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A novel hypoxia-induced miR-147a regulates cell proliferation through a positive feedback loop of stabilizing HIF-1α

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ABSTRACT

Hypoxia is a general event in solid tumor growth. Therefore, induced cellular responses by hypoxia are important for tumorigenesis and tumor growth. MicroRNAs (miRNAs) have recently emerged as important regulators of hypoxia induced cellular responses. Here we report that miR-147a is a novel and crucial hypoxia induced miRNA. HIF-1α up-regulates the expression of miR-147a, and miR-147a in turn stabilizes and accumulates HIF-1α protein via directly targeting HIF-3α, a dominant negative regulator of HIF-1α. Subsequent studies in xenograft mouse model reveal that miR-147a is capable of inhibiting tumor growth. Collectively, these data demonstrate a positive feedback loop between HIF-1α, miR-147a and HIF-3α, which provide a new insight into the mechanism of miR-147a induced cell proliferation arrest under hypoxia.

Abbreviations: miRNA, microRNA; CCK-8, cell counting kit-8; HIF-1α, hypoxia-inducible factor 1 α subunit; HIF-3α, hypoxia-inducible factor 1 α subunit; NC, negative control; MUT, mutated; UTR, untranslated region

Introduction

MicroRNAs (miRNAs or miRs) are a kind of small non-coding RNAs with a length of about 20–24 nucleotides. They participate in a vast majority of physiological and pathological processes, including cell proliferation, differentiation, apoptosis, autophagy, angiogenesis, metabolism and cancer.[1-7] Especially, miRNAs have recently emerged as important regulators of cellular responses under hypoxia. Hypoxia-induced miR-26 production influences myogenic differentiation of embryonic stem cells by targeting HDAC6.[8] MiR-133a attenuates hypoxia-induced apoptosis via inhibition of TAGLN2 expression [9] while miR-195 and miR-26b enhance apoptosis by directly targeting HIF-1α and PTEN respectively.[10,11] MiR-101a exerts anti-fibrotic effects via targeting TGFβRI.[12] MiR-21, miR-322, miR-20s and miR-130/301 influence hypoxia-induced proliferation of pulmonary artery smooth muscle cells.[13-16] We have earlier reported a group of miRNAs which respond to hypoxia induction.[17] Among these, miR-210 and miR-155 are related to cell cycle, proliferation and autophagy.[18,19]

Hsa-miR-147a is related to hsa-miR-210 closely, which differs by one nucleotide in the seed region. MiR-147a was discovered in mouse spleen tissue [20] and subsequently in humans [21] by Thomas Tuschl. Like miR-210, miR-147a inhibits cell proliferation by downregulating cell cycle proteins, such as pRB,

CycB, CycA, Cdk6.[22] MiR-147a is upregulated in Squamous Cell Carcinoma of tongue, human gastric cancer, small cell lung cancer and hepatocellular carcinoma.[23-26] Some reports demonstrate that miR-147a affects cell development, migration and invasion, but has no influence on apoptosis.[5,22,27]

Here we report that miR-147a is a novel hypoxia-inducible miRNA and participates in cell hypoxia response processing. MiR-147a is up-regulated by HIF-1α in Hela cells under hypoxia condition, and then miR-147a increases stabilization of HIF-1α protein via targeting HIF-3α, which is a dominant negative regulator of HIF-1α. Thus, miR-147a plays an important role in inhibiting cell proliferation, one of cellular responses under hypoxic stress. Moreover, overexpressing miR-147a significantly inhibits tumor growth in xenograft mouse tumor models. Collectively, these data demonstrate that the HIF-1α-miR-147a-HIF-3α axis serves as a new mechanism underlying the role of miR-147a in regulating cell proliferation under hypoxia.

Materials and methods

Cell culture

HeLa (human cervical cancer, ATCC, CCL-2) cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Gibco, 12800–017) supplemented with 10% fetal bovine serum (FBS,

PAA, A15–101). Cells were grown under normoxic (21% O_2) or hypoxic (1% O_2) conditions at 37°C in a humidified 5% CO_2 incubator.

Plasmid, miRNA and siRNA transfection

HeLa cells were transiently transfected with Lipofectamine 2000 (Invitrogen, 11668–019) according to the manufacturer's instructions. Plasmid pEGFP-N1-FIH1 (plasmid # 21403) and HA-HIFalpha-pcDNA3 (plasmid # 18949) were purchased from Addgene. A mock-vehicle pcDNA3 and a small RNA with a random sequence were used as negative controls. The plasmid was added to adherent cells in a 6-well plate at a final concentration of 1uM; siRNA or miRNA was suspended for transfection at a final concentration of 100 pmol. MiR-147a mimics, miR-147a inhibitors, negative control (NC) and siRNAs were synthesized and purchased from Shanghai GenePharma (Shanghai, China). The sequences of siRNAs used were:

Si HIF-3α#1: 5′-CCACCACGCCCGACAGUAA-3′,
5′-UUACUGUCGGGCGUGGUGG-3′
Si HIF-3α#2: 5′-GUGCUGGGAUUACUGGUAU-3′,
5′-AUACCAGUAAUCCCAGCAC-3′
Si HIF-1α: 5′-AACUAACUGGACACAGUGUGU-3′
5′-ACACACUGUGUCCAGUUAGUU3′

miRNA targets prediction

We used two conventional online programs to predict the potential targets and precise binding sites of miR-147a: Targetscan (www.targetscan.org) and Findtar (bio.sz.tsinghua.edu.cn).

RNA isolation, reverse transcription and quantitative real-time PCR

After transfection (48h), cells were washed in 1XPBS and RNA was isolated using RNAiso Plus (Takara, D9108B) according to the manufacturer's instructions. Total mRNA was reverse transcribed using a reverse transcription kit (Takara, D6130) with random primers and quantitative real-time PCR of cDNA was performed using SYBR Green Real-time PCR Master Mix (Toyobo, QPK-201). qRT-PCRs of miRNAs were performed according to the manufacturer's instructions using a TaqMan MicroRNA Reverse Transcription Kit (ABI.4366597) and Real-time PCR Master Mix (Toyobo, QPK-101). miRNA probes were purchased from ABI: hsa-miR-147a (ABI, 000469), RNU6 (ABI, 001093). mRNA and miRNA expression levels were normalized to GAPDH and RNU6 respectively.

Western blotting

HeLa cells were lysed with an ice-cold cell lysis buffer (50mM Tris-HCl pH8.0, 4M Urea and 1% Triton X-100), containing protease and phosphatase inhibitors (Roche, 04693132001). Whole cell lysates were equalized and separated by SDS-PAGE and then transferred onto nitrocellulose membrane. Membranes were blocked with 5% milk in Tris-buffered saline plus 0.02% Tween-20 (TBST). Primary antibodies used in this paper were: HIF-1α (BD 610958); HIF-3α (Novusbio 100–2529),

ACTB (CST 3700). After incubation with horseradish peroxidase-coupled secondary anti-mouse (KPL 074–1806) or anti-rabbit antibodies (KPL 474–1506), the membranes were washed and protein bands were detected with ECL blotting detection reagents (KPL 547100).

Luciferase activity assay

HeLa cells were seeded in a 24-well plate. The plasmids pmirGLO-HIF-3α1–3′UTR–WT or pmirGLO-HIF-3α1–3′UTR–MUT were constructed by inserting the wild-type or mutated 3′UTR fragments of HIF-3α into pmirGLO Dual-Luciferase miRNA Target Expression Vectors (Promega, E1330). To detect the reciprocity of miR-147a and HIF-3α1 3′UTR, HeLa cell were co-transfected with WT or MUT UTR luciferase vectors and miRNAs using lipofectamine 2000 according to reference. About 36h later, the cells were collected, firefly and Renilla Luciferase activities were measured by Dual-Luciferase Reporter assay system (Promega, E1960), and normalized to the ratio of firefly and Renilla luciferases. Each experiment was repeated in triplicate.

Cell proliferation assay and colony formation

Cell proliferation was evaluated using cell counting kit-8 (CCK-8 Kit, Dojindo, CK04). HeLa cells were seeded in 96-well plates in triplicate with 2000 cells per well and transfected with miR-NC, miR-147a or siRNA and the number of viable cells was determined by measuring OD_{450} using microplate reader (Epoch, BioTek). For the colony formation assay, HeLa cells were plated on a 6-well plate and transfected with miR-NC, miR-147a or siRNA once in every 3 d after cell adhesion. After culturing for 10 days, cells were fixed with cold methanol and stained with 1% crystal violet for 30 min. The number of colonies was counted assuming >50 cells/colony.

Mice and tumor formation assays

Four week old male BALB/c nude mice were purchased from Guangdong Experimental Animal Center (Guangzhou, China) and kept in a pathogen-free environment for a week at Tsinghua University Shenzhen Graduate School. Approximately 5×10^5 well-conditioned HeLa cells were injected into dorsal flank of nude mice subcutaneously. Ten days after tumor implantation, mice were randomly divided into 3 groups for treatment by either miR-147a, miR-NC. Transferrin-polyethylenimine (TF-PEI) / miRNA-mimics complex were delivered through subcutaneous injections every alternate day. At the end of the experiment (after 5 injections), the mice were killed by breaking the neck, individual tumors isolated and the weight of each animal was recorded.

Statistical analysis

The bar graphs represent mean ± SD from at least 3 independent experiments. Student's t-test was applied for significant difference between 2 groups of data. The results were statistically significant if p-value was <0.05.

Results

Hypoxia induces upregulation of miR-147a

To determine the expression of miR-147a in hypoxia, we cultured HeLa cells at 1% O_2, and subsequently collected the cells for qRT-PCR. The expression level of endogenous miR-147a increased nearly 10 folds after 24h in hypoxia (Fig. 1A). To investigate whether hypoxia-induced upregulation of miR-147a is due to HIF-1α, we transfected cells with pEGFP-N-FIH1, which can inhibit HIF-1α transcription activity.[28] Overexpression of FIH1 dramatically reduced the expression of miR-147a. Knocking down endogenous HIF-1α showed the similar effect (Fig. 1B).

miR-147a inhibits cell proliferation and colony formation

Hypoxia appears to be strongly associated with tumor propagation and malignant progression, thus we were interested to know whether hypoxia-inducible miR-147a can regulate cell proliferation. Hypoxic stress caused cell growth retardation to nearly half of that observed in normoxia (Fig. 1C). Overexpression of exogenous miR-147a remarkably inhibited cell growth in normoxia (Fig. 1D). Conversely, inhibition of endogenous miR-147a accelerated cell proliferation in hypoxia (Fig. 1E). The effect of miR-147a on cell proliferation was also evaluated by colony formation assay. MiR-147a transfected cells formed significantly fewer colonies than the negative control (Fig. 1F).

Overexpression of miR-147a leads to accumulation of HIF-1α

We were keen to understand whether miR-147a mediated cell proliferation inhibition is related to HIF-1α, which is directly involved in hypoxia-induced cell proliferation arrest.[29] HIF-1α is a critical transcriptional regulator and specifically accumulates in low oxygen conditions (Fig. 2A). Overexpression of exogenous miR-147a significantly increased the protein level of HIF-1α (Fig. 2B, 2C), but not the mRNA levels of HIF-1α in both normoxia and hypoxia conditions (Fig. 2D). We also used MG132, a proteasome inhibitor to increase the stability of HIF-1α in normoxia. MiR-147a increased accumulation of HIF-1α even in the presence of MG132 (Fig. 2E). Conversely, blocking endogenous miR-147a decreased the protein expression of HIF-1α (Fig. 2F). Then, we examined the expression of VEGF and GLUT1, which are downstream targets of HIF-1α. MiR-147a increased the expression of VEGF and GLUT1, owing to the accumulation of HIF-1α (Fig. 2G).
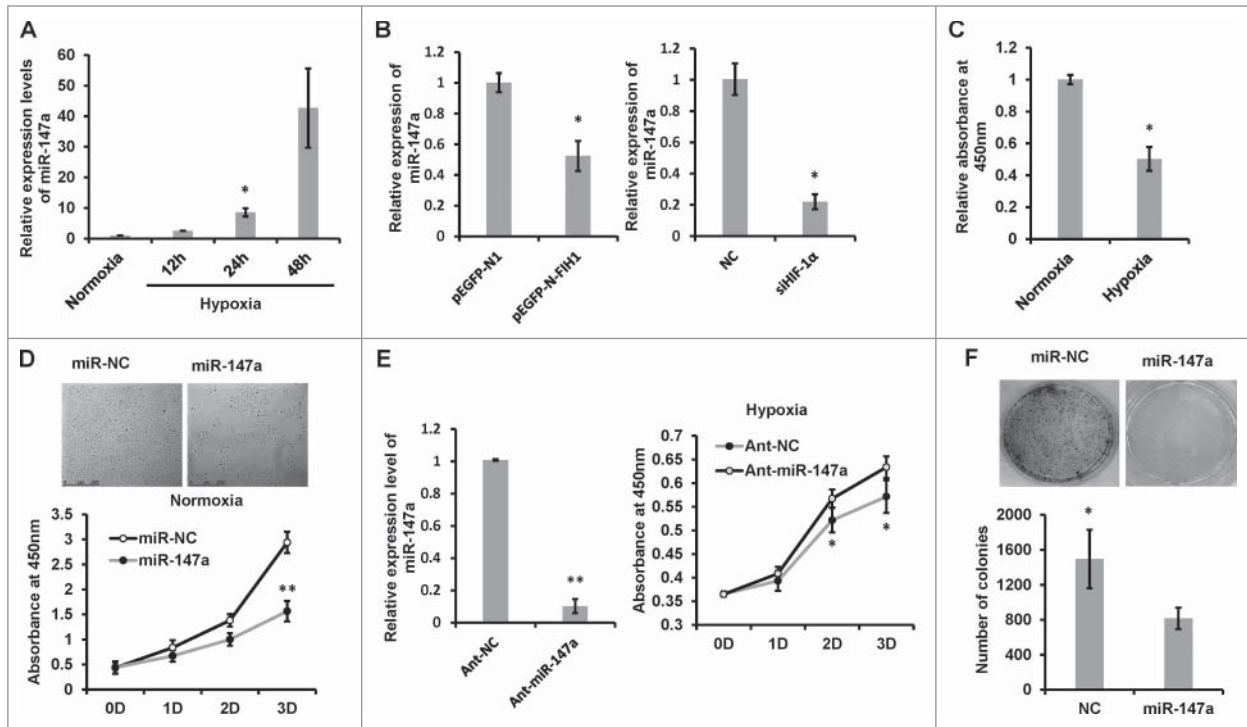

Figure 1. Hypoxia-induced miR-147 inhibits cell proliferation and colony formation. (A) Hypoxia induces miR-147a expression. HeLa cells were exposed to 1% oxygen for 12, 24 and 48 h; real-time PCR analysis of miR-147a levels in HeLa cells was done and compared with normoxia (21% O_2). U6 small nuclear RNA was used as an internal control. Data shown are mean ± SD of 3 independent experiments. *P < 0.05. (B) The expression of miR-147a is regulated by HIF-1α. HeLa cells were transfected with pEGFP-N-FHI plasmid or siHIF-1α for 48h in hypoxia (1% O_2); miR-147a levels in HeLa cells were detected by Real-time PCR. pEGFP-N1 or NC were used as negative controls. Data shown are mean ± SD of 3 independent experiments. *P < 0.05. (C) Hypoxia inhibits cell proliferation. HeLa cells were cultivated in hypoxia (1% O_2) or normoxia (21% O_2) for 48h and viabilities were determined by CCK-8 assay. Data shown are mean ± SD of 3 independent experiments. *P < 0.05. (D) Overexpression of miR-147a inhibits cell proliferation. HeLa cells were transfected with miR-147a or miR-NC in normoxia (21% O_2) for 1, 2 and 3 d and viabilities were determined by CCK-8 assay every day. Representative phase contrast images taken after 48h are shown. Data shown are mean ± SD of 3 independent experiments. *P < 0.05, **P < 0.001. (E) Knockdown of endogenous miR-147a recovery from hypoxia-induced cell growth inhibition. HeLa cells were transfected with single strand ant-miRNA in hypoxia (1% O_2); real-time PCR analysis shows that endogenous miR-147a expression was blocked; viabilities were determined by CCK-8 assay every day. Data shown are mean ± SD of 3 independent experiments. **P < 0.001. (F) Clonogenic assay of HeLa cells transfected with miR-NC or miR-147a. Cells were cultured for 10 d in normoxia and stained with Crystal Violet. The number of colonies were quantified and calculated as mean ± SD of 3 independent experiments (Student t test, *p < 0.05).
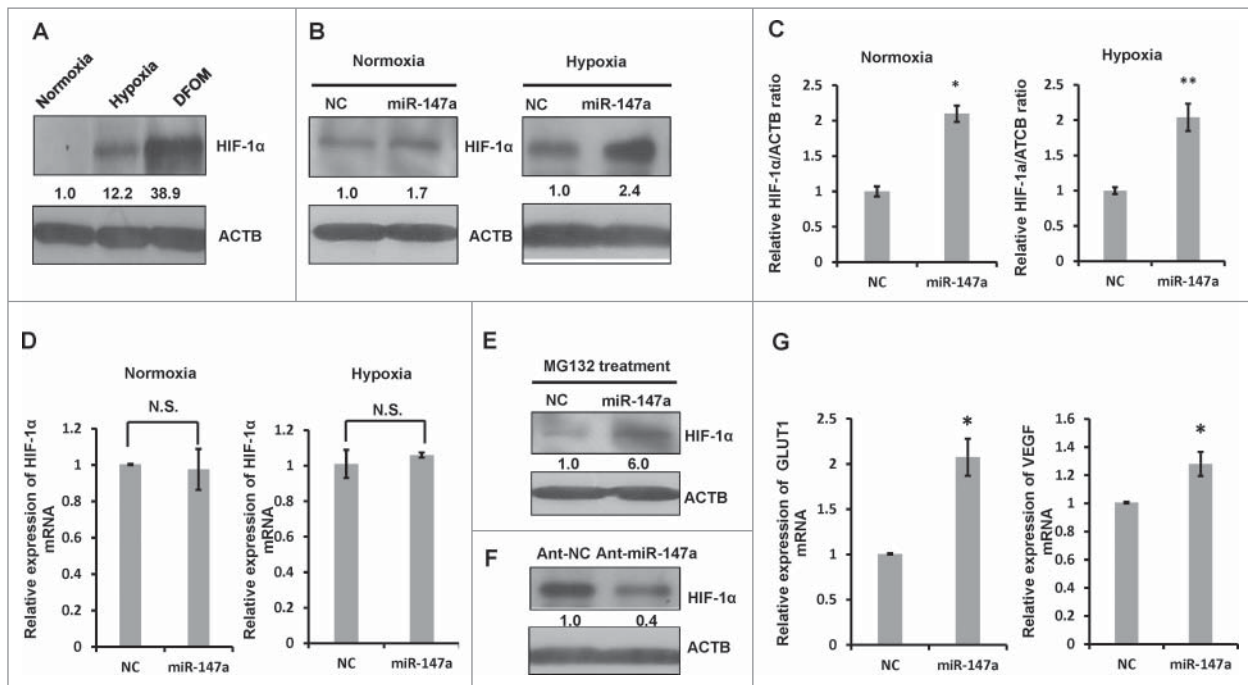
Figure 2. Overexpression of miR-147 leads to accumulation of HIF-1α. (A) Hypoxia induces HIF-1α expression and accumulation. HeLa cells were cultivated in hypoxia (1% O$_2$) or treated with or without DFOM for 24 h in normoxia (21%O$_2$); cells were harvested for western blot. Relative HIF-1α/ACTB ratios were determined by Image J densitometric analysis. (B) Overexpression of miR-147a leads to accumulation of HIF-1α protein. HeLa cells were transfected with NC, miR-147a in normoxia (21%O$_2$) or hypoxia (1% O$_2$). Western blotting was performed to analyze the status of HIF-1α and ACTB. (C) Relative HIF-1α / ACTB ratios from (B) were determined by Image J densitometric analysis. Data shown are mean $\pm$ SD of 3 independent experiments, *P $<$ 0.05, **P $<$ 0.01. (D) Overexpression of miR-147a doesn't effect HIF-1α transcription. HeLa cells were transfected with NC, miR-147a in normoxia (21%O$_2$) or hypoxia (1% O$_2$). Cells were harvested at 48h for qRT-PCR. N.S., not significant. (E) miR-147a enhanced HIF-1α accumulation of MG132 in 21% O$_2$. HeLa cells were transfected with miR-147a or NC. After 36h of transfection, HeLa cells were treated with MG132 (10 $\mu\mu$). The protein level of HIF-1α and ACTB were detected by protein gel blot. Relative HIF-1α/ACTB ratios were determined by Image J densitometric analysis. (F) Blockage of endogenous miR-147a leads to HIF-1α protein reduction. HeLa cells were transfected with Ant-NC or Ant-miR-147a in hypoxia (1% O$_2$). Western blotting was performed to analyze the status of HIF-1α and ACTB. Relative HIF-1α/ACTB ratios were determined by Image J densitometric analysis. (G) Overexpression of miR-147a enhances the mRNA levels of VEGF and GLUT1 in 21% O$_2$, which are regulated by HIF-1α.

miR-147a upregulates HIF-1α via targeting HIF-3α

We used bioinformatics approach [17] to identify the putative miR-147a targets. HIF-3α 3$'$ UTR contains 2 potential miR-147 binding sites (Fig. 3A). To determine if miR-147a regulates the expression of HIF-3α via binding to its 3$'$UTR, we performed luciferase activity assay. We generated luciferase reporters by inserting the 3$'$UTR fragments of HIF-3α with wild-type (WT) or mutated (MUT) miR-147a binding sites into a pmirGLO-luciferase vector. The constructs were then introduced into the cells for luciferase activity assay. As shown in Fig. 3A, miR-147a significantly suppressed luciferase activity when co-transfected with the reporter constructs with wild-type binding sites of miR-147a. By contrast, the reporter vectors carrying mutated binding sites of miR-147a rescued the repressive effects of miR-147a on luciferase activity. Overexpression of exogenous miR-147a significantly reduced HIF-3α protein expression, but had no effect on HIF-3α mRNA (Fig. 3B, 3D). Conversely, inhibition of endogenous miR-147a increased HIF-3α protein level (Fig. 3C). Similar to miR-147, HIF-3α siRNA also increased protein expression of HIF-1α (Fig. 3E, 3F). To determine whether the stabilization of HIF-1α by miR-147a is dependent on its regulation on HIF-3α, we firstly knockdown HIF-3α with HIF-3α siRNA, then, introduced miR-147a into the cells. Compared with HIF-3α knockdown cells, adding miR-147a cannot further increase the accumulation of HIF-1α (Fig. 3G). This result suggests that miR-147a stabilize HIF-1α via HIF-3α.

HIF-3α regulates cell proliferation

To examine whether miR-147a regulate cell proliferation through targeting HIF-3α, we performed colony formation assay. Less colonies were observed in cells transfected with miR-147a or HIF-3α siRNAs (Fig. 4A). HIF-3α siRNAs also inhibited cell proliferation under both normoxia and hypoxia (Fig. 4B). Since siRNA for HIF-1α was reported to accelerate cellular proliferation,[35] we wonder if exogenous HIF-1α increased cell proliferation. We firstly examined the availability of siHIF-1α and HIF-1α pcDNA3.1 (Fig. 4C). In agreement with these observations, exogenous HIF-1α increased cell proliferation, under normoxic and hypoxia conditions (Fig. 4D). We wondered if HIF-1α siRNA can rescue HIF-3α siRNA induced inhibition of cell proliferation. When siHIF-3α and siHIF-1α were co-transfected into HeLa cells, siHIF-3α-induced inhibition of cell proliferation was partially rescued (Fig 4E). These data suggested that HIF-3α may regulate cell proliferation via HIF-1α.

miR-147a suppresses tumor growth in nude mice

In order to determine the anticancer potential of miR-147a and siHIF-3α, we generated xenografts mouse model by subcutaneously injecting HeLa cells into nude mice. Tumor volumes grew sustainably and significantly in the NC group, whereas miR-147a and siHIF-3α groups showed slow tumor growth
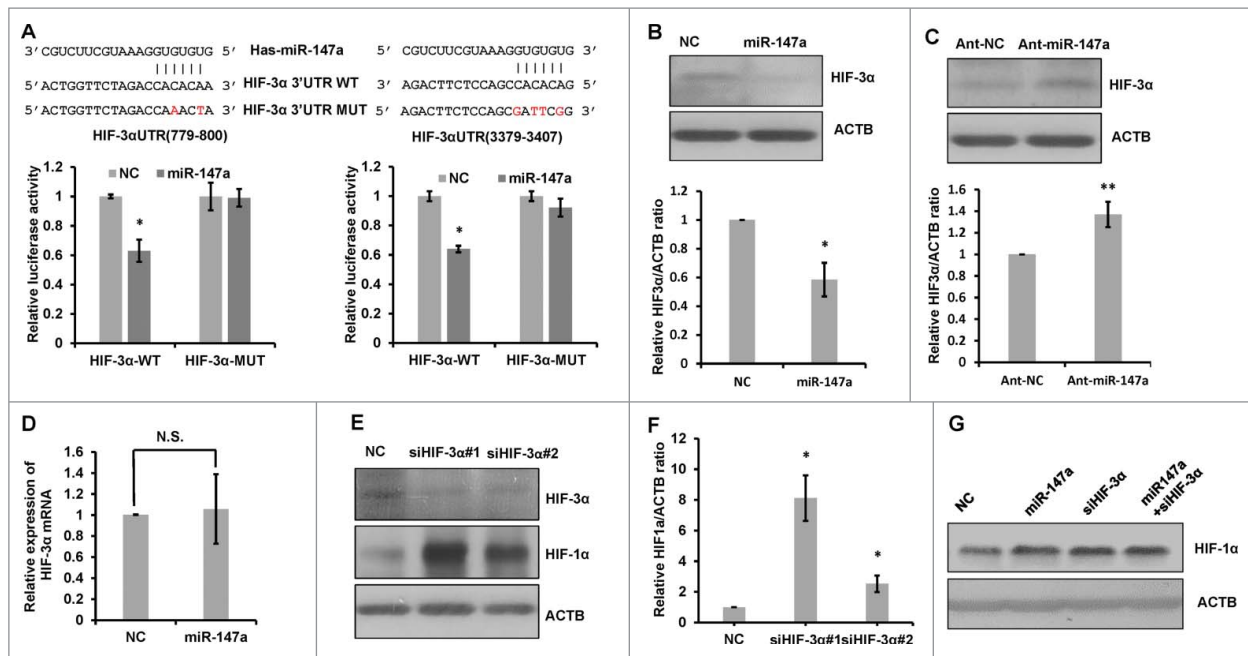
Figure 3. miR-147a upregulates HIF-1α via targeting HIF-3α. (A) miR-147a directly targets the 3′UTR of HIF-3α. Predicted binding sequences between miR-147a and seed matches in HIF-3α 3′UTR. Luciferase reporter assay of wild type (WT) or mutated (MUT) HIF-3α 3′UTR vector co-transfected with NC, miR-147a respectively in normoxia (21%O$_2$). Data shown are mean ± SD of 3 independent experiments, *P < 0.05. (B) Western blot analysis of HIF-3α and ACTB proteins in HeLa cells transfected with NC or miR-147a in normoxia (21%O$_2$). Relative HIF-3α/ACTB ratios were determined by Image J densitometric analysis. Data shown are mean ± SD of 3 independent experiments, *P < 0.05. (C) Western blot analysis of HIF-3α and ACTB proteins in HeLa cells transfected with ant-miR-147a or anti-NC in hypoxia (1% O$_2$). Relative HIF-3α/ACTB ratios were determined by Image J densitometric analysis. Data shown are mean ± SD of 3 independent experiments, **P < 0.01. (D) Overexpression of miR-147a doesn't effect the mRNA level of HIF-3α. HeLa cells were transfected with NC, miR-147a. Cells were harvested at 48h in normoxia (21%O$_2$) for qRT-PCR. N.S., not significant. (E) HIF-1α accumulates when HIF-3α expression is inhibited. HeLa cells transfected with NC or 2 designed HIF-3α siRNAs in hypoxia (1% O$_2$). Western blotting was performed to analyze the protein levels of HIF-1α and ACTB. (F) Relative HIF-1α / ACTB ratios from (E) were determined by Image J densitometric analysis. Data shown are mean ± SD of 3 independent experiments, *P < 0.05, student 2-tailed t test. (G) miR-147a stabilized HIF-1α through HIf-3α. HeLa cells transfected with HIf-3α siRNA firstly and then introduced miR-147a into the cells in hypoxia (1% O$_2$). Western blot analysis of HIF-1α and ACTB proteins.

(Fig. 5A). At the end of the experiments, the nude mice were sacrificed and the tumors were separated to measure tumor mass. Compared with NC (negative control), miR-147a suppressed tumor weight to 43% (P < 0.005), siHIF-3α also moderately decreased tumor mass (Fig. 5B, 5C). Tumor samples with different treatments were collected for Western Blotting assay, the result demonstrated that both miR-147a and siHIF-3α increased protein expression of HIF-1α (Fig. 5D).

Discussion

In this study, we reported that hypoxia-induced miR-147a inhibits cancer cell proliferation, both in vitro and in vivo. It has been reported that miR-147a inhibits cell growth via downregulation of cell cycle proteins, including pRB, cyclin B, cyclin A, CDK2,CDK4 and CDK6.[22,27] But in this study, we find that miR-147a induced cell growth arrest under hypoxia condition also related to HIF-1α accumulation. As a transcription factor, HIF-1α is involved in the regulation for cell growth, angiogenesis, metabolism, apoptosis.[30] Furthermore, there has been reported that HIF-1α accumulation inhibits cell cycle progression and leads to arrest [29] through upregulating cell cycle inhibitors p21 and p27.[31,32] Thus, our results suggest that miR-147a induced cell proliferation arrest could be due to the accumulation and upregulation of HIF-1α protein, which activates the relevant downstream pathway of HIF-1α.

The hypoxia-inducible factors (HIFs) family is an important group of regulators for the cellular oxygen tension response.

There are 4 principal isoforms of HIF (HIF-1α, HIF-2α, HIF-3α and HIF-1β). HIF-1 is a heterodimer containing HIF-1α and HIF-1β, and its expression is regulated by cellular oxygen tension.[33] HIF-3α is a third α-class hypoxia-induced factor isoform. There is cell-specific Characteristics for the expression of HIF-3α, only several cells such as CaKi-1 and HeLa expressing HIF-3α [34] HIF-3α is a negative regulator of hypoxia-inducible gene expression.[35,36] HIF-3α has the capacity of competing with HIF-1α for binding to HIF-1β and causes the degradation of HIF-1α via the ubiquitin pathway. When HIF-3α is silenced, there are more HIF-1β molecules to bind with HIF-1α, thereby increasing stability of HIF- 1α.[37] In this investigation, we discovered that miR-147a induced accumulation of HIF-1α via targeting HIF-3α. Through suppressing HIF-3α expression, miR-147a positively regulates hypoxia-inducible gene expression, including HIF-1α, and finally inhibits cell proliferation.

Our results indicate that the siHIF-3α treatment can increase the level of HIF-1α to a comparable extent as miR-147a treatment does (Fig. 3F). However, the inhibitory effect of siHIF-3α on tumor growth is not as robust as that caused by miR-147a (Fig. 5A). This is due to multi-targets, the prominent feature of miRNAs. In addition to HIF-3α, miR-147a also directly targets some cell cycle-related genes. Thus, the inhibitory effect of siHIF-3α on tumor growth is not as robust as that caused by miR-147a. To further confirm that the suppressive effect of HIF-3α on cell proliferation is related to HIF-1α accumulation, we completed a rescuing experiment via co-transfecting siHIF-3α and siHIF-1α into HeLa cells. The knockdown of
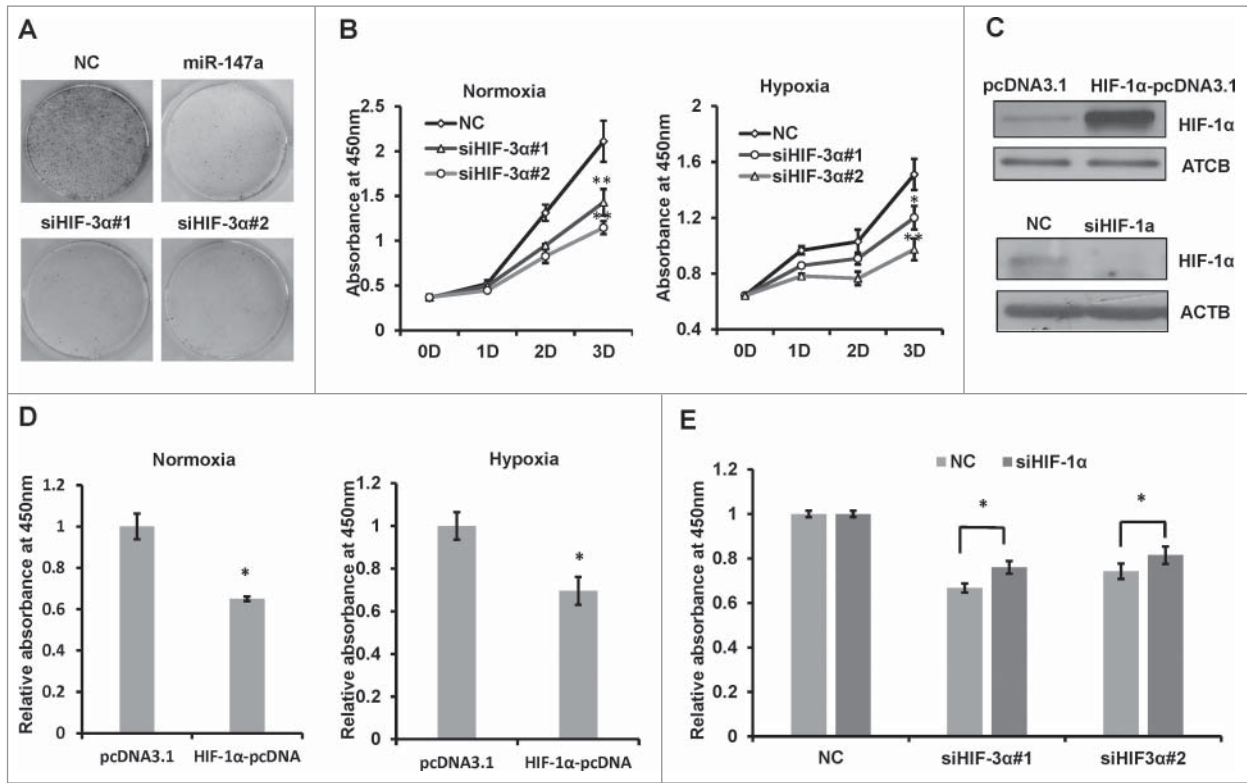
Figure 4. HIF- 3α regulates cell proliferation. (A) Clonogenic assay of HeLa cells transfected with NC, miR-147a or siHIF-3α in normoxia. (B) Inhibition of HIF-3α expression inhibits HeLa cell proliferation. HeLa cells transfected with 2 designed HIF-3α siRNAs or NC for 1, 2 or 3 days; viabilities were determined with CCK-8 assay every day. Data shown are mean ± SD of 3 independent experiments. *P < 0.05, **P < 0.001. (C) Transfection with HIF-1α siRNA reduced HIF-1α synthesis in 1% O₂and transfection with HIF-1α plasmid increased HIF-1α synthesis in 21% O₂ by western blot. (D) Overexpression HIF-1α inhibits cell proliferation in normoxia or hypoxia. HeLa cells were transfected with pcDNA or HIF-1α-pcDNA in 21% O₂or 1%O₂. Expression of HIF-1α was confirmed by protein gel blot and cell viabilities were determined by CCK-8 assay after 3 d. Data shown are mean ± SD of 3 independent experiments. *P < 0.05. (E) CCK-8 assay of HeLa cells co-transfected with siRNAs or NC; cell viability (OD₄₉₀ nm absorbance) was examined at 72h after transfection in hypoxia. Data shown are mean ± SD of 3 independent experiments, *P < 0.05, student 2-tailed t test.
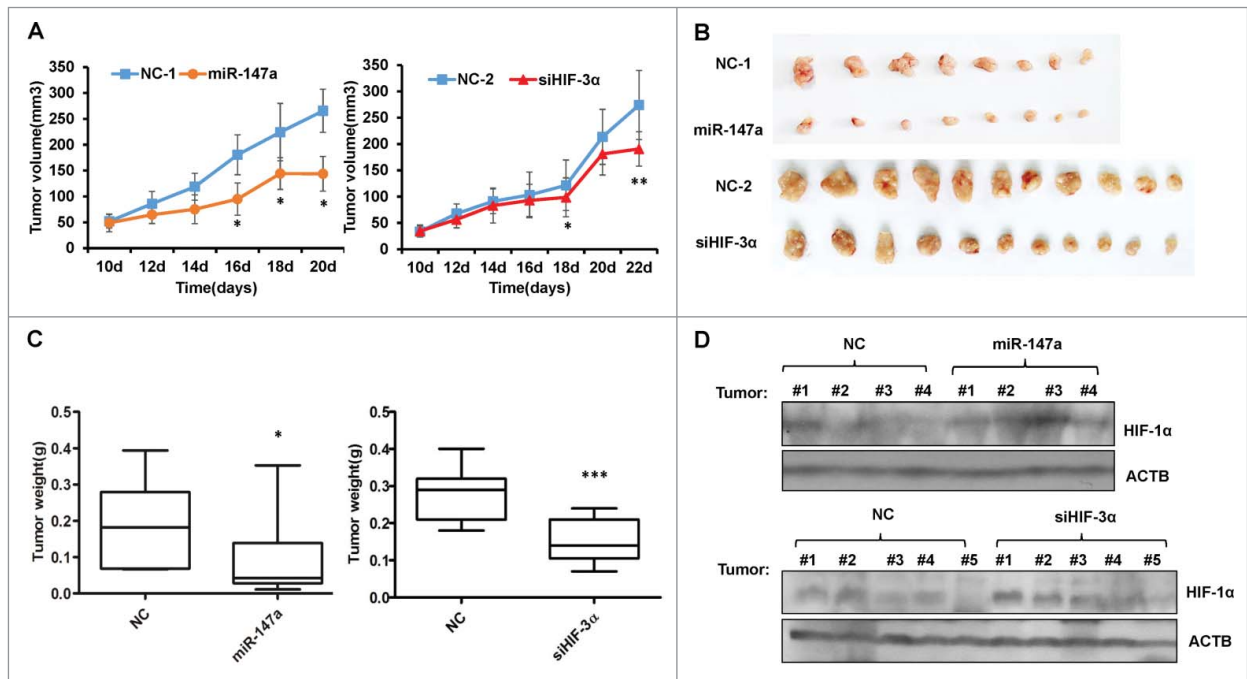

Figure 5. miR-147a suppresses tumor growth in nude mice. (A) Tumor volumes were calculated by the length and width measurement using Vernier calipers every 2 d. The xenografts were established through subcutaneous injection of Hela cells in the flank of nude mice. When the tumors had reached an average volume of 40–50 mm³, tumor-bearing nude mice were treated with miR-147a, HIF-3α siRNAs and NC. Group miR-147a and NC-1 have 5 mice bearing 8 tumors respectively. siHIF-3α and NC-2 have 7 mice bearing 11 tumors respectively. *P < 0.05, **P < 0.001, student 2-tailed t test. (B) Images of tumors from (A). (C) The weight of tumors shown in (B). *P < 0.05, ***P < 0.0001, student 2-tailed t test. (D) Western blot analysis of HIF-1α in tumors treated with NC, miR-147a or siHIF-3α.

HIF-1α can only partially rescue the suppressive effect of siHIF-3α on cell proliferation (Fig. 4E), suggesting there are other mechanisms/pathways that are involved in the siHIF-3α-induced suppression. It was reported that in addition to negatively regulating HIF-1α expression in protein level, HIF-3α also positively regulates expression of HIF-2α. HIF-2α can stimulate proliferation under hypoxic conditions via regulating expression of NANOG.[38] NANOG is involved in cervical tumorigenesis.[39] CDK6 and CDC25A are the downstream cell cycle effectors of NANOG during the transition from G1 to S.[40] Therefore, the knockdown of HIF-1α only partially rescue the suppressive effect of siHIF-3α on cell proliferation.

Under hypoxia condition, HIF-1 binds to hypoxia response elements (HREs) in the promoter region of target genes and activates transcription. Although miR-147a is upregulated during hypoxic stress, we did not find any HRE upstream of miR-147a gene up to 10 kb. Thus, we hypothesized the expression of miR-147a was regulated by HIF-1α indirectly under hypoxic condition. Using ChIPBase (deepbase.sysu.edu.cn/chipbase/index.php), a database for decoding the transcriptional regulation of long non-coding RNA and microRNA genes from ChIP-Seq data, we found that HNF4A was a direct transcription factor of miR-147a from chip-seq data.[41] Hypoxia induced the expression of HNF4A and HIF-1α, so HNF4A and HIF-1α were upregulated in hypoxia.[42] HNF4A interacts with HIF-1α to exert transcription activity.[43] Chip-seq data demonstrated that HNF4A was a direct transcription factor of miR-147a. Taken together, miR-147a is a hypoxia-induced miRNA and regulate by HIF-1α indirectly. Hypoxia induced HIF-1α increases the expression of miR-147a through HNF4A. MiR-147a promotes the stability of HIF-1α by targeting HIF-3α, thus forming a positive feedback loop. The accumulation of HIF-1α increases the expression of downstream gene, which contribute to cell proliferation arrest (Fig. 6). These discoveries provide a new insight into the mechanism of miR-147a induced cell proliferation arrest under hypoxia.
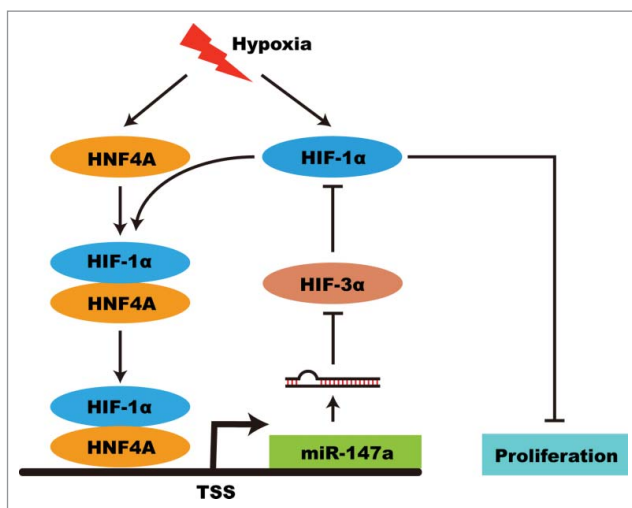
According to our results and other peoples' reports, HIF-1α inhibits cell proliferation in vitro experiments, but promotes tumor growth and angiogenesis in vivo experiments.[44] This is due to that HIF-1α can up-regulate the expression of p21, p27, p53, VEGF, and other gene.[45] In vitro experiment, upregulated p21, p27 and p53 inhibit cell proliferation.[46-48] In vivo experiment, p21, p27, and p53 may also be up-regulated. However, angiogenesis induced by VEGF and other genes play more important roles, and finally promote tumor growth and progression.[49] HIF-1α promotes angiogenesis to improve the blood supply and changes the metabolic model of solid tumor for adapting hypoxia environment in vivo. Proliferation arrest may be necessary to survival under the initial stage of hypoxia condition before the cellular functional alteration derived by HIF-1α.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

Funding

Figure 6. Model of hypoxia-induced miR-147a in regulating cell proliferation and its role in positive feedback. Hypoxia induced HIF-1α and HNF4A expression. HIF-1α increases the expression of miR-147a through interacting with HNF4A. MiR-147a promotes the stability of HIF-1α by targeting HIF-3α. The accumulation of HIF-1α increases the expression of downstream gene, which contribute to cell proliferation arrest.

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