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c-Myc enhances colon cancer cell-mediated angiogenesis through the regulation of HIF-1α

Cheng Chen, Shaoxin Cai, Guihua Wang, Xiaonian Cao, Xi Yang, Xuelai Luo, Yongdong Feng, Junbo Hu *

Cancer Research Institute, Tongji Hospital, Huazhong University of Science and Technology, Wuhan 430030, China

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ABSTRACT

Angiogenesis plays a pivotal role in tumor growth. The hypoxia-inducible factor 1, α subunit (HIF-1α)/vascular endothelial growth factor pathway is the most important pathway for regulating angiogenesis in the tumor microenvironment. c-Myc is an important oncogene that has many biological functions. In this study, we investigated the role of c-Myc in tumor angiogenesis. We found that the overexpression of c-Myc in colon cancer cells could promote the expression of HIF-1α and that of vascular endothelial growth factor. Moreover, we found that c-Myc regulated HIF-1α at the post-transcriptional level. The results revealed c-Myc-dependent regulation of HIF-1α instead of HIF-1α-dependent c-Myc regulation for the first time. They also showed that c-Myc was essential to regulate colon cancer cell-mediated angiogenesis and contributed to tumor growth. This research provides the theoretical basis for clinical trials of new therapeutic targets of c-Myc and HIF-1α in colon cancer cells.

1. Introduction

Angiogenesis is essential to tumor progression: It provides the tumor with oxygen and nutrients by secreting vascular endothelial growth factor (VEGF) [1]. The microenvironment of solid tumors is often exposed to low oxygen tension as a result of an inadequate and poor blood supply [2]. Hypoxia can activate the expression of numerous angiogenic factors such as VEGF by the induction of hypoxia-inducible factor-1 (HIF-1).

HIF-1 consists of two subunits, namely, the α subunit (HIF-1α) and the β subunit (HIF-1β). HIF-1β is constitutively expressed in cells in normoxia and hypoxia. HIF-1α is one of the most important regulators of oxygen homeostasis [1]. Its expression is related to the cellular O_2 concentration. The stability and activity of HIF-1α are regulated by post-translational modifications, such as hydroxylation, ubiquitination, acetylation, and phosphorylation [3]. In hypoxia, HIF-1α is stable and translocates from the cytoplasm to the nucleus, where it dimerizes with HIF-1β and becomes transcriptionally active [4,5]. However, in normoxia, the von Hippel–Lindau protein identifies the hydroxylation of HIF-1α by prolyl hydroxylase enzymes and then leads to rapid proteasomal degradation of HIF-1α through ubiquitination [6].

c-Myc is a major human oncogene that is frequently altered in many forms of cancer [7–10]. It modulates the cell cycle and cell proliferation, increases cell metabolism, and stimulates differentiation, among its many other biological functions [11–14]. However, data about c-Myc and tumor angiogenesis are limited. Many studies have reported that HIF-1α inhibits c-Myc activity via direct interactions under physiologic conditions [15–17]. The present study showed that c-Myc inhibited the degradation of HIF-1α in both normoxia and hypoxia. The results demonstrated, for the first time, that c-Myc enhanced tumor angiogenesis by promoting the expression of HIF-1α in the LoVo cell line.

2. Materials and methods

2.1. Reagents and antibodies

Antibodies for c-Myc, HIF-1α, glyceraldehydes 3-phosphate dehydrogenase (GAPDH), CD31, and VEGF were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The antibody for HIF-1β was purchased from Cell Signaling Technology (Beverly, MA). Cobalt chloride ($CoCl_2$), cycloheximide (Chx), MG132, and DMSO were purchased from Sigma (St. Louis, MO). The enzyme-linked immunosorbent assay (ELISA) kit for VEGF was purchased from R&D Systems (Minneapolis, MN). VEGF receptor (VEGFR) tyrosine kinase inhibitor IV (VEGFRi) was purchased from Santa Cruz Biotechnology (sc-356189, Santa Cruz, CA). Growth factor-reduced Matrigel was purchased from BD Bioscience (San Diego, CA).

2.2. Cell culture and treatment

LoVo cells and human umbilical vein endothelial cells (HUVECs) were obtained from American Type Culture Collection. LoVo cells were cultured at 37 °C in DMEM supplemented with 10% fetal

* Corresponding author.
E-mail address: jbhu@tjh.tjmu.edu.cn (J. Hu).

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bovine serum (Hyclone). HUVECs were cultured at 37 °C in EBM-2 endothelial cell basal medium with SingleQuot Kit (Lonza/Cambrex, Walkersville, MD) as per the manufacturer's instructions. Hypoxic conditions were induced by culturing cells for 2 h in a sealed hypoxia chamber (Billups Rothenberg) after flushing with a mixture of 1% O_2, 94% N_2, and 5% CO_2, as well as by the addition of $CoCl_2$ at the concentration of 200 μM for 4 h.

2.3. Transfection

The c-Myc-overexpressing plasmid (pcDNA3.1-c-Myc plasmid) was constructed in our laboratory. Full-length c-Myc was isolated from a human fetal liver cDNA library and cDNA was subcloned into pCDNA3.1 plasmid. Sequence verified constructs were used in this experiment. c-Myc siRNA (5′-CAGAAATGTCCTGAGCAAT-3′) and non-targeting scrambled siRNA (NS) were purchased from Ribobio (Guangzhou, China). LoVo cells were transfected with the siRNA duplexes and plasmids using Lipofectamine 2000 (Invitrogen, San Diego, CA) according to the manufacturer's instructions.

Stable transfections of pCDNA3.1-c-Myc plasmid and pcDNA3.1 empty vector were performed with Lipofectamine 2000. After transfection for 24 h, LoVo cells were selected with G418 (400 μg/mL).

2.4. Preparation of conditioned medium (CM)

CM was prepared as described previously but with some modifications [18]. Briefly, cells treated differently were seeded at the density of 1×10^5 cells/mL in six-well plates. One day later, the cells were washed three times in phosphate-buffered saline and switched to 2 mL of DMEM without fetal bovine serum. After 24 h of incubation, CM was collected from the six-well plates with a cell density of approximately 90%.

2.5. Real-time PCR assay for c-Myc, HIF-1α, and VEGF

Real-time PCR was used to detect the messenger RNA (mRNA) levels of c-Myc, HIF-1α, and VEGF. Total mRNA was extracted using Trizol (Roche Bioscience, Germany), and reverse transcription was performed using an RT-PCR kit (Transgen, Beijing, China). Real-time experiments were conducted on an ABI-7300 Real-time PCR Detection System using SYBR Green Real-time PCR Master Mix (Toyobo, Shanghai, China). The following PCR conditions were used: 5 min at 95 °C followed by 40 cycles of denaturation for 15 s at 95 °C, annealing for 30 s at 60 °C and primer extension for 40 s at 72 °C. Primers used were: c-Myc CGAGGAGGAGAACTTCTACCAGC and CGAGAAGCCGCTCCACATACAGTCC; HIF-1α GGCGCGAACGACAAGAAAAAG and CCTTATCAAGATGCGAACTCACA; VEGF CGCAGCTACTGCCATCCAAT and GTGAGGTTTGATCCGCATAATCT; GAPDH GGTGTGAACCATGAGAAGTATGACAAC and CCAGTAGAGGCAGGGATGATGTTC. The comparative CT method was used to quantitate the expression of c-Myc, HIF-1α, and VEGF using GAPDH as control [19].

2.6. Measurement of VEGF protein level in CM

The protein level of VEGF in every CM was measured using a sandwich ELISA kit according to the manufacturer's protocol.

2.7. MTT assay

Cell proliferation was measured by MTT assay. Briefly, approximately 10^3 HUVECs were cultured in 96-well plates and the media were replaced 24 h later by various CM. After 48 h of incubation, relative cell numbers were quantified via MTT assay. The medium was replaced with 10 μL of 5 mg/mL MTT in each well. After 4 h of incubation at 37 °C, 100 μL of DMSO was added to each well and the absorbance was measured at 492 nm on a multifunction microplate reader (POLARstar OPTIMA; BMG, Offenburg, Germany).

2.8. Scratch-wound assay

HUVECs were cultured in 24-well plates. A scratch-wound was made using a 200-μL pipette tip when the cell density was approximately 100%. The medium was replaced by CM and 1 μM 5-fluouracil (Sigma) was added after scratch wounding to block cell proliferation. The distance of each scratch closure was obtained by comparing the images from time 0 to the last time point (24 h) and based on the distances measured by software.

2.9. In vitro angiogenesis assay

In vitro angiogenesis was detected by tube formation. Growth factor-reduced Matrigel was placed in 96-well tissue culture plates (100 μL/well) and allowed to form a gel at 37 °C for 30 min. HUVECs (2×10^4 cells) were added into each well and incubated in CM for 24 h. Endothelial tubes were examined under a light microscope every 4 h by inspecting the overall branch points.

2.10. Western blot analysis

Cells were lysed in NP40 with PMSF, and the protein concentration was determined. Proteins were separated on 10% or 12% premade Tris–HCl SDS–PAGE and transferred to a polyvinylidene difluoride membrane (Bio-Rad). Proteins of interest were detected by immunoblotting using specific antibodies.

2.11. Animal studies

Male BALB/c nude mice (4 weeks old) were purchased from the Wuhan Laboratory Animal Center and maintained in the Laboratory Animal Center of Huazhong University of Science and Technology, China. All animal experiments were done in accordance with institutional animal research guidelines approved by the local ethics committee.

Cells were cultured in fresh medium for 24 h and harvested. A total of 5×10^6 cells in 50 μL DMEM were mixed with 50 μl ice-cold Matrigel. Then the mixture was subcutaneously injected into the nude mice. Tumor dimensions and volumes were determined every 7 days. Animals were killed 35 days after injection. The tumor xenografts were then removed and immediately weighed. Next, the tumor xenografts were bisected. Half of each tumor was fixed in 4% paraformaldehyde overnight and analyzed by immunohistochemistry, and the other half was homogenized to generate lysates for Western blot analysis.

Microvascular density (MVD) was determined as described by Weidner et al. [20]. First, the stained sections were screened at 40× magnification under a light microscope to identify the areas of highest CD31-positive vessel density. These areas were then counted at 200× magnification in 10 random fields. Data were collected by two independent observers unaware of the test. The number of microvessels in each field was determined as the MVD.

2.12. Clinical samples

All colon tumors were collected with informed consent of the patients under an institutionally approved protocol at the Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology. Every tumor was the same cell type and confirmed by a pathologist. Fresh tumor tissues were homogenized to generate lysates for Western blot analysis.

2.13. Statistical analysis

Student's *t* test was used for pairwise comparisons. Comparisons between multiple experimental groups were conducted using the Bonferroni test with SPSS 13.0 for Windows. In all cases, $P < 0.05$ was considered statistically significant.

3. Results

3.1. c-Myc suppressed the degradation of HIF-1α

We first detected the mRNA and protein levels of HIF-1α in LoVo cells. Regardless of whether normoxic or hypoxic conditions were used, the mRNA level of HIF-1α did not exhibit variations when c-Myc was overexpressed or knocked down. However, the protein levels of c-Myc and HIF-1α were positively correlated under both normoxia and hypoxia (Fig. 1A–C). As c-Myc could affect the protein level but had no influence on the mRNA level of HIF-1α, we hypothesized that the increase in protein expression is partly due to the decrease in the degradation of HIF-1α. To confirm this, we used Chx to inhibit protein translation. We detected HIF-1α fold induction by comparing the protein levels at 0, 1, 2, and 3 h. Interestingly, we found that overexpression of c-Myc under hypoxia significantly stabilized the HIF1α protein (Fig. 1D), suggesting that c-Myc may affect the degradation of HIF-1α. To further confirm this result, we used the proteasome inhibitor MG132 to inhibit the degradation of HIF-1α. We found that overexpression of c-Myc had no impact on the protein level of HIF-1α (Fig. 1E). Taken together, these results showed that c-Myc was involved in HIF-1α stabilization but not transcription.

We also detected the protein level of HIF-1β and found that c-Myc had no impact on its expression.

3.2. c-Myc regulated the expression of VEGF

As VEGF is one of the most important target genes of HIF-1α [21], we detected the effects of c-Myc on VEGF in the LoVo cell line. We found that overexpression of c-Myc promoted the expression of VEGF at the mRNA level in both normoxia and hypoxia. On the contrary, the mRNA levels of VEGF in normoxia and hypoxia decreased when c-Myc was knocked down (Fig. 2A). Next, we detected the VEGF protein level by Western blot analysis and ELISA. We found that the impact of c-Myc on the VEGF protein level was the same as that on its mRNA level (Fig. 2B and C). Taken together, these results indicated that c-Myc could positively regulate the expression of VEGF at the mRNA and protein levels.

3.3. c-Myc promoted cell proliferation, migration and tube formation by HUVECs induced by CM from LoVo cells

Research has shown that VEGF could promote cell proliferation, migration, and angiogenesis in endothelial cells [22]. We detected the impact of various CM on cell growth, migration, and tube formation in HUVECs. To eliminate the possibility that other factor(s)

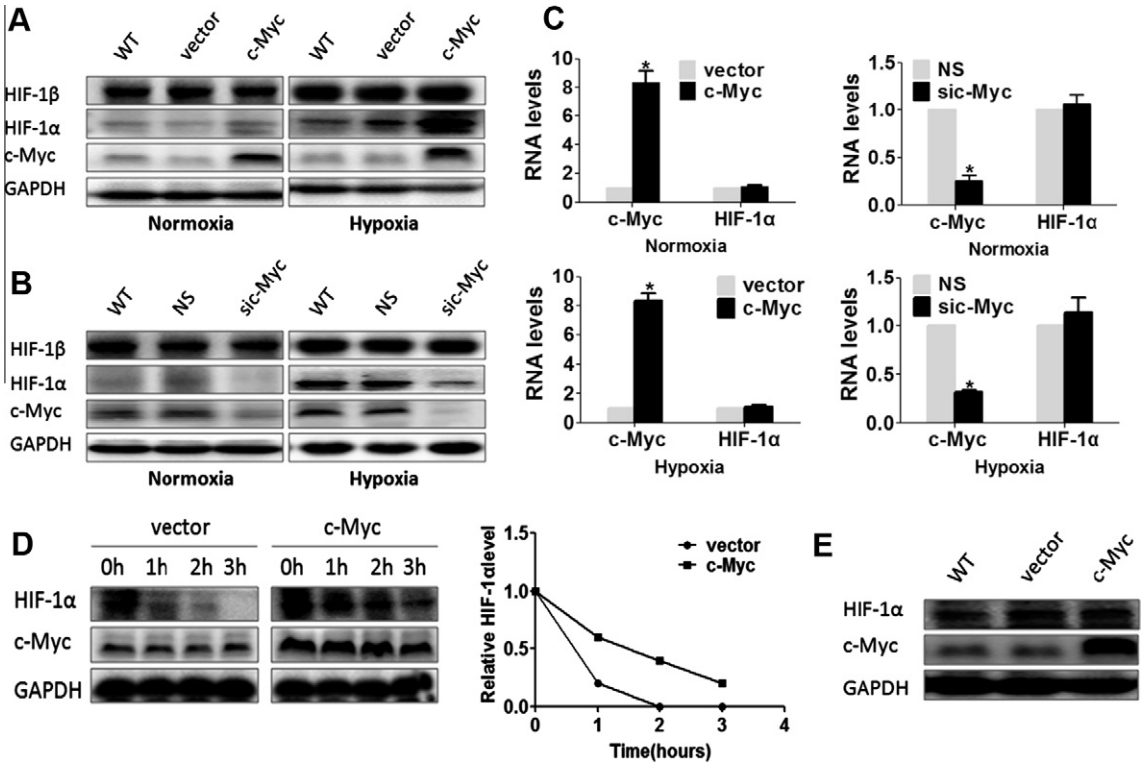

Fig. 1. c-Myc enhances the accumulation of HIF-1α at the post-transcriptional level in both normoxia and hypoxia. (A) Western blot analysis was performed to detect the c-Myc protein level, HIF-1α protein level, and HIF-1β protein level of LoVo cells after they were transfected with pCDNA3.1-c-Myc plasmid (c-Myc). Wide-type (WT) LoVo cells and cells transfected with pCDNA3.1 plasmid (vector) were determined as control. (B) LoVo cells were transfected with siRNA against c-Myc (sic-Myc) or non-targeting scramble (NS). Wild-type cells were used as control. The protein levels of HIF-1α and HIF-1β were detected as described in panel (A). (C) LoVo cells were treated as described in panels (A) and (B). Real-time PCR was performed using RNA harvested from LoVo cells and primers specific to c-Myc, HIF-1α and GAPDH in triplicate. Average c-Myc and HIF-1α RNA levels were normalized to GAPDH. (D) LoVo cells (vector or c-Myc-overexpressing) were treated with the protein translation inhibitor Chx for 1, 2, and 3 h. Hypoxia was established by treating cells with $CoCl_2$ for 3 h prior to Chx treatment. The expression level of HIF-1α was detected by Western blot analysis. Levels of HIF-1α protein were determined by measuring the density of the HIF-1α protein band and normalized to that of GAPDH. The relative HIF-1α protein level at time zero was defined as 1.0. (E) LoVo cells (wild type, vector, and c-Myc-overexpressing) were treated with proteasome inhibitor MG132 under hypoxia. The protein level of HIF-1α was detected by Western blot analysis. All results were obtained from three independent experiments. $^{*}p < 0.05$.

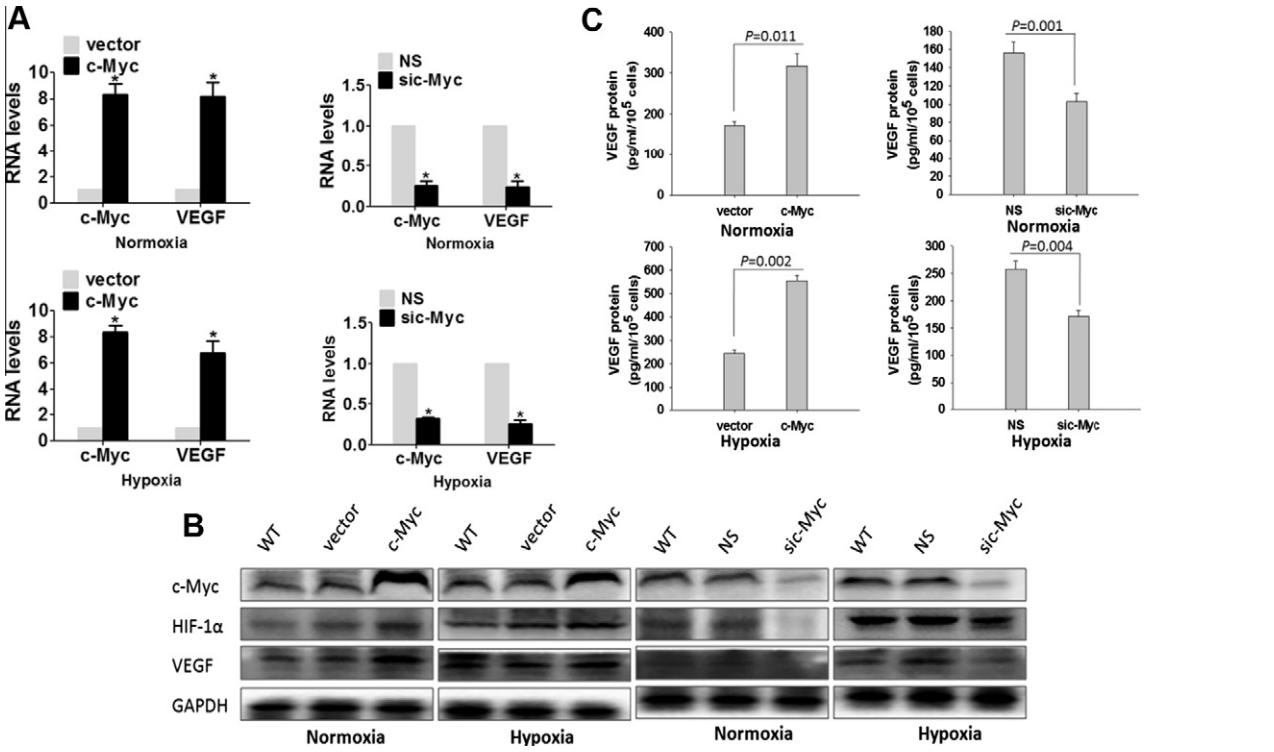

Fig. 2. c-Myc positively regulates the expression of VEGF. LoVo cells were treated as described in Fig. 1. (A) Real-time PCR was performed using RNA obtained from LoVo cells and primers specific to c-Myc, VEGF, and GAPDH in triplicate. Average c-Myc and VEGF RNA levels were normalized to GAPDH. (B) Protein levels of c-Myc, HIF-1α, and VEGF were determined by Western blot analysis. (C) ELISA was used to detect the VEGF protein level of CM obtained in Section 2. All experiments were detected and analyzed in triplicate. $^{*}p < 0.05$.

rather than VEGF in CM was involved in the experiment, we used VEGFRi to block the function of VEGF [23]. Firstly, we checked the effectiveness of VEGFRi by Western blot analysis. We found that VEGFRi could significantly reduce the phosphorylation of VEGFR2 (Fig. 3A). Then, we found that CM secreted by c-Myc-overexpressing LoVo cells could notably promote the proliferation of HUVECs, regardless of whether normoxic or hypoxic conditions were used (Fig. 3B). Next, 24 h after incubation in CM, scratch-wound assay was used to determine the migration ability of HUVECs. We found that CM produced by c-Myc-overexpressing LoVo cells could significantly promote the migration ability of HUVECs in both normoxia and hypoxia (Fig. 3C). Lastly, to determine whether c-Myc could promote angiogenesis, we performed tube formation assay *in vitro*. HUVECs in CM prepared from c-Myc-overexpressing LoVo cells formed more tubes than the control group (Fig. 3D). We also found that the facilitation of CM obtained from cultures of c-Myc-overexpression LoVo cells to the proliferation, migration, and tube formation of HUVECs could be blocked by VEGFRi (Fig. 3B–D). All results were obtained from replicate experiments.

In line with the above-described results, we hypothesized that overexpression of c-Myc in LoVo cells could promote the secretion of VEGF, thereby inducing the proliferation, migration and tube formation of HUVECs.

3.4. c-Myc enhanced tumor xenografts growth and tumor-induced angiogenesis in nude mice

To determine whether c-Myc promoted tumor growth and angiogenesis *in vivo*, we subcutaneously injected nude mice with c-Myc-overexpressing LoVo cells and vector control LoVo cells. We found that the tumors derived from c-Myc-overexpressing LoVo cells were heavier and greater than those from the control group (Fig. 4A–C). Moreover, the MVD of tumors derived from the LoVo cells stably expressing c-Myc was higher than that of tumors from the control group (Fig. 4D). These results suggested that c-Myc may have promoted tumor growth and angiogenesis *in vivo*. To further determine the correlation of c-Myc and tumor angiogenesis *in vivo*, we used Western blot analysis to detect the protein levels of c-Myc and HIF-1α in the tumor xenografts. We found that these were positively correlated (Fig. 4E). This further illustrated that c-Myc could promote tumor angiogenesis *in vivo* through HIF-1α.

3.5. The expression levels of c-Myc and HIF-1α in colon cancer samples

Finally, we explored the correlation of c-Myc and HIF-1α in colon cancer samples. Eighteen human colon cancer samples were tested. Similar to the results of our animal studies, we found that the expression levels of c-Myc and HIF-1α in colon cancer samples were positively correlated (Fig. 4F). This result supports the finding that c-Myc may regulate the expression of HIF-1α *in vivo*.

4. Discussion

Angiogenesis, the process of new blood vessel formation, is important during cancer progression [24]. Similarly, it is indispensible for the development of colon cancer. c-Myc is one of the most important oncogenes, and studies have reported its overexpression in colon cancer [25,26]. It has many biological functions, but data about c-Myc and tumor angiogenesis are limited. In the present study, we found that c-Myc could promote the expression of HIF-1α and VEGF in colon cancer cells. We further demonstrated that c-Myc enhanced colon cancer cell-mediated angiogenesis. We obtained the same results *in vivo* and *in vitro*.

Studies have shown the correlation between c-Myc and HIF-1α. Under physiologic conditions, HIF-1α could counter normal levels

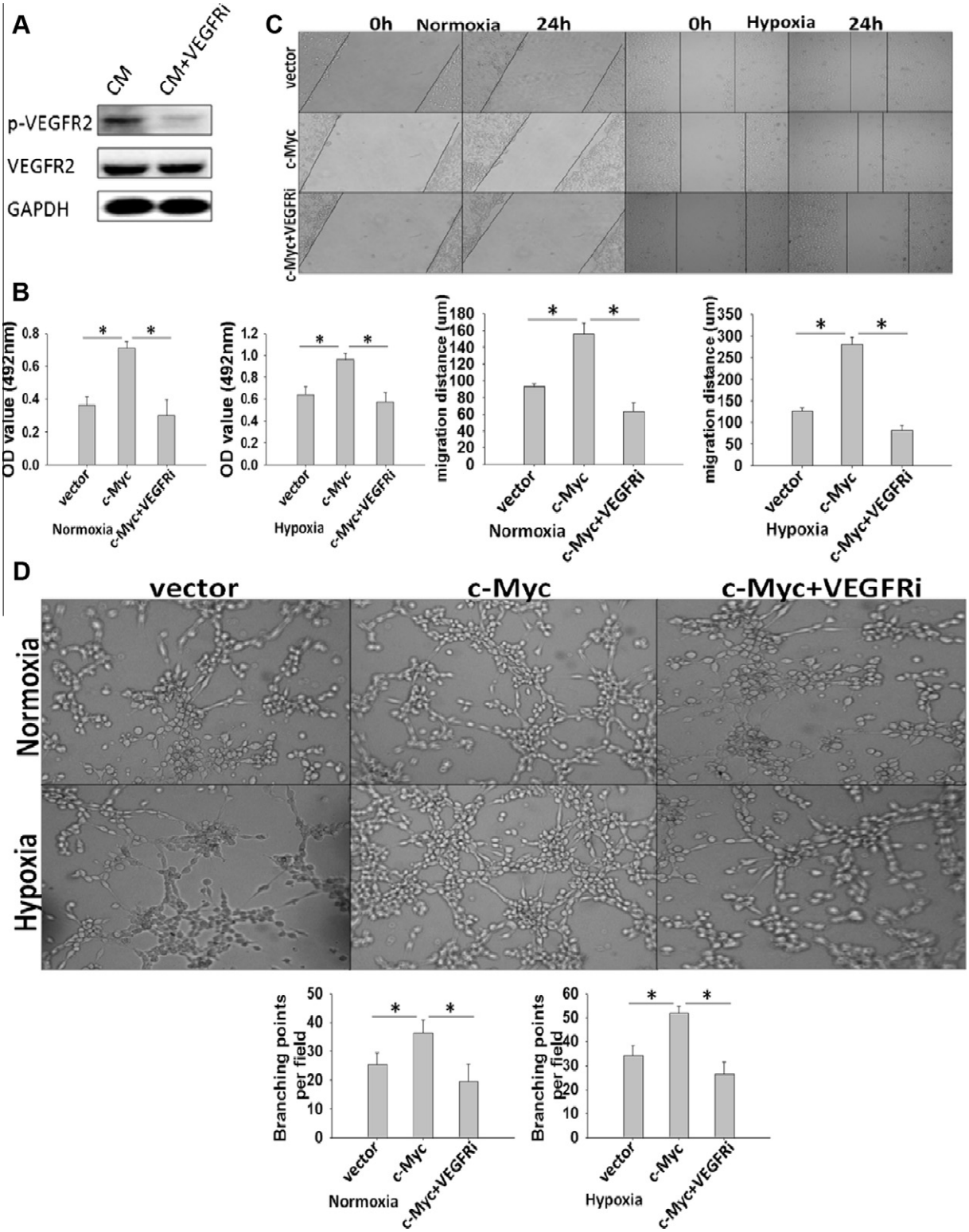

Fig. 3. c-Myc promotes the ability of HUVECs to proliferate, migrate, and form tubes as induced by CM from LoVo cells through VEGF. (A) HUVECs were incubated in CM or CM with VEGFRi, the phosphorylation of VEGFR2 and total VEGFR2 were detected by Western blot analysis. HUVECs were incubated in CM from control LoVo cells, CM from c-Myc-overexpressing LoVo cells or CM from c-Myc-overexpressing LoVo cells with VEGFRi. The abilities of proliferation (B) migration (C) and tube formation (D) were detected. The branching points per field were counted to quantify the cells' tube formation ability. $^{*}p < 0.05$.

of c-Myc by inhibiting its function; however, deregulated oncogenic c-Myc is able to collaborate with HIF-1α [27–29]. The relationship between c-Myc and HIF-1α in colon cancer cells is not clear. In contrast to previous data, the present study found, for the first time, c-Myc-dependent regulation of HIF-1α, instead of HIF-1α-dependent c-Myc regulation, in colon cancer cells regardless of normoxia and hypoxia. We also found that c-Myc and HIF-1α were positively correlated in clinic colon cancer samples.

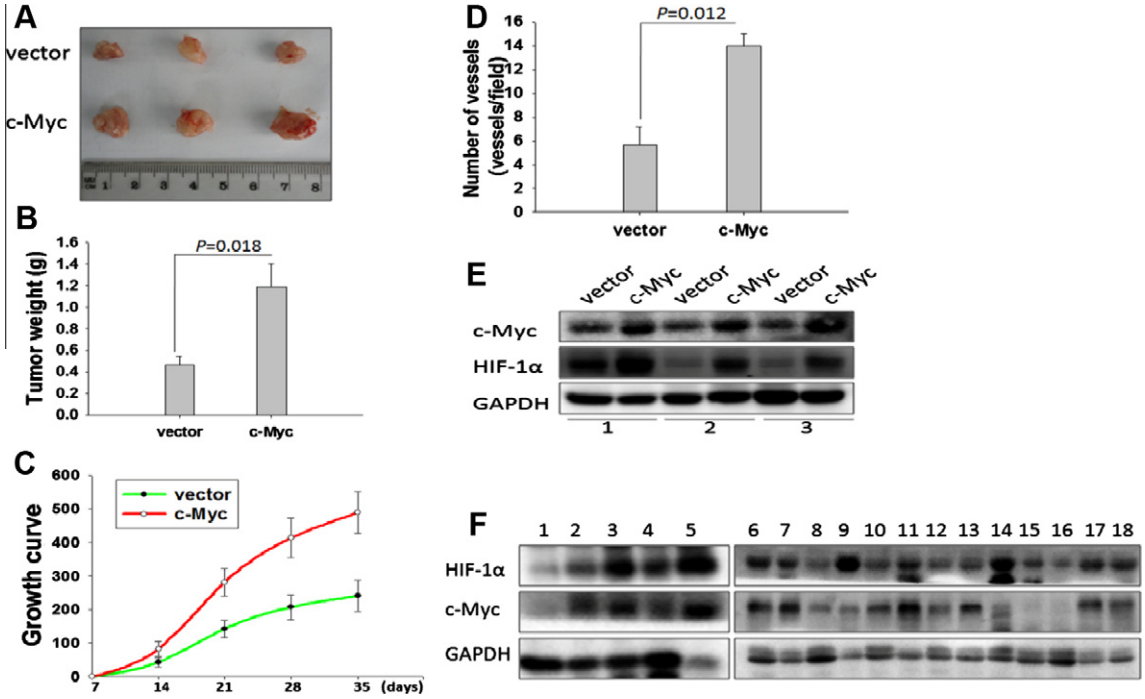

Fig. 4. c-Myc promotes tumor xenograft growth and tumor-induced angiogenesis *in vivo*, and the expression levels of c-Myc and HIF-1α in colon cancer samples are positively correlated. (A–C) Xenograft growth (A) of c-Myc-overexpressing LoVo cells and control LoVo cells (vector) was determined by measuring their tumor weight (B) and tumor volume (C). (D) The number of vessels was determined by MVD as described in Section 2. (E) Correlation of c-Myc and HIF-1α expression in xenograft tumors. Half of each tumor was homogenized to generate lysates for Western blot analysis. (F) The expression levels of c-Myc and HIF-1α in colon cancer samples were detected by Western blot analysis.

Furthermore, we found that c-Myc did not regulate HIF-1α at the transcriptional level. The data demonstrated that c-Myc-induced HIF-1α accumulation was post-transcriptional. Recent study revealed that Myc-dependent stabilization of HIF-1α involved either disruption of binding to the von Hippel–Lindau complex or post-translational protein modifications in breast cancer cells [30]. However the concrete mechanism of c-Myc suppressing HIF-1α degradation remains unresolved, warranting further research.

In the process of tumor growth, because of genetic alterations of oncogenes and tumor suppressor, or in response to the reduced availability of oxygen, tumor cells expression of many of the pro-angiogenic growth factors, which stimulated endothelial cells (ECs) migration and proliferation to sprout and form new vasculature [31,32]. VEGF plays a key role in this manner, which is secreted by tumor cells [33]. VEGF is one of the most important target genes of HIF-1α [21], and we thus detected the impact of c-Myc on VEGF in colon cancer cells. As we speculated, c-Myc could promote the mRNA and protein levels in LoVo cells. As VEGF is a kind of secretory cytokines, we used ELISA to detect the secretion level of VEGF protein in LoVo cells. We found that c-Myc could promote the secretion of VEGF in LoVo cells. We obtained the same results in both normoxia and hypoxia. We then found that CM from c-Myc-overexpressing LoVo cells could facilitate HUVECs proliferation, migration and tube formation. We also found that the inhibitor of VEGFR could block these functions, which further indicated that VEGF was the most important factor involved in the alterations. Therefore, we concluded that c-Myc enhances angiogenesis through the HIF-1α/VEGF pathway.

In summary, this study showed a new c-Myc/HIF-1α-dependent pathway in colon cancer cells that enhances the secretion of VEGF and induction of colon cancer cell-mediated angiogenesis. In this course, c-Myc induced the accumulation of HIF-1α at the post-transcriptional level. The results indicated that c-Myc promoted tumor growth not only through cell proliferation but also partly through the modulation of tumor angiogenesis. We thus identified important roles for c-Myc and HIF-1α in the pathogenesis of colon cancer. This research provides the theoretical basis for clinical trials of new therapeutic targets of c-Myc and HIF-1α in colon cancer cells.

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Contribution: C.C., S.C., G.W., X.C., X.Y., X.L., and Y.F. performed experiments; C.C. analyzed results and made the figures; C.C. and J.H. wrote the paper; J.H. designed the research.

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