Cancer Research

MicroRNA-519c Suppresses Hypoxia-Inducible Factor-1α Expression and Tumor Angiogenesis

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Abstract

Hypoxia-inducible factor-1α (HIF-1α) is widely considered to be one of the key regulators of tumor angiogenesis. The upstream regulation is complex and involves several growth factors, cytokines, and hypoxia. Herein, we have identified miR-519c as a hypoxia-independent regulator of HIF-1α, acting through direct binding to the HIF-1α 3′ untranslated region and leading to reduced tumor angiogenesis. Overexpression of miR-519c resulted in a significant decrease of HIF-1α protein levels and reduced the tube formation of human umbilical vein endothelial cells; similarly, antagomir inhibition of miR-519c increased the level of HIF-1α protein and enhanced angiogenic activity, suggesting an important role of miR-519c in HIF-1α–mediated angiogenesis. Consistent with the overexpression of miR-519c in cancer patients with better prognosis, mice injected with miR-519c–overexpressing cells exhibited dramatically reduced HIF-1α levels, followed by suppressed tumor angiogenesis, growth, and metastasis. In addition, we found that hepatocyte growth factor (HGF), a known HIF-1α inducer, reduced the miR-519c levels through an Akt-dependent pathway. This regulation was post-transcriptional and may be mediated by suppression of miR-519c maturation. Taken together, our findings provide the first evidence that miR-519c is a pivotal regulator of tumor angiogenesis and that microenvironmental HGF contributes to regulating miR-519c biogenesis in cancer cells. *Cancer Res; 70(7); 2675–85. ©2010 AACR.*

Introduction

MicroRNAs (miRNA) are a new class of endogenous, small, 19- to 25-nucleotide noncoding RNAs, which facilitate gene repression and play an important role in physiologic and pathologic processes, including development, virus infection, and cancer (1–3). To date, >500 miRNAs have been identified; however, most of their functions are unclear (4). miRNAs can regulate the angiogenic process either through a direct effect on the endothelial cells or through an indirect effect on the tumor cells (5). In human umbilical vein endothelial cells (HUVEC), miR-221/222 represses c-kit, leading to angiogenic inhibition (6), whereas miR-15b and miR-16 cause indirect effects on tumor angiogenesis (7). However, little is known about the role of miRNAs in angiogenesis.

The activation of angiogenesis is a critical step in cancer development (8), involving several kinds of stimulation, such as hypoxia, cytokines, and growth factors (7, 9–10). Hypoxia-inducible factor-1α (HIF-1α) is a pivotal transcription factor that regulates angiogenesis by inducing the expression of vascular endothelial growth factor (VEGF), interleukin-8 (IL-8), and basic fibroblast growth factor (bFGF; ref. 11). Under hypoxic conditions, HIF-1α is stabilized and translocated into the nucleus, activating a broad array of downstream genes (12 13). In addition, several studies have shown that *de novo* synthesis of HIF-1α is regulated through oxygen-independent mechanisms involving oncogene activation (e.g., H-Ras, myc, and src), tumor suppressor genes (e.g., *p53, PTEN*, and *VHL*), and a variety of growth factors [e.g., hepatocyte growth factor (HGF), EGF, and Cyr61; refs. 11, 14–16]. We hypothesized that miRNAs may have a role in regulating HIF-1α expression at the posttranscriptional level and that the dysregulation of miRNAs might contribute to the onset of tumor angiogenesis.

The present study focuses on miRNA-mediated regulation of HIF-1α in human cancers. We have identified miR-519c as a key regulator of HIF-1α–induced tumor angiogenesis both *in vitro* and *in vivo*.

Materials and Methods

Computational analysis. We used three databases, TargetScan (17), RNAhybrid (18), and PicTar (19), which predict the

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binding potential of miRNA to the 3′ untranslated region (3′UTR) region of HIF-1α. The binding potential is calculated based on the extent of complementarity between the sequences at the 5′-end of the miRNA and the binding region in the 3′ UTR of the mRNA. These databases predicted miR-20a, miR-130a, miR-519a, miR-519c, and miR-338 as potential miRNAs to target HIF-1α mRNA. The suggested binding sites of these five miRNAs to the 3′UTR region of HIF-1α mRNA are shown in Supplementary Fig. S1.

Plasmid constructs. miRNA constructs expressing miR20a, miR130a, miR-519a, miR-519c, and miR-338 were designed by our laboratory, and DNA was synthesized by a biotech company (Mission Biotech). In brief, the pre-miRNA was ligated into a BLOCK-iT Pol II miR RNAi expression vector. A 1,175-bp HIF-1α 3′UTR containing target sites for five candidate miRNAs was amplified by PCR and cloned into pmiR-REPORT miRNA Expression Reporter Vector (Ambion), which can be used to conduct accurate, quantitative evaluations of miRNA function. The following primers were used to amplify HIF-1α 3′UTR: 5′-GGGTTCGAACCCCGAAAAAGA-3′ (forward) and 5′-TTCGGGCCCAAACGCAGAATATATTCC-3′ (reverse). To generate mutations in the predicted target site for miR-519c, five nucleotides, corresponding to the seed sequences of miR-519c and HIF-1α mutant 3′UTR (Mut-3′ UTR), were deleted using a QuikChange site-directed mutagenesis kit (Stratagene). miRNA knockdown was purchased from Dharmacon, AntagomiR-519c, AAAGUGCAUCUUUUUA-GAGGAU. Dominant-negative mutant HIF-1α (DN-HIF1α) carries both the deletions of the basic DNA binding domain (amino acids 4–27) and the COOH-terminal transactivation domain (amino acids 390–826), thus effectively inhibiting HIF-1 activity (20).

Cell culture. CL1-0 and CL1-5 lung adenocarcinoma cell lines were previously generated by our collaborator, Dr. P.C. Yang at National Taiwan University (Taipei, Taiwan). In brief, a Transwell chamber was used to generate cell lines with increased migratory potential. CL1-0 is the parent cell line and CL1-5 is the subline with the highest migratory potential (21). Four lung cancer (H1299, PC14, H928, and A549) and five breast cancer (MCF-7, MDA-MB231, MDA-MD431, T47D, and SKBR3) cell lines were obtained from the American Type Culture Collection and cultured according to the supplier's recommendations.

Isolation of RNA, reverse transcription, primer design, and real-time PCR quantification. Total RNA was isolated from frozen primary tumor and cancer cell lines using Trizol (Invitrogen), and cDNA was synthesized following standard protocols. Gene-specific primers or random hexamers with Taqman miRNA were obtained from Applied Biosystems. The primary (pri–miR-519c), precursor (pre–miR-519c), and mature mi519c sequences were designed with BLOCK-iT Pol II miR RNAi Expression Vector kits according to the manufacturer's instructions. Real-time PCR was performed using an Applied Biosystems 7900 Fast Real-time PCR system with miRNA-specific primers and Taqman Universal PCR Master Mix, on AmpErase UNG (Applied Biosystems). Values represent the average of two independent experiments, normalized to the endogenous control gene (*RNU-6B*).

Western blotting. Western blotting was performed with primary antibodies for mouse anti-human HIF-1α (BD Pharmingen), rabbit anti–human-HIF-1β, rabbit anti–human-HIF-2α, mouse anti-human p-MET, rabbit anti-human p-ERK, rabbit anti-human p-AKT (S473), mouse anti-human p-ERK, and mouse anti-human actin (Santa Cruz Biotechnology). Monoclonal rabbit anti–phospho-S6K (T389) was from Cell Signaling Technology.

Specimens and immunohistochemistry. All lung adenocarcinoma specimens were obtained from the pathology archive of National Taiwan University Hospital. The histologic differentiation and clinical stage was determined according to WHO criteria. The antibodies included anti-human HIF-1α antibody (Santa Cruz Biotechnology) and anti-CD31 antibody (R&D Systems). Immunodetection was performed with an EnVisionTM dual-link system-HRP detection kit (DAKO Corp).

Mouse model. Five-week-old male BALB/c nude mice (n = 20; National Health Research Institute) were injected s.c. with (*a*) CL1-5/vector (n = 10) or (*b*) CL1-5/miR-519c (n = 10; 10^7 cells in serum-free RPMI 1640 and Matrigel, v/v 1:1). Tumor volume was calculated weekly according to width^2 × length × 0.5. Mice were euthanized 6 wk postinjection, tumors were removed and weighed, and tumor segments were fixed in 10% neutral buffered formalin. In the metastasis models, mice were injected with CL1-5/vector or CL1-5/miR-519c cells (10^6 cells in serum-free RPMI 1640) by tail vein injection. Mice were euthanized 1 mo postinjection, and all organs were examined for metastasis formation.

Angiogenesis assay. Matrigel plugs (100 μL; Becton Dickinson Labware) were presoaked in concentrated (50×) conditioned medium from CL1-0/anti–miR-519c, CL1-0/DN-HIF-1α, or CL1-0/DN-HIF-1α +anti-miR519 cells. This was done alone or in combination with either 5 μg IgG or human VEGF-A–specific goat polyclonal IgG-neutralizing antibody (R&D Systems). Plugs were s.c. injected into 8-wk-old female C57BL/6 mice (n = 8 per condition). After 7 d, when the vessels had matured *in vivo* (22), mice were euthanized and Matrigel plugs were removed and photographed. Blood vessels were quantified by measuring the hemoglobin in the plugs at an absorbance of 540 nm using Drabkin Reagent kit 525 (Sigma). All mouse studies were performed using protocols approved by the Institutional Animal Care and Use Committee of the College of Medicine of National Taiwan University.

Results

To identify miRNAs involved in regulating HIF-1α, we used three target prediction algorithms, miRanda (17), RNAhybrid (18), and PicTar (19), to assay miRNA binding sites of HIF-1α 3′UTR. miRNAs with target sites present in at least two algorithms, including miR-20a, miR-130a, miR-519a, miR-519c, and miR-338, were selected as candidate miRNAs (Supplementary Fig. S1). We found that miR-519c and miR-20a, but none of the other candidates, suppressed the luciferase activity of HIF-1α 3′UTR in 293T cells (Fig. 1A, left). Because miR-20a has already been identified as a HIF-1α regulator (23), we investigated the regulatory role of miR-519c. We designed a mutant HIF-1α 3′UTR, which carried a 5-bp deletion

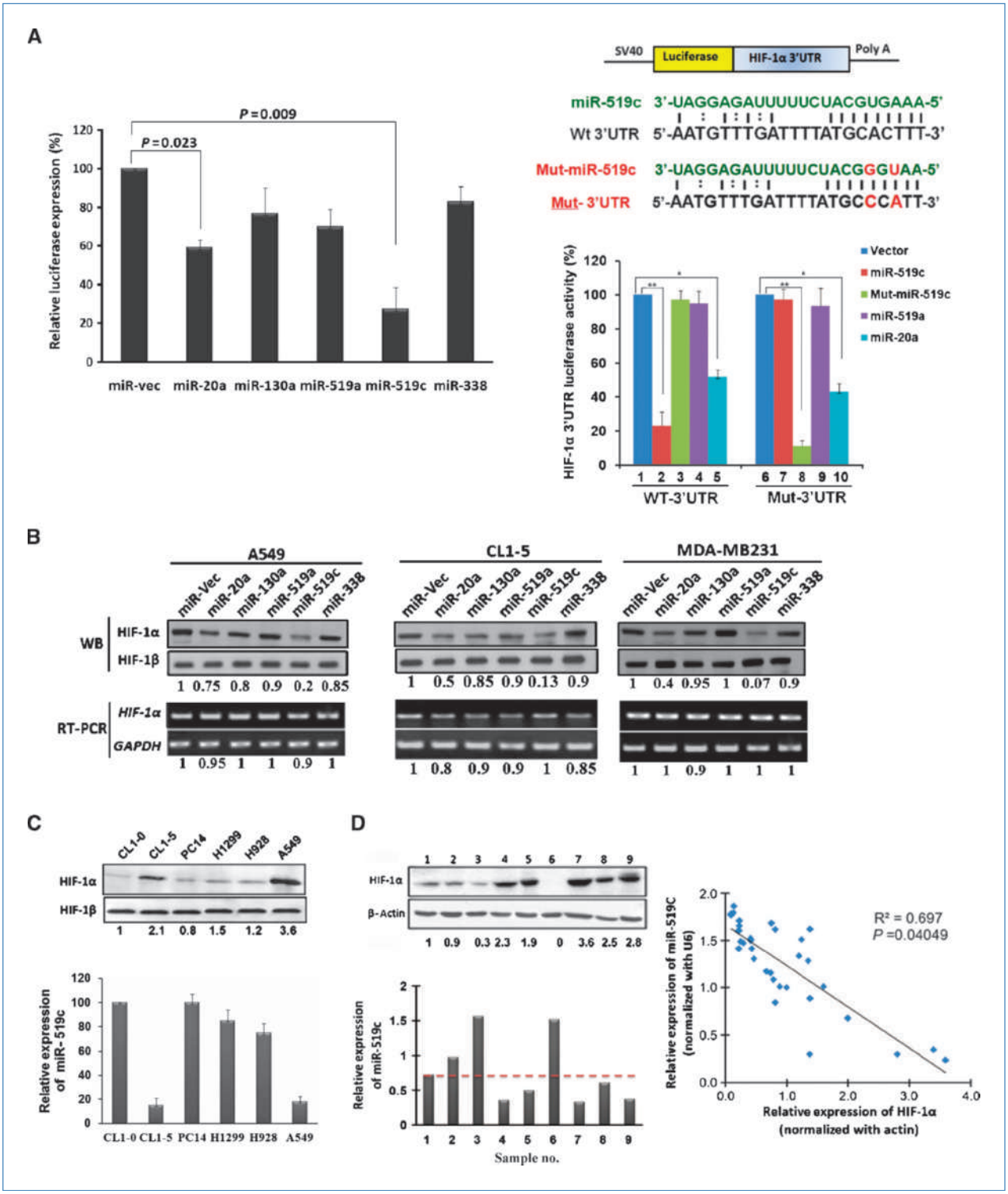


Figure 1. Analysis of miRNA binding site in HIF-1α. A, the 3′UTR of HIF-1α enables miR-519c regulation. Left, 293T cells were cotransfected with the indicated miRNA and HIF-1α 3′UTR. Right, 3′UTR mutants were constructed, without 5 bp of the miRNA::mRNA interaction site. All of the experiments were performed twice in triplicate (n = 3). B, HIF-1α expression in the presence of candidate miRNAs. Individual miRNA was introduced into cells. Western blot (WB) and reverse transcription-PCR (RT-PCR) were conducted at 72 h after transfection. GAPDH, glyceraldehyde-3-phosphate dehydrogenase. C, reciprocal expression of miR-519c and HIF-1α protein levels in six lung cancer cell lines. Top, Western blot analysis of HIF-1α expression. Bottom, miR-519c expression by real-time PCR. D, miR-519c expression in primary lung tumor samples. Left, correlation between HIF-1α protein level and miR-519c miRNA expression in lung cancer patients (1–9). Right, inverse correlation between the expression of miR-519c and HIF-1α in 64 human lung adenocarcinomas.

in the seed region of the target site for miR-519c. We observed a significant decrease in luciferase activity in cells expressing miR-519c (Fig. 1A, right, columns 1 and 2). In contrast, luciferase activities of mutant 3′UTR were unchanged in miR-519c expressed cells, whereas miR-20a was still capable of such inhibition (Fig. 1A, right, column 6 and 7).

To confirm the binding of miR-519c to the 3′UTR region of HIF-1α, we generated a mutant form of miR-519c (mut-miR-519c) harboring corresponding mutations to the mutant HIF-1α 3′UTR. As expected, mut-miR-519c dramatically inhibited the luciferase activity of the mutant form of HIF-1α (Fig. 1A, right, column and 6 and 8). Furthermore, Western blot analysis showed that expression of both miR-519c and miR-20a suppressed the endogenous HIF-1α levels in several types of cancer cell lines, including A549, CL1-5, and MDA-MB-231. In contrast, HIF-1α mRNA levels were unchanged in all cell lines (Fig. 1B), indicating that miR-519c regulates HIF-1α in a post-transcriptional manner. Next, we obtained a panel of six lung and five breast cancer cell lines and evaluated the levels of HIF-1α and miR-519c (Supplementary Fig. S2A; Fig. 1C). Cell lines with low levels of HIF-1α (CL1-0, PC14, H1299, H928, MDA-MB483, T47D, and SKBR3) expressed high levels of miR-519c, whereas cell lines expressing high levels of HIF-1α (CL1-5, A549, MDA-MB231, and MCF-7 cells) had low miR-519c levels (Supplementary Fig. S2A; Fig. 1C). To determine the role of miR-519c in primary tumor samples, we assayed 64 human lung adenocarcinoma and 34 breast cancer tissues. As expected, we detected a reverse correlation between the expression of HIF-1α and miR-519c (Supplementary S2B; Fig. 1D), indicating that miR-519c has a clinically relevant role in regulating HIF-1α expression. HIF-1α has been reported to upregulate several critical angiogenic proteins, such as VEGF, bFGF, and IL-8 (11). To further elucidate the role of miR-519c in HIF-1α–induced angiogenesis, we measured the levels of VEGF, bFGF, and IL-8 in conditioned medium from CL1-5 cells transfected with miR-519c or a HIF-1α construct without 3′UTR (HIF-1$\alpha^{\Delta 3'UTR}$). As expected, miR-519c significantly decreased the levels of these proteins, whereas coexpressing HIF-1$\alpha^{\Delta 3'UTR}$ restored this effect (Fig. 2A). In addition, tube formation was reduced by 75% in HUVECs treated with CL1-5/miR-519c conditioned medium. Tubular formation was restored in the presence of HIF-1$\alpha^{\Delta 3'UTR}$, indicating the essential role of HIF-1α in miR-519c–suppressed tube formation (Fig. 2B). To investigate whether miR-519c could regulate HIF-1α–dependent angiogenesis under endogenous conditions, we blocked the function of miR-519c using antisense oligonucleotides (anti–miR-519c). Inhibition of miR-519c was sufficient to increase the levels of HIF-1α as well as the secretion of angiogenic factors in CL1-0 cells (Fig. 2C). Consistently, the tube-forming ability of HUVECs was also significantly increased in response to anti–miR-519c treatment. All these effects could be reversed by coexpressing a dominant-negative HIF-1α (DN-HIF-1α) construct, emphasizing the regulatory role of miR-519c in HIF-1α–dependent angiogenic induction.

Regulation of HIF-1α can occur through oxygen-dependent (e.g., hypoxia) and oxygen-independent mechanisms involving a variety of growth factors (e.g., EGF, HGF, and Cyr61; refs. 24–26). To further delineate the expression of miR-519c in hypoxia, we determined the miR-519c levels of CL1-0 cells cultured in hypoxic conditions. Although the HIF-1α level was rapidly induced by hypoxia, the levels of miR-519c were not changed (Supplementary Fig. S3), indicating that miR-519c is regulated in a hypoxia-independent manner. To identify potential growth factors involved in the regulation of miR-519c, we treated cells with HGF, EGF, or Cyr61 and measured the levels of miR-519c using qPCR. In response to HGF, miR-519c expression was reduced in a dose-dependent manner, whereas no significant change was noted following treatment with other growth factors (Fig. 3A). In contrast, miR-20a expression was not affected by HGF. We further investigated whether HGF-suppressed miR-519c expression was required for HIF-1α induction. Restoring miR-519c in CL1-5 cells (CL1-5/miR-519c) completely abolished the effect of HGF on HIF-1α expression (Fig. 3B), indicating an essential role of miR-519c in HGF-mediated HIF-1α regulation. Similarly, a small tyrosine inhibitor, SU11274, targets the kinase domain of the HGF receptor (MET) and completely shuts down the effect of HGF on miR-519c and HIF-1α expression. Similar results were found in cells treated with the phosphoinositide 3-kinase (PI3K) inhibitor LY294002, but not in cells treated with the mitogen-activated protein (MAP)/extracellular signal-regulated kinase kinase (MEK) inhibitor U0126 or the mammalian target of rapamycin (mTOR) (Fig. 3C). To determine the correlation of HGF and miR-519c expression in non–small cell lung carcinoma primary tumor specimens, we compared the levels of miR-519c in tumors with high and low levels of HGF. We observed a significant inverse correlation between expression of HGF and miR-519c ($P < 0.005$, Fisher's exact test; Supplementary Fig. S4A), confirming the HGF-dependent regulation of miR-519c in human tumors. Moreover, consistent with a previous study (27), expression of HGF was predominantly in the lung tumor cells, with a significantly lower expression level in the surrounding stroma cells (Supplementary Fig. S4B), indicating that HGF regulates miR-519c mainly through an autocrine manner in lung cancer. To confirm the role of AKT in the regulation of miR-519c, we expressed dnAKT into CL1-0 cells in the absence and presence of HGF. The suppressive effect of HGF on miR-519c expression was abolished by introducing dominant-negative Akt (dnAkt) in CL1-0 cells (Fig. 3D, left). In contrast, active AKT (myrAKT) failed to reverse the effect of MET inhibition in CL1-5 cells, indicating that AKT was not sufficient to regulate the miR-519c activity (Fig. 3D, right).

Several studies have suggested that regulation of miRNAs occurs at both the transcriptional and posttranscriptional levels (28–30). To understand the precise mechanism of HGF-mediated miR-519c reduction, we analyzed the expression of pri–miR-519c, pre–miR-519c, and mature miR-519c after 6 hours of HGF treatment. Interestingly, only the mature form of miR-519c was significantly reduced by HGF treatment (Fig. 4A). We further investigated whether HGF could induce the degradation of miR-519c in an expression time course. In the presence of the transcription inhibitor actinomycin D, treatment of HGF had no effect on miR-519c expression (Fig. 4B), indicating that HGF-mediated miR-519c

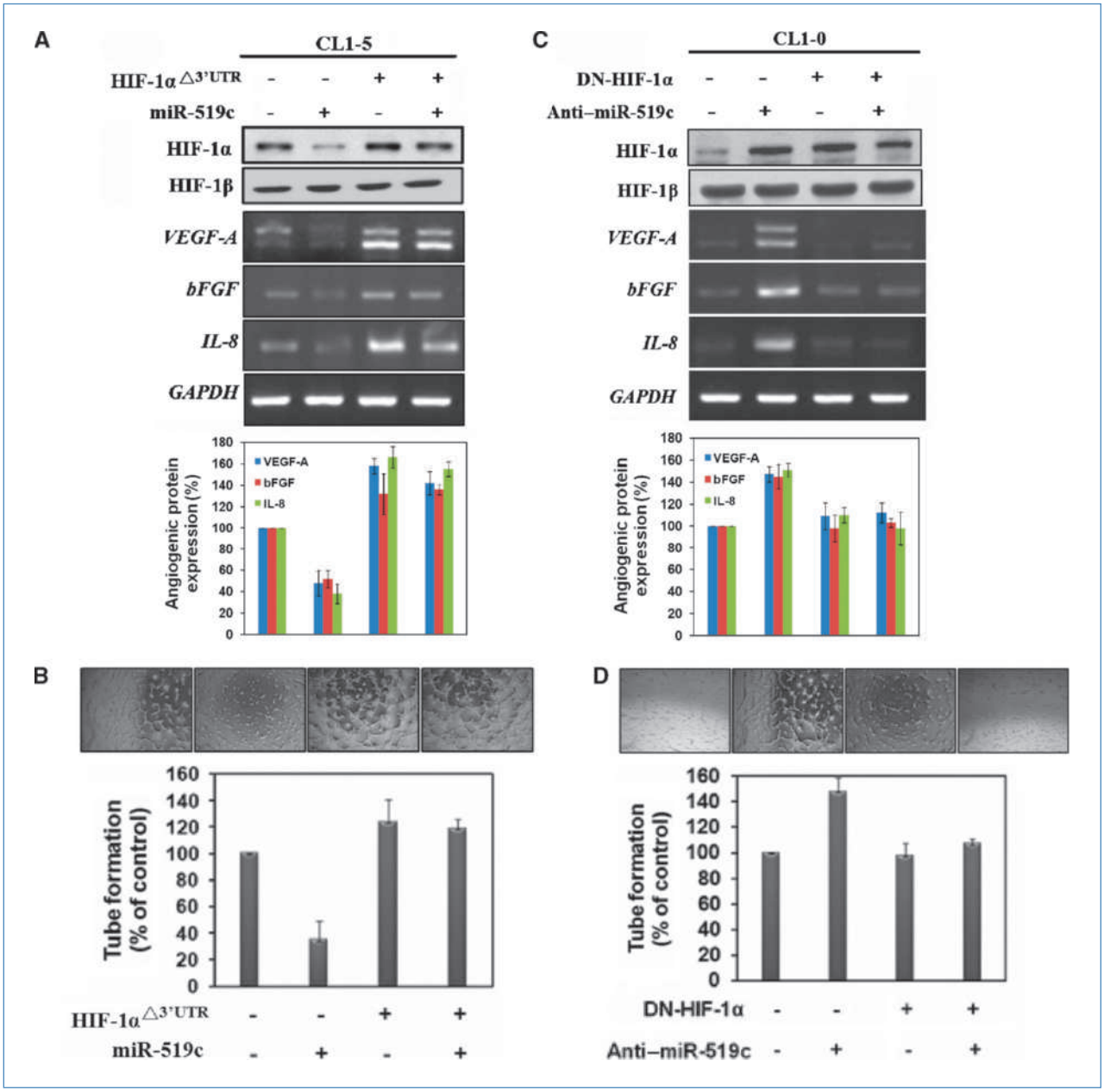
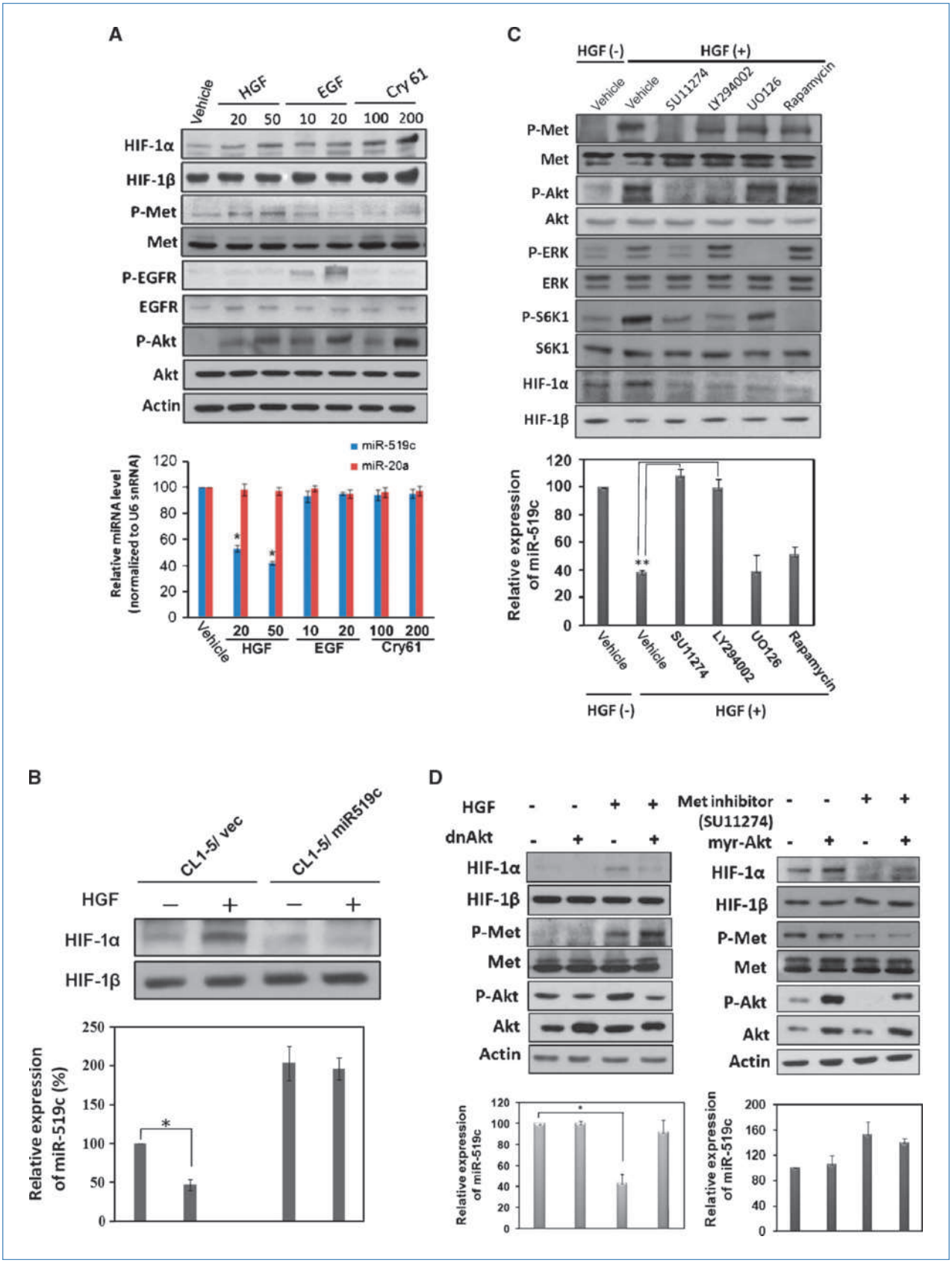


Figure 2. miRNA-519c suppresses HIF-1α, leading to reduced tumor angiogenic activity. A, top, Western blot analysis of CL1-5 cells transfected with HIF-1α expression vector (wt HIF-1α) and miR-519c expression vector (mir-519c), as indicated. Bottom, expression of angiogenic factors VEGF-A, IL-8, and bFGF in the conditioned medium of cells in the top panel as determined by ELISA. Means and upper 95% confidence intervals of four experiments are shown. *, $P = 0.043$, two-sided Student's t test. B and D, miR-519c antagonized the antiangiogenic activity of HIF-1α. HUVECs were cultured together with conditioned medium from the transfected cells, as indicated, and seeded on a "growth factor–reduced" Matrigel basement membrane matrix for 8 h. C, top, Western blot analysis of HIF-1α protein in CL1-0 transfectant, which overexpressed dominant-negative HIF-1α expression vector (DN-HIF-1α) and antisense RNA against miR-519c (anti–mir-519c).

reduction is not controlled by accelerating miR-519c degradation. This result tempted us to ask whether HGF could regulate the *de novo* synthesis of miR-519c. Upon addition of the RNase inhibitor (RNasin), HGF significantly decreased the accumulation of mature miR-519c without affecting the levels of pri–miR-519c or pre–miR-519c expression (Fig. 4C). These data support that miR-519c expression is suppressed by HGF by inhibiting mature miR-519c biogenesis, without affecting its precursors.

To test the involvement of miR-519c in HIF-1α–dependent angiogenesis *in vivo*, mice were s.c. injected with Matrigel plugs presoaked in conditioned medium. As expected, the angiogenic activity of CL1-0/anti–miR-519c was higher than that of control cells, as shown by the increased concentration of hemoglobin in the Matrigel plugs (Fig. 5A); moreover, expression of DN-HIF-1α significantly abolished this effect. We concluded that miR-519c downregulates angiogenesis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Vehicle
HGF
20 50
EGF
10 20
Cry61
100 200
HIF-1α
HIF-1β
P-Met
Met
P-EGFR
EGFR
P-Akt
Akt
Actin
miR-519c
miR-20a
Relative miRNA level (normalized to U6 snRNA)
Vehicle
HGF
EGF
Cry61
C
HGF (-)
HGF (+)
Vehicle
SU11274
LY294002
UO126
Rapamycin
P-Met
Met
P-Akt
Akt
P-ERK
ERK
P-S6K1
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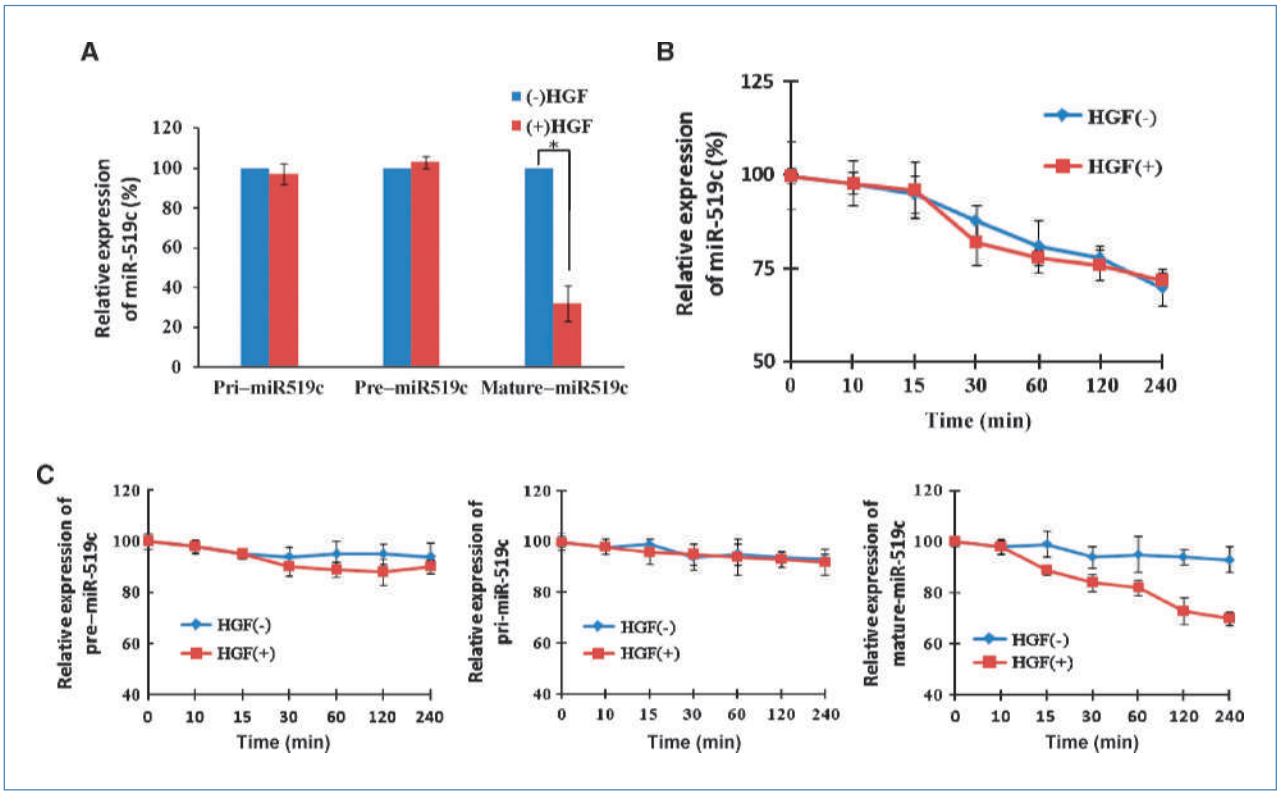

Figure 4. Posttranscriptional regulation of miR-519c biogenesis by HGF. A, expression of primary transcripts (pri–miR-519c), precursors (pre–miR-519c), and mature miR-519c normalized to U6 snRNA in CL1-5 cells stimulated with HGF (2 h; *, $P < 0.05$; $n = 3$). B, steady-state levels of miR-519c after HGF treatment of CL1-5 cells. CL1-5 cells were treated for different times with 5 nmol/L actinomycin D. Points, means of two to three experiments repeated in triplicate; bars, SD. C, time course of pri–miR-519c, pre–miR-519c, and mature miR-519c expression in CL1-5 upon stimulation with HGF in the presence of RNasin (100 units/mL).

both *in vitro* and *in vivo* through the inhibition of HIF-1α. To elucidate the role of miR-519c in tumor angiogenesis, we injected CL1-5/vector and CL1-5/miR-519c cells s.c. into the flanks of nude mice. CL1-5/miR-519c cells displayed a slower growth rate and reduced tumor size (Fig. 5B). These tumors were dramatically infiltrated with more blood vessels, as evidenced by CD31 staining (Fig. 5C, top). Quantitative analysis showed that only 18.48 vessels per field were found in CL1-5/miR-519c tumors, whereas CL1-5/vec tumors had 57.65 vessels per field (Fig. 5C, bottom). To examine the role of miR-519c in tumor metastasis, CL1-5/vector and CL1-5/miR-519c were injected into the tail vein of tumor-bearing Balb/nude mice. Mice injected with CL1-5/miR-519c showed fewer macroscopically visible nodules in the lungs (Fig. 5D), indicating that miR-519c is an angiogenic inhibitor and may suppress metastasis *in vivo*. Similar results were obtained *in vitro*—miR-519c expression significantly inhibited the migration and invasion abilities of CL1-5 cells, whereas inhibition of miR-519c promoted these activities (Supplementary Fig. S5B). These results suggest that miR-519c is a regulator of both tumor invasion and angiogenesis.

To further evaluate the role of miR-519c in regulating tumor angiogenesis and metastasis, we investigated the correlation between miR-519c level and the clinical outcome of lung cancer patients. One hundred thirty-five primary human lung cancer specimens and their nontumor counterparts were collected and assayed. Consistent with the aforementioned results, miR-519c and tumor status exhibited an inverse association in patient

Figure 3. HGF-dependent inhibition of miRNA-519c through the Akt pathway. A, top, immunoblots of HIF-1α, MET, EGFR, and p-AKT in CL1-0 cells after 2-h treatment with recombinant HGF, EGF, or Cyr61. β-Actin was used as a loading control. Bottom, qPCR analysis of miR-519c and miR20a (*, $P < 0.05$; $n = 3$). B, miR-519c downregulation is essential for HGF-induced HIF-1α expression. Both CL1-5/vec and CL1-5/miR-519c cells were treated with vehicle control and HGF for 2 h. *, $P < 0.05$. C, immunoblots for PI3K/Akt/S6 kinase. CL1-0 cells were pretreated with SU11274 (MET inhibitor, 10 μmol/L), LY294002 (PI3K inhibitor, 20 μmol/L), UO126 (MEK inhibitor, 20 μmol/L), and rapamycin (mTOR inhibitor, 5 nmol/L) for 30 min before stimulation with 50 nmol/L HGF for 2 h. D, left, the HGF-induced reduction of miR-519c is dependent on Akt activation. Top, immunoblots for HIF-1α, Met, and Akt. Bottom, qPCR analysis of miR-519c expression after introducing dominant-negative Akt (dnAkt) in CL1-0 cells. *, $P < 0.05$. Right, activation of Akt alone could not regulate miR-519c expression. CL1-5 cells were transfected with 3 μg of active Akt (myrAkt) and treated with SU111274 (10 μmol/L) as indicated. Cell extracts were analyzed by Western blotting (top) with the respective antibodies, as indicated on the right and described in text. Bottom, qPCR analysis of the corresponding miR-519c expression.

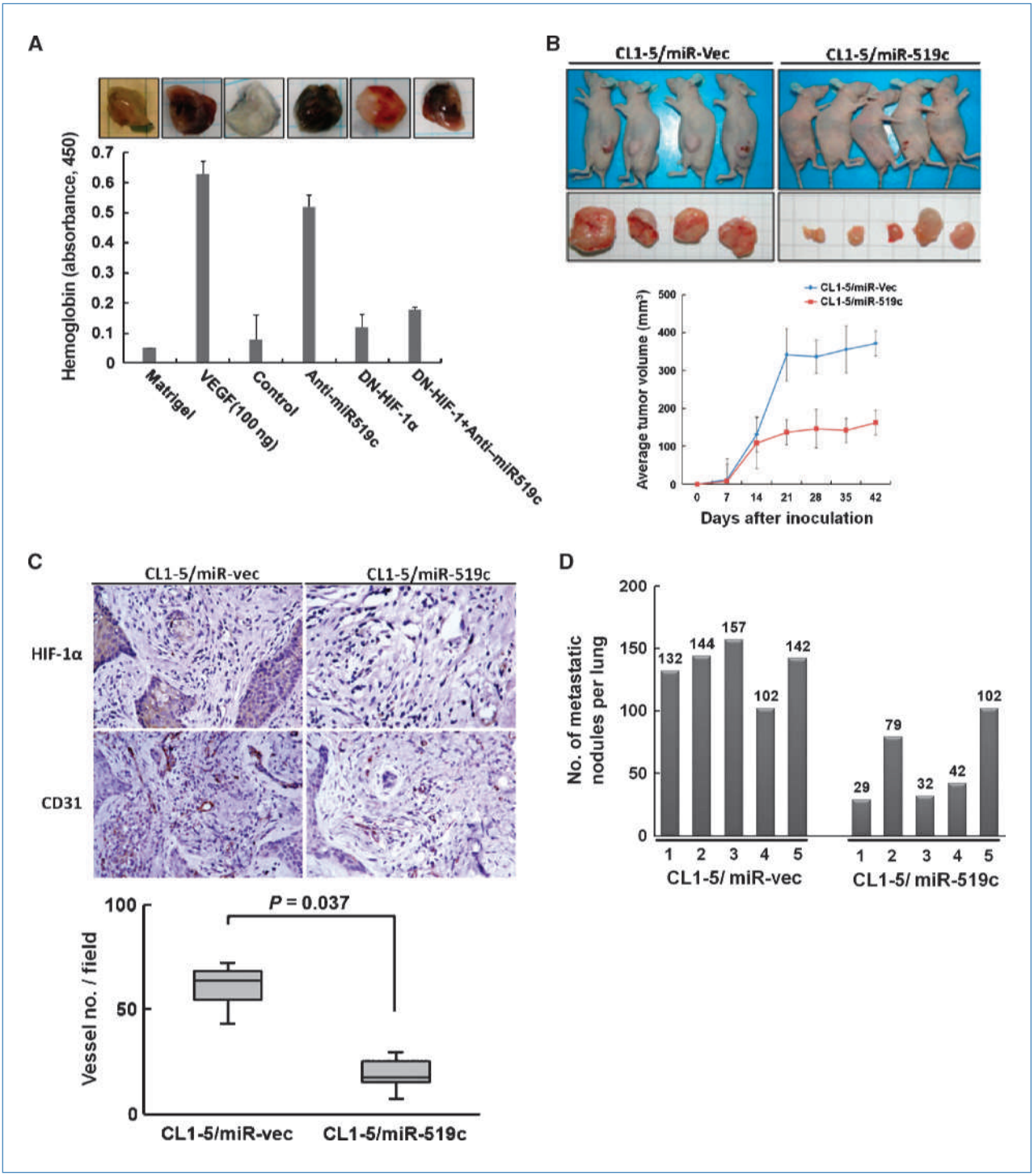

Figure 5. Tumor formation and angiogenesis affected by miR-519c overexpression. A, Matrigel (100 μL) was presoaked in concentrated (10-fold) conditioned medium of CL1-5 cells treated with IgG, anti–miR-519c transfectants, VEGF, DN-HIF-1α transfectants, or double transfectants with DN-HIF-1α and anti–miR-519c, and injected s.c. into C57BL/6 mice ($n = 8$). Top, 7 d after the injection, the Matrigel plugs were removed and photographed. Bottom, neovessels in plugs were measured by assaying hemoglobin levels (absorbance at 540 nm) using Drabkin reagent kit 525 (Sigma). The means and upper 95% confidence intervals of eight data points per group are shown. B, miR-519c expression and tumor formation. CL1-5 cells transfected with miR-519c (red symbols) or a control vector (blue symbols) were injected s.c. into nude mice. Bottom, at the end of the study, tumors were drastically smaller in the miR-519c group (top; $P < 0.05$; $n = 10$). C, sections of comparable tumors were stained against CD31 and HIF-1α. We noticed a significant decrease in the number of CD31-positive vessels in the miR-519c–transfected group compared with vector control. D, numbers of lung micrometastases per lung in individual mice that received tail vein injection of miR-519c–transduced or vector group cells.

Table 1. Clinicopathologic characteristics of patients with high and low expression of miR-519c

Characteristic	Patients with high miR-519c expression (%)	Patients with low miR-519c expression (%)	*P*
Training data set (*n* = 135)	52	83	
Age (mean ± SD)	65.7 ± 10.3	67.3 ± 9.3	0.503
Gender, no. of patients			0.57
Male	14	35	
Female	38	48	
Stage, no. of patients			
I–II	37 (71)	24 (25)	0.008*
III–IV	12 (29)	59 (75)	
Tumor status, no. of patients			
T_1	41 (65)	29 (32)	0.028*
T_2–T_4	11 (35)	54 (68)	
Lymph nodal status, no. of patients			
N_0	26 (50)	48 (58)	0.86
N_1–N_3	26 (50)	35 (42)	

*$P < 0.05$. Significances of association were determined using a Chi-square test.

samples (Supplementary Fig. S6A; Table 1, $P = 0.008$). In addition, similar results were found in tumor stages (Supplementary Fig. S6B; Table 1, $P = 0.028$). Taken together, the above results indicate that miR-519c expression is closely associated with tumor progression in human cancer tissues.

Discussion

HIF-1α and HIF-2α are known to have some nonoverlapping biological roles due to their unique target genes and different oxygen requirements for activation (31). High HIF-2α expression correlates with tumor angiogenesis and patient mortality in several primary and metastatic human cancers (32–36). Hence, HIF-2α may play a critical role in tumor formation/angiogenesis. Considering the suppressive effect of miR-519c in tumor angiogenesis, we determined whether miR-519c regulated HIF-2α. In contrast to HIF-1α, we did not find any predicted association between HIF-2α and miR-519c in any of the target screening systems (miRanda, RNAhybrid, and TargetScan). In agreement with these findings, overexpression of miR-519c did not alter the HIF-2α protein levels in CL1-5 and MDA-MB-231 cells (Supplementary Fig. S7). Apparently, miR-519c regulates HIF1α without affecting HIF2α.

The finely tuned mechanisms of HIF-1α regulation include stabilization, phosphorylation, modifications of redox conditions, and interactions with coactivators. It is well established that von Hippel-Lindau–mediated ubiquitination plays a major role in the regulation of HIF-1α through proteasome-dependent degradation under normoxia, whereas oxygen depletion results in stabilization and activation. In contrast, *de novo* synthesis of HIF-1α is controlled in an oxygen-independent manner by growth factors, cytokines, and other signaling molecules through activation of the phosphoinositide 3-kinase or MAP kinase pathways (26). Recent reports indicate that miRNAs are involved in HIF-1α regulation. For instance, miR-92-1 has been shown to target pVHL to indirectly suppress HIF-1α degradation (28). In addition, the *miR-17-92* gene is a target for c-*myc* and is involved in the downstream regulation of HIF-1α (23). Similar to these results, we found that miR-519c can regulate HIF-1α in an oxygen-independent manner, as hypoxia has no effect on miR-519c expression. Although progress has been made toward understanding the regulation of miRNA, little is known about the microenviromental factors affecting the expression of miRNAs. Herein, we illustrated a novel mechanism by which the HGF/Met axis mediates the inhibition of miR-519c through the Akt signaling pathway, providing a new paradigm for the regulation of miRNAs by microenvironmental factors. The HGF/Met signaling pathway plays an important role in tumor growth and angiogenesis. Our present work indicates that miR-519c is an important regulator in the HGF/c-Met signaling pathway.

The regulation of miRNA involves several posttranscriptional factors and their exact role in this process is still unknown. Generally, miRNA is thought to be controlled in the transition between pri-miRNA and mature miRNA (28–30). For instance, BMP-2 reduces the miR-206 level by inhibiting the processing of pri–miR-206 and, simultaneously, the accumulation of pri–miR-206 (37). In our study, HGF blocked the production of miR-519c without affecting pre/pri-miRNA levels. Importantly, we showed that this was not due to increased degradation of the mature miRNA but to an effect on transcriptional regulation. Similar findings have been reported in other miRNA models. For example, although both pre–miR-21 and mature miR-21 accumulations are enhanced by SMAD4-induced maturation of pri–miR-21, transforming growth factor-β or BMP4 does not alter the pri–miR-21 level (38). In addition, *lin-28* blocks pri-*let*-7 processing without altering the level of pri–*let*-7 (39). It is possible that, in these cases, sufficient transcriptional regulation may keep the amount of miRNA precursors constant; thus, reduced consumption might be paralleled by decreased transcription. However, the precise mechanism needs to be investigated further.

Although miRNAs play important roles in cancer development, little is known about their role in angiogenesis (2, 40, 41). In the present study, we showed that miR-519c regulates angiogenesis both *in vitro* and *in vivo* through the inhibition of HIF-1α. Our *in vitro* data correlate with the overexpression seen in early-stage lung cancer patients, indicating that miR-519c is a tumor suppressor involved in tumor progression. HIF-1α has been implicated in a wide range of biological processes, including angiogenesis, apoptosis pH regulation, metabolism, cell proliferation/survival, invasion, and metastasis (42–44). In our animal experiments, tumors overexpressing miR-519c lacked metastatic activity. Considering that metastasis is the leading reason for cancer-related deaths, it is of great importance to further evaluate miR-519c as a potential cancer therapeutic. The exact role of miR-519c in tumor development remains to be investigated, but it is likely to involve other downstream targets. Besides HIF-1α, a recent report suggests that the miR-519 family represses RNA binding proteins (HuR) and recapitulates cell proliferation in several human carcinoma cells (45). Further, we also noticed several genes, such as *IGF, Bcl2*, and *Myc*, which may exert their regulatory activity through miR-519c. Because all of these genes have been documented as promoters of cancer progression, further studies are required to delineate the tumor-suppressive functions of miR-519c.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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