT-3’ and 5’-AGC TAT CCA AAA AGA GGA AGA ACT ATG AAC ATA ATC TCT TGA ATT GTG GAT-3’ and 5’-AGC TAT CCA AAA AGA GGA AGA ACT ATG AAC ATA ATC TCT TGA ATT GTG GAT-3’ and 5’-AGC TAT CCA AAA AGA GGA AGA ACT ATG AAC ATA ATC TCT TGA ATT GTG GAT-3’ and 5’-AGC TAT CCA AAA AGA GGA AGA ACT ATG AAC ATA ATC TCT TGA ATT GTG GAT-3’ and 5’-AGC TAT CCA AAA AGA GGA AGA ACT ATG AAC ATA ATC TCT TGA ATT GTG GAT-3’ and 5’-AGC TAT CCA AAA AGA GGA AGA ACT ATG AAC ATA ATC TCT TGA ATT GTG GAT-3’ and 5’-AGC TAT CCA AAA AGA GGA AGA ACT ATG AAC ATA ATC TCT TGA ATT GTG GAT-3’ and 5’-AGC TAT CCA AAA AGA GGA AGA ACT ATG AAC ATA ATC TCT TGA ATT GTG GAT-3’ and 5’-AGC TAT CCA AAA AGA GGA AGA ACT ATG AAC ATA ATC TCT TGA ATT GTG GAT-3’ and 5’-AGC TAT CCA AAA AGA GGA AGA ACT ATG AAC ATA ATC TCT TGA ATT GTG GAT-3’ and 5’-AGC TAT CCA AAA AGA GGA AGA ACT ATG AAC ATA ATC TCT TGA ATT GTG GAT-3’ and 5’-AGC TAT CCA AAA AGA GGA AGA ACT ATG AAC ATA ATC TCT TGA ATT GTG GAT-3’ and 5’-AGC TAT CCA AAA AGA GGA AGA ACT ATG AAC ATA ATC TCT TGA ATT GTG GAT-3’ and 5’-AGC TAT CCA AAA AGA GGA AGA ACT ATG AAC ATA ATC TCT TGA ATT GTG GAT-3’ and 5’-AGC TAT CCA AAA AGA GGA AGA ACT ATG AAC ATA ATC TCT TGA ATT GTG GAT-3’ and 5’-AGC TAT CCA AAA AGA GGA AGA ACT ATG AAC ATA ATC TCT TGA ATT GTG GAT-3’ and 5’-AGC TAT CCA AAA AGA GGA AGA ACT ATG AAC ATA ATC TCT TGA ATT GTG GAT-3’ and 5’-AGC TAT CCA AAA AGA GGA AGA ACT ATG AAC ATA ATC TCT TGA ATT GTG GAT-3’ and 5’-AGC TAT CCA AAA AGA GGA AGA ACT ATG AAC ATA ATC TCT TGA ATT GTG GAT-3’ and 5’-AGC TAT CCA AAA AGA GGA AGA ACT ATG AAC ATA ATC TCT TGA ATT GTG GAT-3’ and 5’-AGC TAT CCA AAA AGA GGA AGA ACT ATG AAC ATA ATC TCT TGA ATT GTG GAT-3’ and 5’-AGC TAT CCA AAA AGA GGA AGA ACT ATG AAC ATA ATC TCT TGA ATT GTG GAT-3’ and 5’-AGC TAT CCA AAA AGA GGA AGA ACT ATG AAC ATA ATC TCT TGA ATT GTG GAT-3’ and 5’-AGC TAT CCA AAA AGA GGA AGA ACT ATG AAC ATA ATC TCT TGA ATT GTG GAT-3’ and 5’-AGC TAT CCA AAA AGA GGA AGA ACT ATG AAC ATA ATC TCT TGA ATT GTG GAT-3’ and 5’-AGC TAT CCA AAA AGA GGA AGA ACT ATG AAC ATA ATC TCT TGA ATT GTG GAT-3’ and 5’-AGC TAT CCA AAA AGA GGA AGA ACT ATG AAC ATA ATC TCT TGA ATT GTG GAT-3’ and 5’-AGC TAT CCA AAA AGA GGA AGA ACT ATG AAC ATA ATC TCT TGA ATT GTG GAT-3’ and 5’-AGC TAT CCA AAA AGA GGA AGA ACT ATG AAC ATA ATC TCT TGA ATT GTG GAT-3’ and 5’-AGC TAT CCA AAA AGA GGA AGA ACT ATG AAC ATA ATC TCT TGA ATT GTG GAT-3’ and 5’-AGC TAT CCA AAA AGA GGA AGA ACT ATG AAC ATA ATC TCT TGA ATT GTG GAT-3’ and 5’-AGC TAT CCA AAA AGA GGA AGA ACT ATG AAC ATA ATC TCT TGA ATT GTG GAT-3’ and 5’-AGC TAT CCA AAA AGA GGA AGA ACT ATG AAC ATA ATC TCT TGA ATT GTG GAT-3’ and 5’-AGC TAT CCA AAA AGA G
TTG AAT ATA CGT GAA TGT GGC CTG TGC GG-3'; (3) 5'-GAT CCC GAC TGA TGA CCA GCA ACT TGA TTC AAG AGA TCA AGT TGC TGG TCA TCA GTG TAC TTT TTG GAT-3' and 5'-AGC TAT CCA AAA AGA CTG ATG ACC AGC AAC TTG ATC TCT TGA ATC AAG TTG CTG GTC ATC AGT CGG-3'.

Transfection. Eca109 and TE13A cells were transfected with HIF-1α shRNA constructs or control HIF-1α Neo construct using Lipofectamine 2000 (Invitrogen) following the manufacturer’s instruction and stable knockdown cells were selected by 400 μg/ml G418 for 2 weeks and HIF-1α expression level was detected by protein gel blot. The stable cell lines in which HIF-1α was efficiently knockeddown were named as H315 derived from Eca109 cell line and R12 derived from TE13 cell line, and the stable control cell lines were named as Eca109 Neo and TE13 Neo.

MTT assay. Eca109, TE13, H315, R12, Eca109 Neo and TE13 Neo cells were seeded into 96-well-plates at 1 x 10^4 cells/well (100 μl) and cultured for 7 days. In the following days, the medium was removed and 20 μl MTT solution (500 μg/ml) was added to each well followed by 4 h incubation. MTT solution was replaced by DMSO to dissolve blue formazan crystals and absorbance was measured at 490 nm using a microplate reader.

Flow cytometry analysis of apoptosis. Eca109, TE13, H315, R12, Eca109 Neo and TE13 Neo cells were seeded into 100 mm dishes and grown to 90% confluence. Then the cells were collected by digestion with EDTA-free trypsin (Invitrogen). The cell pellet was washed with cold PBS twice and 1–5 x 10^5 cells were resuspended in 250 μl Annexin V binding buffer (10 mM Hepes pH 7.4, 150 mM NaCl, 2.5 mM CaCl2, 1 mM MgCl2 and 4% BSA). Next the cells were stained with Annexin V FITC for 15 min in the dark and subjected to flow cytometry analysis within 1 h.

Protein gel blot. Eca109, TE13, H315, R12, Eca109 Neo and TE13 Neo cells were collected and resuspended in RIPA buffer (50 mM Tris HCl pH 6.8, 1% Triton X 100, 0.5% Sodium deoxycholate, 150 mM NaCl and 5 mM EDTA and protease inhibitor). Alternatively, mouse tumor tissues were dissected, homogenized using a homogenizer and resuspended in RIPA buffer. Total protein was isolated from the lysate and quantitated by BCA reagent (Pierce). About 40 μg protein was loaded, separated by 10–15% SDS-PAGE and transferred to PVDF membranes (Millipore). Next, the membranes were incubated with specific antibody for HIF-1α (Chemicon), VEGF (R&D), MMP2 (Abcam), p53 (Cell Signaling Technology), bcl-2 (Cell Signaling Technology) or α-tubulin (Sigma) at 4°C overnight. The membranes were developed using ECL kit (Pierce) and exposed to x-ray film.

Tumorigenicity in nude mice. About 100 μL viable Eca109, H315 and Eca109 Neo cells at suspensions (4 x 10^5/mL) were
injected subcutaneously into the flanks of 6-weeks-old nude mice BALB/c-nu. Tumor size was measured in three dimensions every two days, and the tumor volume was calculated by the formula (length x width x height x/6). The animals were killed when the tumor volume was large enough and the tumors were dissected, weighed and subjected to protein gel blot as described above.

Animal experiments were approved by the Institutional Animal Care and Use Committee and conducted in accordance with Institutional guidelines.

**Statistical analysis.** The experimental data were expressed as mean (X) ± standard deviation (SD). Statistical analysis was performed using SPSS11.0 software (SPSS Inc.) and p < 0.05 was considered significant.

**References**